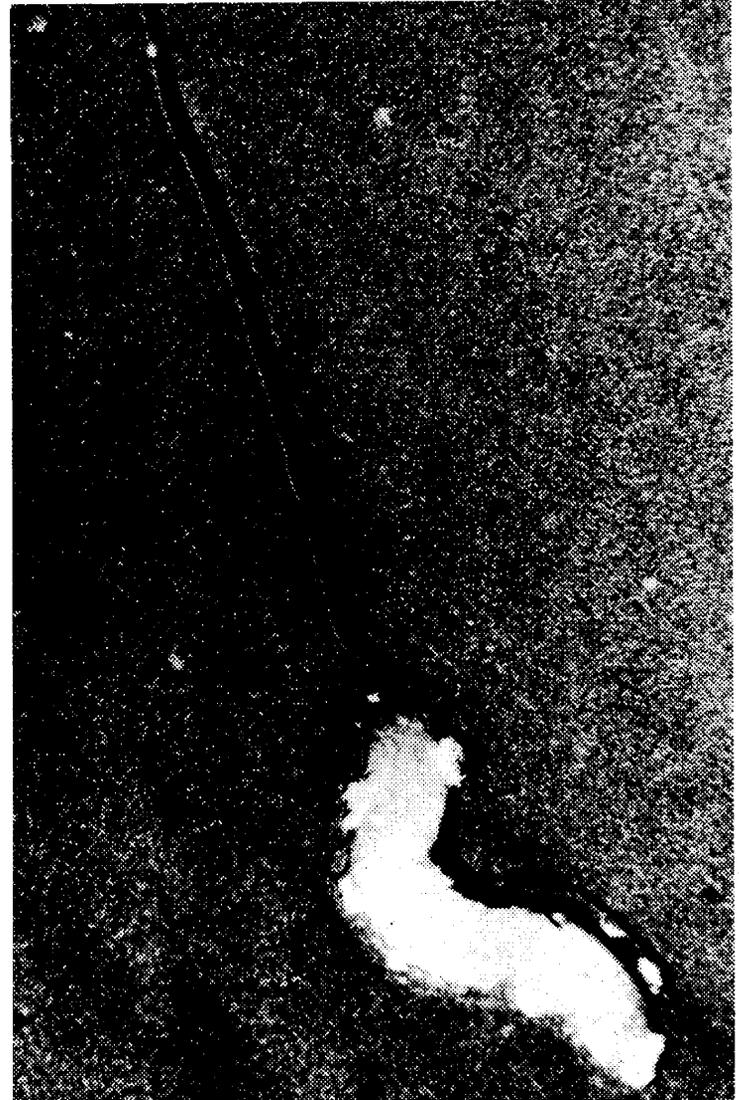


Proceedings of 1982

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INTERNATIONAL CONFERENCE ON MICROBIAL ENHANCEMENT OF OIL RECOVERY



May 16-21, 1982
Shangri-La, Afton, Oklahoma

Sponsored by

Engineering Foundation,
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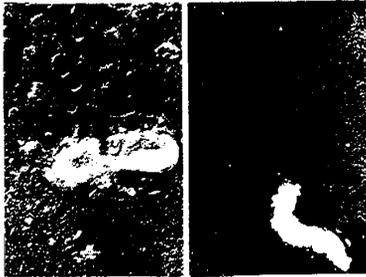
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Bacterium from
3,000 feet, Wil-
mington Field, Cal-
ifornia

Desulfovibrio

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Courtesy Drs. Edward A.
and Mary M. Grula
Oklahoma State University
Stillwater, Oklahoma

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International Conference on
Microbial Enhancement of Oil Recovery**

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Introduction

An International Conference on Microbial Enhancement of Oil Recovery (MEOR) was held May 16-21, 1982, at Afton, Okla. This Conference was sponsored by the U.S. Department of Energy, the University of Oklahoma Energy Resources Center, and the Engineering Foundation. Its objective was to bring together microbiologists and engineers from around the world who are diligently trying to develop methods for the application of microbial systems to the petroleum industry (32 scientists from 12 foreign countries attended). This is actually a resurgence of interest in this field of endeavor that had its beginnings in the 1930's with Dr. ZoBell's pioneering work.

The conference generated 32 papers, nine poster presentations, and a short course on the fundamentals of petroleum reservoir geology. It

showed that a new, more fundamental, approach is being taken in the search for ways to apply microbes to oil recovery. Great effort is being expended by microbiologists to understand the complex subsurface environment of a petroleum reservoir in relation to microbial metabolism; and by engineers to understand the fundamental activities of microbes before attempting to bring the two systems together.

The Conference was organized into seven formal sessions and three informal poster sessions that followed consecutively during the five days of the Conference. A review of the papers revealed that they fall naturally into the four divisions which are used in the organization of the Proceedings. The seven sessions and their chairmen were:

TITLE OF SESSION

- I. Introductory Statements
- II. Overall Perspectives
- III. Microbial Activities
- IV. *In Situ* Approaches
- V. Heavy Oils
- VI. Bioproducts for Microbial EOR
- VIIa. Potential Reservoir Damage
- VIIb. Potential Approaches
- Poster Sessions

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The chairmen of each session began the task of searching for papers to fit their specialized needs about one year before the Conference was held and assisted throughout in the organization and conduct of the Conference.

Some very difficult questions were asked on both sides that could not be answered at the Conference; however, those questions very vividly pointed out the areas where research to acquire new knowledge is necessary before microbes can

be generally applied for petroleum recovery and processing operations. The unanswered questions did not dampen the enthusiasm of the participants, and the Conference ended on a general note of optimism in the belief that microbial enhancement of oil recovery, and other applications of microbial systems in the oil industry, will become an important part of petroleum technology. Before the Conference ended, the groundwork was laid for another Conference to be held in the Spring of 1984.

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Microbes and Their Metabolites

Numerous microbial cultures (pure and mixed) are capable of synthesizing a variety of biochemical products from crude petroleum and its distillate fractions. The range of metabolic products from microbial consumption of petroleum is very broad, depending on environmental conditions (pressure, temperature, salinity, pH and the presence or absence of oxygen), supporting nutrients available for cell metabolism (nitrogen, phosphorus, minerals, etc.), and the specific bacteria interacting with the petroleum.

In very general terms the metabolic products may be gases (methane, hydrogen, carbon dioxide, hydrogen sulfide), low molecular weight carboxylic acids (formic, acetic, propionic, valeric), solvents (alcohols, aldehydes, ketones), polymers (proteins, polysaccharides), surface active compounds that are generally polyanionic lipids, and many other compounds ranging from simple to very complex macromolecules.

The chemical structures of most of the high molecular weight polymers produced by microbes have not been completely elucidated because of their molecular diversity and complexity. However, the renewed emphasis on these products for possible use in petroleum recovery, processing, transportation, and storage applications should result in greater emphasis on the determination of the chemical structure and properties of the microbial products.

In MEOR research, the study of specific microorganisms, or types of microorganisms, and their metabolic products is done for one of three reasons: 1) for the surface production of various compounds which, when injected into a petroleum reservoir, will enhance oil production, 2) for the injection of cells into a reservoir for *in situ* production of metabolic products which will enhance oil recovery, or 3) for the study of the microbial ecology of the reservoir.

Slodki and Camus presented a brief history on the development of xanthan gum, which is a biopolymer produced by *Xanthomonas campestris*, that has good rheological properties for polymer floods of oil reservoirs. They then discussed the biodegradability of the biopolymer. Unsterile solutions of the gum were found to be remarkably stable, which suggested to them that xanthan-degrading microorganisms are not abundant in nature. By means of culture enrichment procedures, a pure culture of a salt-tolerant *Bacillus* sp. was isolated that produced a salt-tolerant degradative enzyme.

A more prolific mixed culture producing xanthanase was obtained by adding a *Flavobacterium* sp. to the *Bacillus* culture although the *Flavobacterium* does not, by itself, produce any xanthanase. A non-salt tolerant xanthanase was found in a second *Bacillus* species. The enzymes were partially purified and characterized. Determination of the products of the enzymatic action led them to conclude that the xanthanase preparations must be mixtures of enzymes that attack all the side chain linkages in xanthan gum.

Several papers were presented on bioemulsifiers and biosurfactants. Gutnick, Zosim, and Rosenberg reported on the interaction of emulsan with hydrocarbons. Emulsan is a high molecular weight (M.W. = 10^6), water soluble, extracellular bioemulsifier produced by *Acinetobacter calcoaceticus* RAG-1. It is a d-galactosamine-containing polyanionic polysaccharide which is initially released from the cell surface as a protein complex. Removal of the protein yields a polymer, called apoemulsan, which retains emulsifying activity. Studies on the interaction of emulsan and apoemulsan revealed several interesting properties. The value of emulsan lies in its ability to form very stable emulsions with crude oils. The authors found that the higher the molecular weight of the hydrocarbon the more effective emulsan is in the formation of stable emulsions. Furthermore, the emulsion is non-wetting (non-adhesive) to metal surfaces which suggests several uses for transportation and storage of crude oils.

The bacterial strain Rsan ver [*Pseudomonas* sp.] was reported by Guerra-Santos et al to produce surface active compounds equally well with glucose or hydrocarbon as a substrate. The behavior of the culture and surfactant production were studied in a bioreactor, in batch culture, and in continuous culture. The surface tension of surfactant from batch culture growth was considerably less than that obtained in continuous culture.

The effect of surfactant hydrophile-lipophile balance (HLB) on bitumen recovery and surface activity of *Corynebacterium fascians* was reported by Zajic et al. The highest total bitumen recovery by hexadecane-grown bacteria occurred at HLB values of 5.5 and 16. Hydrophobic values (HLB 0.5 to 11) stimulated the growth and surface activity of *C. fascians* grown on such water-soluble substrates as sucrose. HLB values of 0.5, 16, and 29 improved the emulsification properties of the sucrose-grown bacteria. Membrane filtrates of culture broths to which a surfactant had been

added showed that the bacterial cells, *per se*, contribute highly to the total surface activity of the culture.

According to Rosenberg et al two general types of interaction between microorganisms and hydrocarbons have been postulated: 1) adherence of cells directly to large oil droplets, and 2) release of extracellular surfactants or emulsifiers that greatly increase the hydrocarbon:aqueous interfacial area. In the case of the hydrocarbon-degrading *Acinetobacter calcoaceticus* RAG-1, both types of interaction are operative. It was demonstrated that adherence is a prerequisite for growth on hexadecane under two conditions: low initial cell density and limited emulsification of the substrate, which are the conditions in most natural environments. Bioemulsification, however, is a cell-density-dependent phenomenon. Adherence of microorganisms to hydrocarbons was found to be neither an exclusive property of hydrocarbon-degrading microorganisms nor is it restricted to those hydrocarbons that a microorganism can metabolize.

Wang reported on a bacterial strain which produces extracellular polysaccharide from crude oil or heavy liquid paraffin as the carbon source. This was found to be a new species and was named *Brevibacterium viscogenes*. The yields of polysaccharide were 8 gm/l from 12 percent (W/V) crude oil and 12 gm/l from 4 percent (W/V) heavy liquid paraffin. The conversion rate of added paraffin was over 40 percent. The physical properties of this polysaccharide are comparable to those of xanthan and are considerably better than partially hydrolyzed polyacrylamide or carboxymethylcellulose. When the polysaccharide was used as a driving solution, an injection volume, corresponding to 20 percent of the pore volume, enhanced oil recovery to about 9 percent of the initial reserves.

The environmental parameters of the reservoir will limit the types of microorganisms which can be used for *in situ* processes. McInerney discussed the physiological types of microorganisms that would be useful in *in situ* processes. The limiting environmental factors that would affect either injectivity of cells or their growth and metabolic activities include permeability, temperature, pressure, salinity, salt composition, pH, and the nature of the residual oil. Most reservoirs are essentially anaerobic and, since there is no well documented record of truly anaerobic utilization

of hydrocarbons, an external food supply would be necessary. This could add the parameter of nutrient limitation as a mechanism for controlling growth and metabolism of injected cells. The metabolism of various types of microorganisms and the potential role of metabolic types in MEOR were discussed.

Because of the anaerobic environment in reservoirs, a potentially useful group of microorganisms for MEOR would be the *Clostridia*. E. Grula et al reported on a screening program designed to isolate species of *Clostridium* that would have useful characteristics for *in situ* processes. They looked for the following characteristics: 1) production of large amounts of gas (primarily CO₂), 2) production of large amounts of low molecular weight organic acids, 3) production of large amounts of low molecular weight organic solvents (primarily alcohols and acetone), and 4) production of large amounts of low molecular weight nonionic emulsifiers that would form oil-in-water emulsions. Techniques were devised for rapid screening, and several potentially very useful *Clostridium* strains were isolated. E. Grula et al emphasized the difficulty of isolating species of *Clostridium* that performed well metabolically in high (5.0-17.5 percent) concentrations of sodium chloride.

Knowledge of the role of microorganisms in the genesis of oil is useful in developing microbial processes for enhanced oil recovery.

The distribution of microorganisms and the rate of bacterial methanogenesis in flooded oil fields was reported by Ivanov and Belyaev. It was found that the population of microorganisms and the rate of methanogenesis increased as stratal waters were freshened. This correlated with the decrease of organic carbon and the increase of bicarbonate concentration, as well as with methane isotopic composition becoming less and the bicarbonate carbon isotopic composition becoming greater. The process of bacterial oil oxidation took place in the zone of contact between the injected fresh waters and stratal waters of oil fields. The products of aerobic oil destruction stimulated the activity of methane-producing bacteria. Methods were reported to induce a considerable stimulation of activity of the stratal microflora in oil fields. This activation of microbial processes resulted in the increase of concentration of soluble organic compounds and the content of newly-produced methane in stratal water, which could enhance oil recovery.

Production, Composition, and Biodegradation of Xanthan Gum (Polysaccharide B-1459)¹

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When research was undertaken in 1955 at NRRC on development of commercially feasible, fermentative conversion of corn sugar (dextrose) to extracellular polysaccharides, the stated objective was to replace imported plant gums in food and industrial applications. In retrospect, it is doubtful that any measurable replacement resulted from the establishment of a growing microbial gum industry whose major product is NRRC's xanthan gum from the bacterium *Xanthomonas campestris* NRRL B-1459.

We have seen, instead, new uses arise that exploit the unique physical properties of microbial gum dispersions. Initial commercialization of xanthan gum depended in large measure on its use as a food additive. FDA approval was obtained for such uses not precluded by Standards of Identity Regulations. Since that time, FDA approval has become a much more lengthy and costly process. Consequently, new microbial gums have gone into industrial uses, which, like those for xanthan gum, will continue to grow. Applications in drilling and recovery operations represent, by far, the largest potential market for such gums.

Among the remarkable rheological properties exhibited by aqueous dispersions of xanthan gum are relatively high viscosity of low concentrations and rapid shear-thinning that is instantaneously reversible. In addition to this pseudoplastic behavior, rheological yield is exhibited by gum solutions of 0.75 percent concentration or greater. Pseudoplasticity and yield are characteristic of many of the extracellular gums that were studied at NRRC.

Additional properties of xanthan gum dispersions important to their use in enhanced oil recovery were summarized by Sandvik and Maerker:¹⁰ low sensitivity of viscosity to pH, salinity, divalent metal ions and temperature; resistance to chemical and shear degradation; and relatively low adsorption of the polysaccharide by formations.

The structure of xanthan gum, as determined by Jansson, Keene and Lindberg,⁶ is that of a β -(1-4)-linked cellulosic backbone chain that regularly bears trisaccharide side chains on alternate glucosyl residues (Fig. 1).

These side chains consist of two mannose residues interspersed by a glucuronic acid residue. Note that every mannosyl residue involved in branching bears

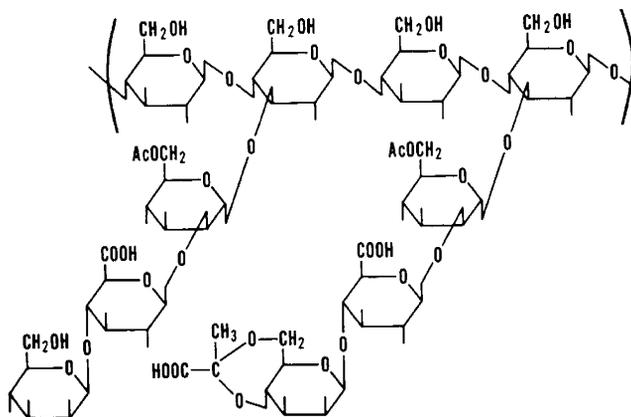


FIG. 1.

an acetyl group; on average, every other mannose end group has a pyruvic acetal substituent. Recent work in Argentina⁵ has shown that the pentasaccharide repeat unit is first assembled on a pyrophosphate-lipid precursor and then polymerized on the cell membrane surface to xanthan gum.

Transfer of the pyruvic acetal substituent to the terminal mannosyl from phosphoenolpyruvic acid occurs at the pentasaccharide pyrophosphate-lipid level. No information is available about the stage of biosynthesis at which *O*-acetylation takes place. It is likely an early one (sugar-nucleotide level?) because gum produced under various nutrient-limiting conditions in chemostat cultures has, in contrast to pyruvic acetal, invariant amounts of the *O*-acetyl substituent.⁴

Research at NRRC subsequent to development of the xanthan gum fermentation and study of the gum's properties, composition, and structure revealed a number of factors essential to both use and production. This research covered aspects of strain and product variability and their control, development of a synthetic medium, and, finally, microbial degradation. The remainder of this essay will deal with these topics.

X. campestris is known to be a phenotypically variable organism. In both continuous¹¹ and batch-type¹ fermentations, low yields of product having inferior quality were traced to generation of small colony-forming variants. That is not the full story, because large-colony variants also were isolated that made gum in high yield but of poor rheological quality. The

¹Dedicated to Dr. Allene R. Jeanes: Thirty years of continuing publication on microbial polysaccharides.

only detectable difference was a lower pyruvate content in gum of inferior quality.

Sandford et al.⁸ demonstrated the relationship between lower viscosities of dilute xanthan gum solutions and decreased pyruvic acid content (2.5–3.0 percent vs. 4.0–4.8 percent). The maximum viscosity difference was displayed by 0.05 percent (w/w) polysaccharide dispersions. Concentrations of this order are employed as mobility control agents in waterflooding. Dispersions (1 percent) of atypical xanthan gum also fail to give the large increases in viscosity usually observed between 50–70°C in the absence of salt.

This effect is attributed to an increase in hydrodynamic volume resulting from an uncoiling of the polymer's helical conformation and spreading of the side chains from the backbone. Microbiological procedures for ensuring reproducible fermentations and high quality of product have been reviewed¹² and are given along with other detailed information in USDA Bulletin ARS-NC-51.⁷

While developing a synthetic medium for xanthan gum production, Cadmus et al.² noted increasing pyruvic acetal content as the fermentation progressed. The suggestion that batch cultures contain mixtures of gum having different degrees of substitution by pyruvic acid was confirmed by Sandford et al.⁹ The main point of our work on a synthetic medium is that any change in a medium or fermentation condition must be examined not only with respect to its effect on yield but also with careful attention to quality of the product.

Aside from a long-term study of the stability of *X. campestris* in lyophil storage, our recent work on biodegradation represented the completion of work on xanthan gum at NRRC.³ Even though there is a report that the gum is to some degree, in the unordered conformation, subject to attack by cellulase, we have found unsterile solutions to be remarkably stable.

This experience suggests to us that xanthan-degrading organisms are not abundant in nature. Our objectives were: to obtain enzyme(s) that could alter xanthan and related polysaccharide structures for subsequent chemical or biological modification; to have means, through an enzymic fragmentation analysis, of comparing related polysaccharides from related species; and, possibly, to obtain an agent that could modify the viscosity of suspensions injected into underground oil- or gas-bearing formations.

By means of enrichment culture (the polysaccharide as sole carbon source), a pure culture of salt-tolerant (4 percent NaCl) *Bacillus* sp. that produced a salt-tolerant degradative enzyme was obtained from soil collected from inside a decaying tree trunk. A more prolific enzyme-producing mixed culture, consisting of this organism and a *Flavobacterium* sp., was isolated from the same source. Although the *Flavobacterium* does not by itself produce a xanthanase, production of xanthanase by the *Bacillus* is greatly enhanced by the associative growth. Several other soil bacteria elicited a similar effect. Another xanthanase-producing *Bacillus* was isolated from sewage sludge, but it did not form a salt-tolerant enzyme.

Partial purification of the enzymes was achieved by ion-exchange chromatography on diethylaminoethyl cellulose. Higher salt concentrations were required to

elute the salt-tolerant enzyme. Enzyme recoveries greater than 80 percent and 6- to 13-fold increases in specific activity were obtained by this procedure.

Both enzymes exhibited maximum activity, as measured by release of reducing sugar, at pH 5.4 and were inactive below pH 4 or above pH 8. Some difference was noted with regard to pH of maximum stability at 25°C. The salt-tolerant enzyme was stable over the range pH 6.0–7.5; the other enzyme was stable from pH 4.8–6.0. Even though the salt-tolerant enzyme displayed maximum activity in dilute buffer (0.05 M sodium acetate, pH 5.4) over the temperature range 42–48°C in the presence of 0.1 M NaCl, the temperature-activity curve was identical to that of the other enzyme, for which the maximum was 42°C.

A principal drawback to the use of the enzymes in certain situations would be temperature stability. The salt-tolerant enzyme loses activity rapidly beyond 42°C; the other enzyme is somewhat more stable. In the presence of 0.25 percent substrate, the salt-tolerant enzyme is stabilized at 42°C. Whereas the enzyme from the non-tolerant *Bacillus* is inactive in the presence of 4 percent NaCl, the enzyme from the salt-tolerant organism is stabilized significantly up to 45°C by that concentration of salt.

All of the enzyme preparations bring about a dramatic loss in viscosity of xanthan gum dispersions. For example, a 1 percent dispersion is reduced in viscosity from 1,750 mPa·S to 10 mPa·S in 18 h at 37°C. The yield of reducing sugar indicates a 38 percent conversion. Even so, a residual high-molecular-weight polysaccharide is precipitable from the digest by ethanol. The fragment, which is resistant to further attack by fresh enzyme, is principally a *D*-glucan (ca. 90 percent *D*-glucose content). Methylation/fragmentation analysis showed it to be the (1–4)-linked, cellulosic backbone of the xanthan gum.

Paper chromatographic examination of the supernatant alcoholic liquors revealed the same four components in all digests. These products were isolated by elution from thick chromatographic paper and chemically identified as *D*-glucuronic acid, pyruvylated *D*-mannose and 6-*O*-acetyl *D*-mannose. Identification of the position of *O*-acetyl substitution in the latter was accomplished by glc-ms of the 6-*O*-acetyl-2,3,4,5-tetra-*O*-trideuterioacetyl aldonitrile derivative.

It is unlikely that the various cleavages of individual side-chain sugar units could have been brought about by a single enzyme. Consequently, the xanthanase preparations must be mixtures of enzymes that attack all the side-chain linkages in xanthan gum, including the one involving (1–3)-linkage of acetylated mannose to the glucosidic backbone. Because glucose is not one of the low-molecular-weight products and the (1–4)-linked backbone remains intact, a cellulase type of activity is likely absent or limited.

REFERENCES

1. Cadmus, M. C., Rogovin, S. P., Burton, K. A., Pittsley, J. E., Knutson, C. A., and Jeanes, A. 1976. *Can. J. Microbiol.* 22:942–948.
2. Cadmus, M. C., Knutson, C. A., Lagoda, A. A., Pittsley, J. E., and Burton, K. A. 1978. *Biotechnol. Bioeng.* 20:1003–1014.

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3. Cadmus, M. C., Jackson, L. K., Burton, K. A., Plattner, R. D., and Slodki, M. E. 1982. *Appl. Environ. Microbiol.* 44: in press.
4. Davidson, I. W. 1978. *FEMS Microbiol. Lett.* 3:347-349.
5. Ielpi, L., Couso, R. O., and Dankert, M. A. 1981. *FEBS Lett.* 130:253-256; *Biochem. Biophys. Res. Commun.* 102:1400-1408.
6. Jansson, P.-E., Kenne, L., and Lindberg, B. 1975. *Carbohydr. Res.* 45:275-282.
7. Jeanes, A., Rogovin, P., Cadmus, M. C., Silman, R. W., and Knutson, C. A. 1976. ARS-NC-51, U.S. Department of Agriculture, Peoria, Ill. 14 pp.
8. Sandford, P. A., Pittsley, J. E., Knutson, C. A., Watson, P. R., Cadmus, M. C., and Jeanes, A. 1977. *Extracellular Microbial Polysaccharides, ACS Symp. Ser. No. 45*, P. Sandford and A. Laskin, eds., American Chemical Society, Washington, D.C., pp. 192-210.
9. Sandford, P. A., Watson, P. R., and Knutson, C. A. 1978. *Carbohydr. Res.* 63:253-256.
10. Sandvik, E. J., and Maerker, J. M. 1977. *Extracellular Microbial Polysaccharides, ACS Symp. Ser. No. 45*, P. Sandford and A. Laskin, eds., American Chemical Society, Washington, D.C., pp. 242-264.
11. Silman, R. W., and Rogovin, P. 1972. *Biotechnol. Bioeng.* 14:23-31.
12. Slodki, M. E., and Cadmus, M. C. 1978. *Adv. Appl. Microbiol.* 23:19-54.

The Interaction of Emulsan with Hydrocarbons

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ABSTRACT

The growth of microorganisms on hydrocarbons is generally accompanied by the emulsification of the carbon source in the aqueous media. In the case of the hydrocarbon-degrading bacteria *Acinetobacter calcoaceticus* RAG-1 this process is brought about by a high molecular weight (M.W. = 10^6), water soluble extracellular bioemulsifier termed emulsan. Emulsan is a d-galactosamine-containing polyanionic (pK'3.05) polysaccharide in which both acetate and long chain fatty acids are bound via O-acyl and N-acyl linkages.

Emulsan is initially released from the cell surface as a protein complex; removal of the protein yields a polymer, termed apoemulsan, which retains emulsifying activity.

Studies on the interaction of emulsan and apoemulsan with hydrocarbons reveal several interesting properties:

1. Hydrocarbon substrate specificity; the polymer works best in the presence of a mixture of aliphatic and cyclic or aromatic hydrocarbons.

2. Emulsion stability; in addition to enhancing the formation of emulsions, emulsan stabilizes preformed emulsions at weight ratios of oil to emulsan as high as 800:1.

3. Reversibility; emulsions stabilized by emulsan separate by creaming rather than by coalescence. The creams (emulsanosols) readily disperse in water to yield emulsions which exhibit the same characteristics as the original emulsions.

4. Emulsan binding at the oil-water interface; immunological analysis of emulsanosols demonstrates that emulsan concentrates at the oil-water interface independent of the pH of the aqueous phase.

5. Binding of cations at the oil-water interface; an aqueous solution of emulsan differs from emulsan which is bound to an oil droplet in that the latter binds cations such as Rhodamine B. This hydrocarbon-mediated cation binding is pH-dependent, decreasing rapidly below the pK' of emulsan.

INTRODUCTION

The growth of microorganisms on hydrocarbons is often accompanied by the emulsification of the insoluble carbon source in the culture medium.^{5,6,13} In many cases, this has been attributed to the production of an extracellular bioemulsifier. We have previously reported the purification,¹⁰ partial chemical characterization,^{2,14} and substrate specificity⁹ of the emulsifier of *Acineto-*

bacter RAG-1. The polymeric substance (referred to as emulsan) had an average molecular weight of 9.9×10^5 and intrinsic viscosity of 505 cm³ per g.

Olefin-free hexadecane (99 percent purity) was obtained from Fluka Chemical Co., Switzerland. Other paraffins and aromatic hydrocarbons were reagent grade products of either Merck or Aldrich Chemicals.

Emulsan contains (a) a polysaccharide backbone composed of N-acetyl D-galactosamine, N-acetylaminouronic acid, an unidentified amino sugar, (b) esterified fatty acids (0.5 μ moles per mg) consisting primarily of *a* and *B*-hydroxydodecanoic acid, and (c) proteins which can be removed by phenol treatment without destruction of emulsifying activity or loss of viscosity. The experiments presented here were performed to further investigate the manner by which emulsan interacts with hydrocarbons and the properties of the emulsan-hydrocarbon complex.

MATERIALS AND METHODS

Olefin-free hexadecane (99 percent purity) was obtained from Fluka Chemical Co., Switzerland. Other paraffins and aromatic hydrocarbons were reagent grade products of either Merck or Aldrich Chemicals. Kerosine and gas-oil were obtained from the Haifa Refinery, Haifa, Israel. Agha Jari crude oil was obtained from the Ashkelon-Eilat Pipeline Co., Israel. Rhodamine B (C₂₈H₃₁ClN₂O₃) recrystallized from ethanol, had a molar extinction coefficient of 0.9×10^5 at 560 nm, in close agreement with reported values.¹² TM buffer consisted of 0.02 M Tris (hydroxymethyl) amino-methane hydrochloride buffer, pH 7.2, and 10 mM MgSO₄. Glycerol (anhydrous) was a highly purified, redistilled product of Merck. Tritiated water (0.5 mCi per ml) was obtained from Nuclear Research Center, Negev, Israel.

Emulsan, the extracellular emulsifying agent of *Acinetobacter calcoaceticus* RAG-1 (ATCC 31012), was purified from a cell-free supernatant obtained from an ethanol grown culture both by ammonium sulfate precipitation¹⁰ and cetyltrimethyl ammonium bromide fractionation (I. Belsky, unpublished). The emulsan used in these studies contained 17 percent protein. Apoemulsan, prepared by a modification¹⁴ of the hot phenol method, contained less than 5 percent protein. Methyl cellulose (degree of substitution of 1.6) was a product of BDH (England). Xanthan⁸ (XFL-14630) was a product of XANCO Oil Field Products, Inc. The viscosities of 0.5 mg per ml solutions of emulsan, apo-

emulsan, methyl cellulose and xanthan at 30°C were 0.98, 1.40, 1.20 and 2.47 centipoise, respectively.

Emulsion stability measurements—Hydrocarbon-in-water emulsions were prepared by ultrasound treatment, using the Braun Labsonic 1510 instrument. Ten ml of the hydrocarbon-water mixture in a cellulose nitrate tube (Beckman) was exposed to sonic oscillation for 15 sec at a constant power value of 30 W. Unless otherwise stated, the emulsifiers were added prior to ultrasound treatment. The emulsions that were formed were transferred immediately to calibrated Klett tubes and turbidity measured using a Klett-Summerson photoelectric colorimeter fitted with a green filter. Reported turbidity values were corrected using a standard calibration curve prepared by serially diluting a hexadecane-2-methylnaphthalene emulsion. Stabilities of emulsions were determined by measuring the turbidity after standing undisturbed at 25 ± 2°C for 1 to 120 h. The percent stability is defined as the Klett units after 24 h divided by the turbidity immediately after sonication times one hundred.

Dye binding studies—A spectrophotometric method was developed for quantitative estimation of Rhodamine B binding to emulsan-stabilized hexadecane droplets. Emulsions were prepared in the presence of varying concentrations of the dye as described above. The emulsions were transferred to glass test tubes and allowed to stand undisturbed for 21 h (equilibrium was reached during the first 15 h). Samples of the emulsions were then centrifuged at 8,000 × g for 20 min at 25°C. Under these conditions the droplets rose to the surface and did not interfere with absorbance readings of the aqueous phase. The residual dye (equilibrium concentration of the dye in the aqueous phase) was determined from trichromic readings ($2A_{560} - A_{540} - A_{580}$) by a comparison with a standard calibration curve of known concentrations of the dye in TM buffer. When Rhodamine B concentration exceeded 0.01 mg per ml, dilutions were made in the buffer before determining absorbance.

General analytical methods—Protein concentrations were determined by the method of Lowry et al⁷ using bovine serum albumin as a standard. Viscosity was measured in an Ostwald-Fenske microviscometer (water value, 55.1 sec) at 30°C, calibrated with 20 percent and 50 percent glycerol. Emulsan concentration was determined by a standard emulsification assay¹⁰ and by the microplate modification of the enzyme-linked immunosorbent assay (ELISA test)⁴ in which anti-emulsan IgG prepared against purified emulsan was first covalently linked to alkaline phosphatase with glutaraldehyde. This material was then bound to varying quantities of emulsan previously immobilized on the walls of an IgG-coated microplate. The ELISA test yielded the same quantitative results regardless of whether the antigen was emulsan or apoemulsan (S. Goldman, in preparation). Size distribution of hexadecane droplets stabilized by emulsan was determined both by microscopy (see Results) and using a Coulter Counter Model ZB.

RESULTS

RAG-1 emulsan enhanced both the formation and stability of hexadecane-in-water emulsions prepared by ultrasonic treatment. Using a volume fraction (V_f) of hexadecane of 0.01, the initial turbidities varied from

520 Klett units (no emulsan) to 2600 Klett units (0.1 mg emulsan per ml). On standing, the hexadecane droplets in the control coalesced and rose rapidly to the surface; by 10 h the control emulsion was completely broken. In the presence of either 0.05 and 0.1 mg per ml emulsan (hexadecane:emulsan weight ratios of 155 and 77, respectively) there was a small decrease in turbidity for 2 h, after which the emulsions remained relatively stable for the next 40 h.

With a higher ratio of hexadecane to emulsan of about 400 (0.02 mg emulsan per ml) the turbidity dropped sharply during the initial 17 h of standing and then remained relatively stable for the remainder of the experiment. Slightly more stable emulsions were obtained when emulsan was added immediately after, rather than prior to ultrasonic treatment. (This might be due to an approximately 10 percent decrease in viscosity of the emulsan during the sonic treatment.)

The effect of emulsan on the formation and stability of different hydrocarbon-in-water emulsions is summarized in Table 1. Although the stability of all hydrocarbon emulsions tested was enhanced in the presence of 0.05–0.1 mg emulsan per ml, the effect varied significantly with the particular hydrocarbon examined. In general, the higher the molecular weight of the liquid hydrocarbon, the more effective emulsan was in inducing and stabilizing the emulsion. This was true both for aliphatic and aromatic hydrocarbons, even though the initial turbidities of aromatic hydrocarbon emul-

TABLE 1—Various Hydrocarbon-in-Water Emulsions Stabilized by Emulsan^a

Hydrocarbon	Emulsan (mg/ml)	Turbidity (Klett Units)		Apparent stability, ^b percent
		t = 0 hr	t = 24 hr	
Aliphatics				
Octane	0.1	1200	112	9.3
Dodecane	0.1	1700	850	50
Tetradecane	0.1	2720	1500	55
Hexadecane	0.1	3150	1900	60
Aromatics				
Toluene	0.05	3600	165	4.6
p-Xylene	0.1	4400	475	11
	0.05	3080	260	8.4
2-Methylnaphthalene	0.05	7600	4000	53 ^c
Tetrahydro-naphthalene	0.05	7600	3800	50
Mixtures				
Hexadecane:				
2-Methyl-naphthalene	0.05	17000	12000	71
Kerosine	0.05	6700	3400	51
Gas-oil	0.05	3250	2650	85
Crude oil	0.05	6200	4400	71

^a Emulsions were prepared as described in Methods with V_f of hydrocarbon of 0.01.

^b Apparent stability is defined as the turbidity of the emulsion at 24 hr divided by turbidity at t = 0 times 100. Controls for each hydrocarbon (without emulsan) yielded 24-hr turbidity values that were less than 3 percent of the corresponding values for the emulsan experiment.

^c This experiment was performed at 35°C to avoid solidification of the hydrocarbon.

sions were much higher than aliphatic hydrocarbon emulsions. Mixtures containing both aliphatics and aromatics were invariably better substrates for emulsan than the individual hydrocarbons by themselves.

For example, initial turbidities of 2-methylnaphthalene and hexadecane emulsions (formed in the presence of 0.05 mg emulsan per ml) were 7600 and 3150, respectively, whereas for the 1:1 mixture of the two hydrocarbons the initial turbidity was 17,000; similarly the stabilities of the individual hydrocarbons were 60 percent and 53 percent after 24 h, while the stability of the emulsified mixture was 71 percent. Emulsions prepared from each of the complex hydrocarbon mixtures—crude oil, gas-oil and kerosine—were stabilized by 0.05 mg/ml emulsan.

Table 2 and Figure 1 compare two RAG-1 emulsifier preparations with two polymeric emulsifiers, methylcellulose and xanthan. Both emulsan and apoemulsan (deproteinized emulsan) were more effective than the two commercial emulsifiers in stabilizing hexadecane-in-water emulsions over the entire concentration range studied. It should be noted that native emulsan (containing approximately 20 percent protein) is more effective than apoemulsan in forming emulsions.¹⁴ However, if the hydrocarbon-in-water emulsion is prepared by ultrasound treatment, the protein does not play a crucial role in emulsion stabilization. At a V_f of hexadecane of 0.01 and an emulsifier concentration of 0.1 mg per ml the turbidities of emulsions (after standing for 120 h) stabilized by emulsan, xanthan and methylcellulose were 1420, 160 and 155 K.U., respectively (Fig. 1, initial period).

The lowering of turbidity may be the result of (a) coalescence of the hydrocarbon droplets giving rise to a complete phase separation, or (b) creaming of stabilized hydrocarbon droplets which float to the surface. In the case of creaming, the hydrocarbon droplets would be expected to reform a homogenous emulsion with similar stability characteristics upon gentle agitation. The results summarized in Fig. 1 (second period) illustrate the turbidities of partially broken emulsan mixtures which were agitated by hand and subsequently allowed to stand for an additional 120 h. Breakage of xanthan-stabilized emulsions was primarily brought about by coalescence of oil droplets since breakage could not be reversed. Upon gentle mixing the turbidity rose only to 305 Klett units and then dropped rapidly to 150 Klett units. Microscopic examination provided further evidence that breakage of xanthan-stabilized emulsions was due to coalescence rather than by flocculation. In contrast, breakage of emulsan-stabilized emulsions was due to creaming,

TABLE 2—Stabilization of Hexadecane-in-Water Emulsions by Different Emulsifiers^a

Emulsifier	Apparent Stability (Percent)		
	(0.02 mg/ml)	(0.05 mg/ml)	(0.1 mg/ml)
Emulsan	13	63	74
Apoemulsan	14	62	75
Methylcellulose	4	13	27
Xanthan	10	19	21

^a Experiments were performed as described in Methods with a V_f of hexadecane of 0.01. Apparent stabilities of emulsions prepared without emulsifier were less than 2 percent.

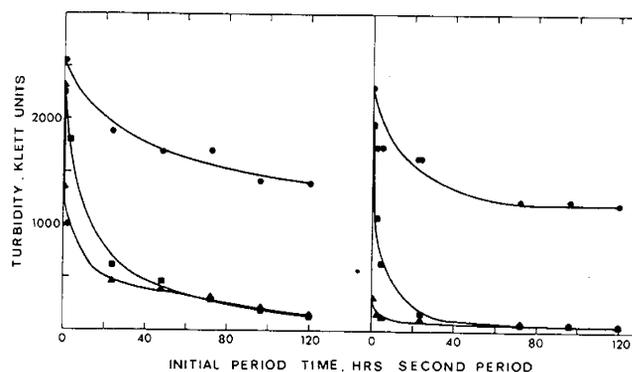


FIG. 1—Stability and reversibility of hexadecane-in-water emulsions. The emulsions were prepared using 1 mg emulsan (●), 1 mg methylcellulose (■) or 1 mg xanthan (▲) (see Methods). After measuring the decrease in turbidities of the emulsions for 120 h, the samples were mixed gently by hand and turbidities determined for an additional 120 h (second period).

since gentle mixing totally reversed the process. (2250 Klett units initially compared to 2300 Klett units after mixing at 120 h.) It can be seen that breakage during the second period was similar to that which was observed in the initial 120 h. Furthermore, cream obtained by centrifugation of emulsan-stabilized emulsions readily became suspended in aqueous solution to reform emulsions which did not differ significantly from those obtained initially by ultrasonic treatment. The methylcellulose-stabilized emulsion was an intermediate case; breakage was due to both creaming and coalescence. The hydrocarbon upper layer reformed an emulsion when mixed with water to yield a turbidity similar to that produced by ultrasonic treatment. However, emulsion breakage was much more rapid during the second period than during the initial 120 h.

A photomicrograph of an emulsan-stabilized hexadecane-in-water emulsion is shown in Fig. 2 (lower

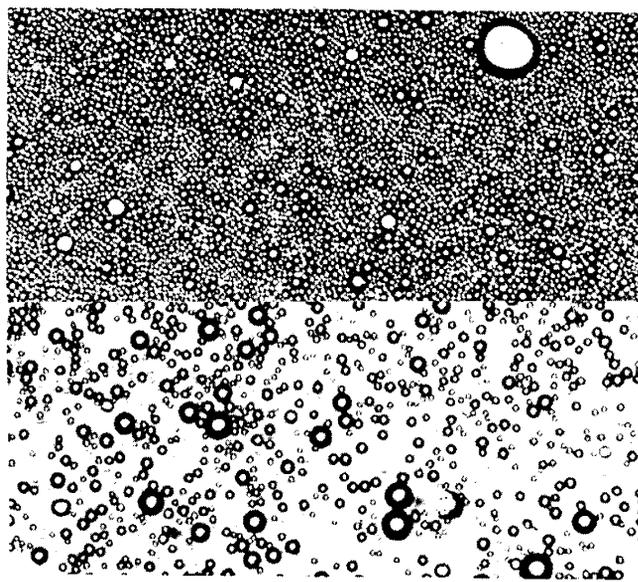


FIG. 2—Light microscopy of hexadecane-in-water emulsions stabilized by emulsan. Hexadecane (0.2 ml) in 10 ml TM buffer was emulsified by sonic oscillation for 15 sec in the presence of 0.2 mg emulsan. The lower field shows the emulsion immediately after sonic treatment; the upper field shows the cream formed after several days standing. Magnification in both cases is 389 X.

field). Analysis of a number of such micrographs yielded an average droplet diameter of 4.0 microns when the weight ratio of hexadecane to emulsan was 155 (Fig. 3). Emulsions produced under similar conditions yielded an average droplet diameter of 3.6 microns when measured with a Coulter counter. Ninety percent of the droplets had a diameter of less than 6.1 microns. Upon prolonged standing the droplets rose and concentrated at the surface forming a cream (Fig. 2 upper field). After standing for 1 week, the cream was separated from the aqueous phase. Less than 10 percent of the emulsan was found in the aqueous phase as determined both immunologically (see Materials and Methods) and by the standard emulsifier assay.¹⁰

The water content of the different samples of cream, calculated from the density of the cream at 20°C compared to the known densities of water and hexadecane was found to be 40-60 percent. Following centrifugation at 8,000 × g for 40 min, more viscous creams were obtained which consisted of about 65 percent hexadecane and 35 percent water. Even after the centrifugation procedure there was no coalescence of oil droplets. The water content of the cream was also determined using tritiated water as a tracer. With a V_f of hexadecane of 0.1 and an emulsan concentration of 0.5 mg per ml, the resulting cream contained 43 percent water after standing 3 days and 30 percent water after standing 10 days and then centrifuging at 8,000 × g for 30 min.

A qualitative experiment demonstrating binding of the water soluble dye Rhodamine B to emulsan-stabilized hexadecane droplets is shown in Table 3. When the hexadecane emulsion was prepared in the presence of both emulsan and Rhodamine B, the dye was rendered nondialyzable for at least 24 h. However, the dye was released during the first hour of dialysis if either emulsan or hexadecane was omitted, or if the emulsion was stabilized by methylcellulose instead of emulsan. Even when much higher concentrations of methylcellulose were used to stabilize the emulsions, the dye was not retained in the dialysis bag. Thus, Rhodamine

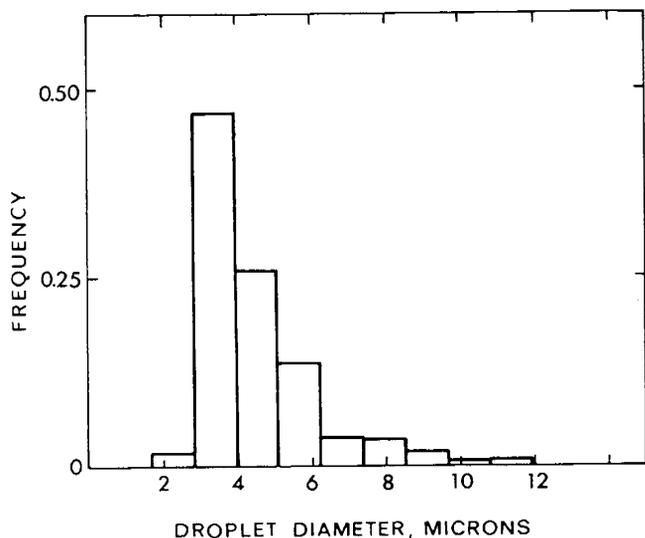


FIG. 3—Size distribution of hexadecane-in-water droplets stabilized by emulsan. Emulsions were prepared in 10 ml TM buffer containing 0.1 ml hexadecane and 0.5 mg emulsan. Diameters of 1724 droplets were determined from enlargements of micrographs.

TABLE 3—Binding of Rhodamine B to Emulsified Hexadecane Stabilized by RAG-1 Emulsan

Reaction mixture ^a	Observation after 24 h of dialysis ^b	
	Inside (10 ml)	Dialysate (800 ml)
1. Complete	Intense pink, turbid	Colorless
2. Complete minus hexadecane	Colorless, clear	Faint pink
3. Complete minus emulsan	Colorless, two phases	Faint pink
4. Complete minus hexadecane and emulsan	Colorless, clear	Faint pink
5. Complete minus emulsan plus 1.0 mg methylcellulose	Colorless, turbid	Faint pink

a The complete reaction mixture consisted of 0.1 ml hexadecane, 1.0 mg emulsan and 0.1 mg Rhodamine B in a final volume of 10 ml TM buffer.

b After exposing the mixture to sonic oscillation as described in Materials and Methods, the entire volume was placed in a cellophane bag and dialyzed for 24 h against 800 ml distilled water.

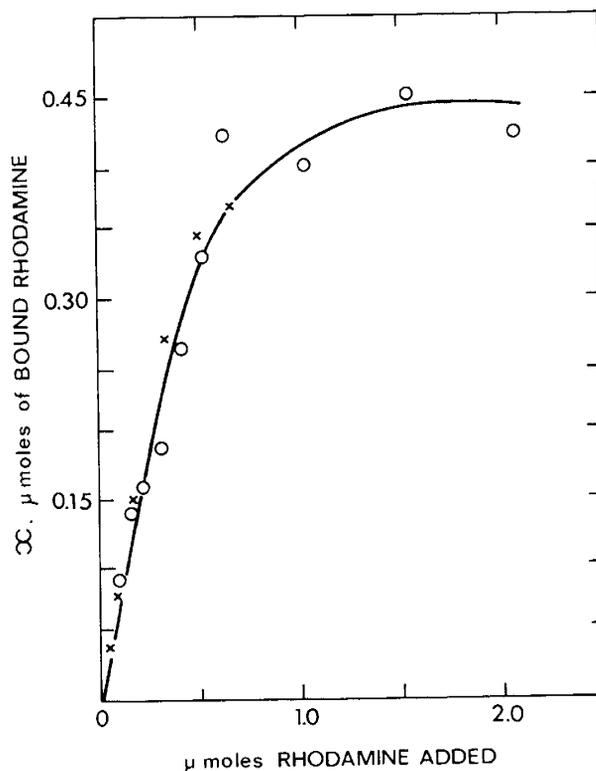


FIG. 4—Adsorption of Rhodamine B to hexadecane-in-water droplets stabilized by emulsan. Experiments were performed with (a) V_f of hexadecane of 0.01 and emulsan concentration of 0.1 mg per ml (x) or (b) V_f of hexadecane of 0.02 and emulsan concentration of 0.02 mg per ml (o). Rhodamine binding was measured spectrophotometrically following separation of the "cream" from the aqueous phase (see Materials and Methods).

binding was a property of the emulsan-hydrocarbon complex and not due simply to the greater hydrocarbon-water interface.

Adsorption of Rhodamine B to hexadecane-in-water droplets stabilized by emulsan was further investigated by determining residual Rhodamine spectrophotometrically following separation of the cream from the aqueous phase (Fig. 4). At low concentrations of Rhodamine, more than 80 percent of the dye was adsorbed. Since the cream phase represented less than 5 percent of the total volume of liquid, the effective concentration of dye into the cream was greater than 75-fold. At saturating concentrations of dye, about 2.2 μ moles of dye were bound per 1.0 mg emulsan.

insert fig 4,5,6

The data in Fig. 4 were replotted according to the Freundlich empirical adsorption equation¹¹ in logarithmic coordinates. With initial dye concentrations from 0.2 to 6 μ moles, a straight line was obtained, indicating a single type of binding. The apparent dissociation constant for the Rhodamine-emulsan complex was 3.4×10^{-6} .

The influence of pH on the binding of Rhodamine B to hexadecane-in-water droplets stabilized by emulsan is shown in Fig. 5. Below pH 4, the dye was desorbed from the interface. Release of the dye was not a result of emulsan coming off the oil-water interface (Fig. 6). Emulsan remained bound to the oil droplets over the entire pH range studied (pH 2-9).

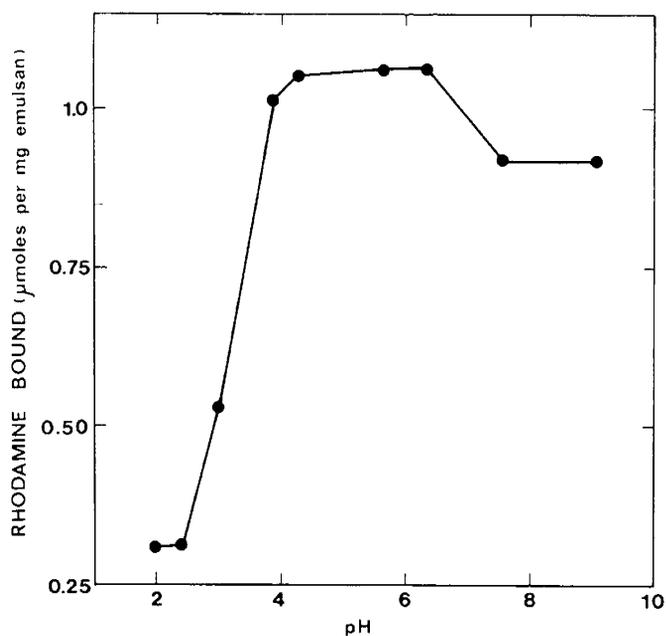


FIG. 5—Effect of pH on the adsorption of Rhodamine B to hexadecane-in-water droplets stabilized by emulsan. Experiments were performed with emulsions containing V_f of hexadecane of 0.01, emulsan concentration of 0.05 mg per ml and 0.1 mM Rhodamine. pH of the mixtures was adjusted before the emulsion was formed by sonic oscillation. Rhodamine binding was measured spectrophotometrically.

DISCUSSION

The data presented here demonstrate that emulsan, the water soluble, extracellular bioemulsifier of *Acinetobacter calcoaceticus* RAG-1 stabilizes a wide variety of hydrocarbon-in-water emulsions. The emulsan binds tightly to the surface of hydrocarbon droplets, presumably forming a strong polymeric film on the droplet surface which prevents coalescence. This provides a rationale for the previously reported finding that the average size of hydrocarbon droplets formed in water by mechanical agitation depended upon the weight ratio of hydrocarbon to emulsan, rather than simply on emulsan concentration.¹⁰ Furthermore, the tight binding of emulsan to hexadecane-water interfaces explains the failure to find emulsan in clarified culture broths of hexadecane-grown *Acinetobacter* RAG-1.

The stability-reversibility phenomenon, as summarized in Fig. 2, may be considered to reflect the affinity of the various emulsifiers for hexadecane droplets; xanthan < methylcellulose < emulsan. This relationship may be due to the presence of hydrophobic groups (or sites) on the structure of the three polysaccharides. Emulsan is the most hydrophobic of the gel polymers, containing C_{12} fatty acid side chains, in addition to N-acyl groups.

From the weight ratio of hydrocarbon to bound emulsan and the average droplet diameter (as determined by two independent methods—microscopy and Coulter-counter techniques), it is possible to estimate the thickness of the emulsan film around the oil droplet. For a weight ratio of 155, the mean droplet diameter was 3.8 microns. The volume of hexadecane per droplet, V_H , is

$$\frac{4}{3} \pi r_H^3$$

where r_H is the radius of the inner hexadecane sphere; the volume of emulsan per droplet, V_E , is

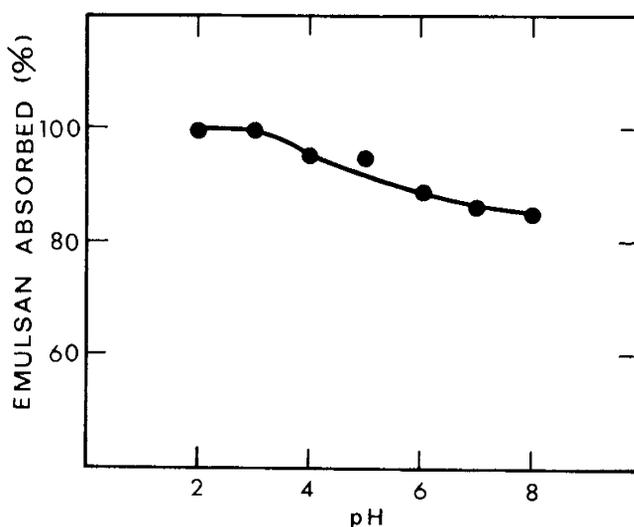


FIG. 6—Effect of pH on adsorption of emulsan onto hexadecane/water interfaces. Experiments were performed with emulsions of hexadecane V_f of 0.01 and emulsan initial concentration of 0.05 mg per ml. Residual emulsan in the aqueous phase was determined immunologically (see Materials and Methods).

$$\frac{4}{3}\pi r_T^3 - \frac{4}{3}\pi r_H^3 = \frac{4}{3}\pi \left(r_T^3 - r_H^3 \right),$$

where $r_T = 1.9$ microns is the radius of the total droplet (hexadecane sphere plus emulsan shell).

Dividing,

$$\frac{V_H}{V_E} = \frac{r_H^3}{6.86 - r_H^3};$$

$$\text{also, } \frac{V_H}{V_E} = \frac{m_H d_E}{m_E d_H}$$

where the ratio of masses $m_H/m_E = 193$ (corrected for fraction of emulsan bound) and the densities of emulsan and hexadecane are 1.404 and 0.773 g/cm³, respectively.¹⁴

$$\frac{V_H}{V_E} = 350.5 = \frac{r_H^3}{6.86 - r_H^3}$$

or $r_H = 1.898$ microns and the thickness of the emulsan film, $r_T - r_H$, equals 0.002 microns or 20 Å. In the same way, for a weight ratio of hexadecane to emulsan of 39 which gave a mean droplet diameter of 2.0 microns, it was found that the film thickness was 42 Å. These values are minimum estimates since they assume the tightest possible packing of the emulsan molecules. The data are consistent with early results showing that polysaccharides tend to lie flat on the oil-water interface.¹ In general, coalescence is inversely related to the thickness of the film formed on the droplet; strong polymeric films with a thickness greater than 5 Å have been shown to prevent coalescence.³

Hexadecane droplets stabilized with emulsan can be separated from the bulk water phase by centrifugation. We refer to this cream phase, consisting of small hydrocarbon droplets coated with emulsan and water, as "emulsanosol." With a weight ratio of hexadecane to emulsan of 155, the resulting emulsanosol contained 30-50 percent water. Not surprisingly, emulsanosols have properties characteristic of neither the pure hydrocarbon nor aqueous solutions of emulsan. One such interesting property is the ability to bind and thereby concentrate the water-soluble dye Rhodamine B. At saturating concentrations of the dye, 2.2 μmoles were bound per mg emulsan in the emulsanosol. Since emulsan contains only 1.5 micro equivalents of carboxyl residues per mg,¹⁴ simple ionic binding between the tertiary amine groups of Rhodamine and the carboxyl groups could not explain the phenomenon. Rather, a particular conformation of emulsan on the interfacial film in contact with the hydrocarbon may better explain the binding properties of emulsanosols.

The fact that the anionic nature of emulsan also plays a crucial role in binding Rhodamine can be inferred from the observation that protonation of the carboxyl group of emulsan (pK = 3.05) blocked dye binding.

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REFERENCES

1. Adam, N. K. 1933. Surface films of cellulose derivatives on aqueous solutions. *Trans. Faraday Soc.* 29: 90-106.
2. Belsky, I., D. L. Gutnick, and E. Rosenberg. 1979. Emulsifier of *Arthrobacter* RAG-1: Determination of emulsifier-bound fatty acids. *FEBS Letters* 101:175-178.
3. Biswas, B., and D. A. Haydon. 1962. Coalescence of droplets stabilized by viscoelastic adsorbed films. *Koll. Z. und Zeitschr. fur Polymere* 1262, 185: 31-38.
4. Clark, M. F., and A. N. Adams. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virology*, 34: 475-483.
5. Erickson, L. E., and T. Nakahara. 1975. Growth in cultures with two liquid phases: hydrocarbon uptake and transport. *Proceed. Biochem.*, 10: 9-13.
6. Gutnick, D. L., and E. Rosenberg. 1977. Oil tankers and pollution: a microbiological approach. *Ann. Rev. Microbiol.* 31: 379-396.
7. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 193: 265-275.
8. Morris, E. R. 1977. Molecular origin of xanthan solution properties. In: *Extracellular Microbial Polysaccharides* (P. A. Sandford and A. Laskin, Eds.), American Chem. Soc. Symp. 45, Washington, D.C., pp. 81-89.
9. Rosenberg, E., A. Perry, D. T. Gibson, and D. L. Gutnick. 1979. Emulsification of *Arthrobacter* RAG-1: Specificity of hydrocarbon substrate. *Appl. Environ. Microbiol.*, 37: 409-413.
10. Rosenberg, E., A. Zuckerberg, H. Rubinowitz, and D. L. Gutnick. 1979. Emulsifier of *Arthrobacter* RAG-1: Isolation and emulsifying properties. *Appl. Environ. Microbiol.*, 37: 402-408.
11. Shaw, D. J. 1970. In: *Introduction to Colloid and Surface Chemistry* (Butterworths, London, second edition), pp. 120-132.
12. Singleterry, C. R., and L. A. Weinberger. 1951. The size of soap micelles in benzene from osmotic pressure and from the depolarization of fluorescence. *J. Amer. Chem. Soc.*, 73: 4574-4579.
13. Zajic, J. E., and C. J. Panchal. 1976. Bioemulsifiers. *CRC Crit. Rev. Microbiol.* 5: 39-66.
14. Zuckerberg, A., A. Diver, Z. Peeri, D. L. Gutnick, and E. Rosenberg. 1979. Emulsifier of *Arthrobacter* RAG-1: chemical and physical properties. *Appl. Environ. Microbiol.* 37: 414-420.

Growth and Biosurfactant Production of a Bacteria in Continuous Culture

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In applications of microbiology, the most distinctive element is usually the biological one: the exploitation of a living organism for the manufacture of a useful substance. The methods of genetic engineering promise to increase the efficiency and the versatility of the organisms even further. It must be kept in mind, however, that a biological process can attain its full utility only when it is adapted to a context of production.

A certain environment needs to be maintained in a reactor in order to favor the desired biochemical transformations of the raw material. It follows that the organism with its biological potential is only one factor in the success of a process; the contribution of process engineering is also essential. In relation to biosurfactant production, the ultimate aim is a maximum con-

version of the carbon source into the surface-active compound.

The organism—The bacterial strain Rsan ver (*Pseudomonas sp.*) was isolated from soil samples from the vicinity of an oil refinery. Table 1 shows some of the substrates assimilated by the strain. The most important property of this isolate was that it produced surface-active compounds equally well when growing on the expense of glucose or hydrocarbon. The production of biosurfactants from carbohydrate substrates offers some advantages as compared to hydrocarbon substrates. From an engineering point of view, hydrocarbon substrates require more sophisticated equipment and more power input in order to achieve an adequate dispersion of the insoluble hydrocarbons. Biologically, carbohydrate-grown cells are easier to handle. These were the principal reasons for the further consideration of Rsan ver in biosurfactant production.

The bioreactor—A major problem in cultures with a biosurfactant producing microorganism is the exces-

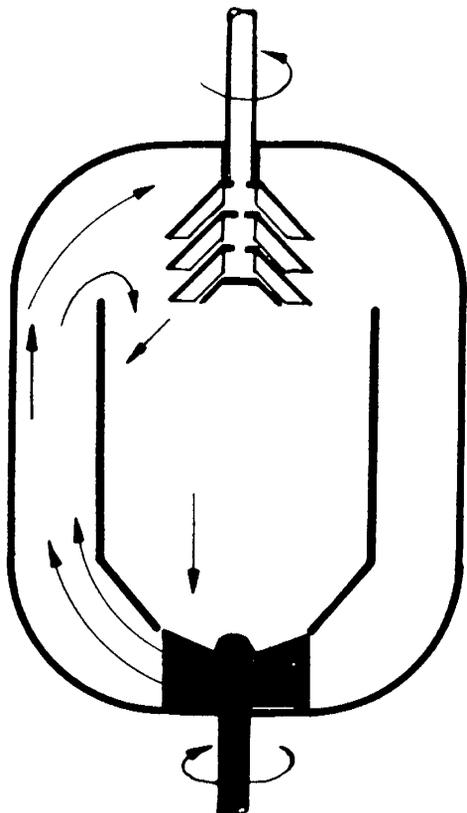


FIG. 1—Basic design of a completely filled bioreactor. The construction consists of a stirrer, a foam separator and a draft tube. The arrows in the reactor mark the circulation of the culture liquid.

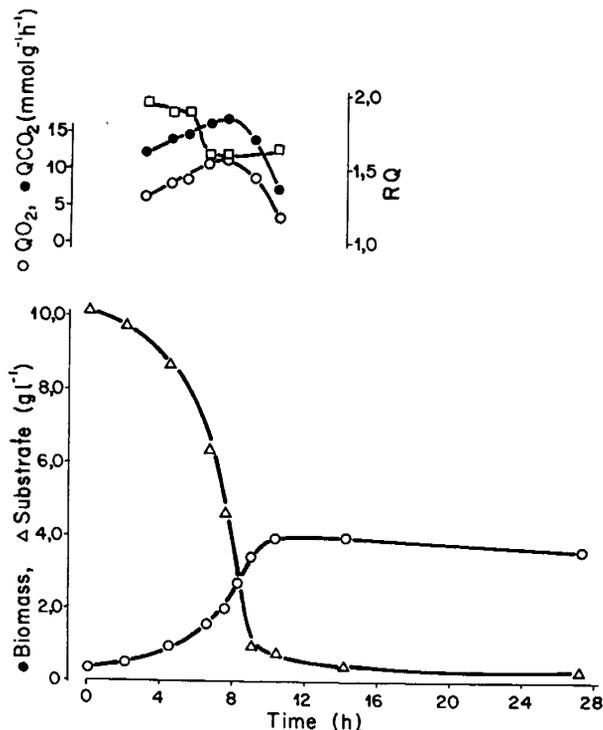


FIG. 2—Growth of Rsan ver in batch culture with glucose as the carbon source.

sive foam formation. With the conventional flat blade turbine, the cultivation of *Rsan ver* could not be carried out because of foam formation. With the completely filled bioreactor (Fig. 1) where a foam separator belongs to the standard equipment, it was possible to carry out the culture of the biosurfactant producing organism.

Batch culture—The growth of *Rsan ver* is shown in Fig. 2. Glucose is used with the concomitant formation of biomass. The maximal growth rate was approximately 0.35 h^{-1} . The O_2 uptake rate was between 3.5 and $11.3 \text{ mmol g}^{-1} \text{ h}^{-1}$ and the CO_2 production rate in the range of 7.5 and $16.8 \text{ mmol g}^{-1} \text{ h}^{-1}$ yielding RQ values between 1.5 and 2.0. The yield of biomass with respect to glucose was at 40 percent relatively low. When the culture was left for a further 12 h, after exhaustion of glucose the biomass decreased only slightly, indicating a slow autolysis of the cells.

Surface tension, interfacial tension, reciprocal CMC—When the medium was inoculated, there was an immediate drop in surface tension from the value of water (72 mNm^{-1}) to approximately 32 mNm^{-1} . The interfacial tension and the surface tension remained low during the whole cultivation time (Fig. 3). The course of the reciprocal CMC indicates that surface active compounds are formed during the growth of *Rsan ver*. It is low after inoculation, which is a consequence of the diluting effect accompanying the inoculation. During the growth it increases steadily and reaches a maximum when the maximal biomass concentration is reached. It is remarkable that the reciprocal CMC drops only slightly when the cells are kept in the bioreactor after glucose is consumed completely. This indicates that the produced biosurfactant is not used by the cells under starving conditions. The reciprocal CMC is relatively low, but one has to consider that only 1 percent glucose was used as a carbon source.

Carbon recovery in the spent medium—From the culture liquid, the cells were separated and the distribution of carbon determined by total carbon analy-

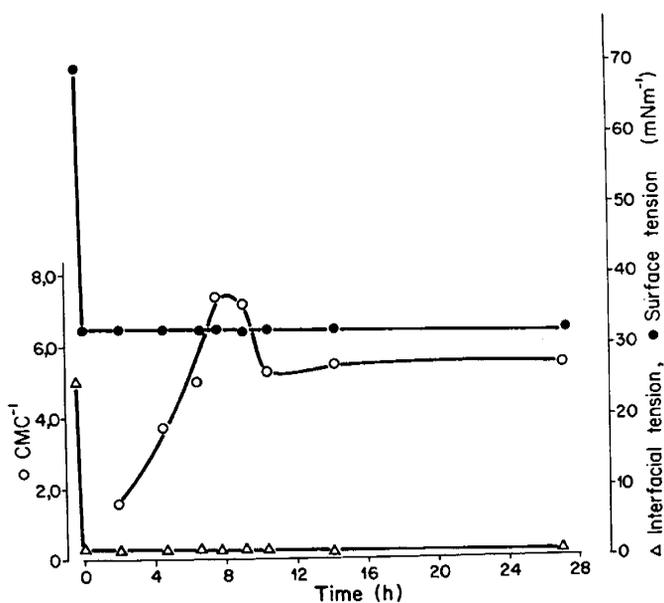


FIG. 3—Development of surface tension, interfacial tension and reciprocal CMC during the growth of *Rsan ver* in batch culture.

TABLE 1—Properties of the Organism Used for Biosurfactant Production

Substrate	Growth	Surface-Active Compounds
Glucose.....	+	+
Hydrocarbon	+	+
Acetate.....	+	n.d.
Citrate	+	n.d.

n.d.: not determined

TABLE 2—Growth Parameters and Properties of the Culture Liquid Relevant to Surfactant Activity as a Function of Dilution Rate

D (h ⁻¹)	X (g l ⁻¹)	S (g l ⁻¹)	QO ₂ QCO ₂		RQ (-)	ST (mN m ⁻¹)	IT (mN m ⁻¹)	CMC ⁻¹ (-)	C _{prod} (g l ⁻¹)
			(mmole g ⁻¹ h ⁻¹)	(mmole g ⁻¹ h ⁻¹)					
0.16	1.71	0.16	9.0	15.1	1.68	44	13	1	0.02
0.19	1.71	0.13	13.6	19.0	1.40	42	12.5	1	0.01
0.22	1.68	0.12	16.0	22.2	1.38	45	14.0	1	0.03
0.25	1.81	0.26	14.6	20.7	1.42	35	5.5	3.0	0.17
0.28	1.81	0.74	15.3	22.9	1.50	35	5.5	4.2	0.19

ST, surface tension; IT, interfacial tension; CMC⁻¹, reciprocal critical micelle concentration; X, biomass; S, substrate; QO₂ and QCO₂, oxygen uptake rate and CO₂ production rate respectively; RQ, respiratory quotient; D, dilution rate; C_{prod}, carbon in product.

sis (Fig. 4). The curve of the total carbon in the medium versus time declined more slowly than that of the carbon in glucose. This indicates that a product is formed by the cells. After having reached a peak at the end of growth, it amounts to about 15 percent of the total carbon present at the beginning of the culture. These observations are in accordance with the relatively low yield in biomass mentioned above.

Continuous culture—The growth experiments were expanded to continuous culture in order to investigate the kinetics of growth and biosurfactant production. The results are summarized in Table 2. Regarding the results of the continuous culture, it becomes obvious that the surfactant production was practically absent at low dilution rates. The surface tension and the interfacial tension were lowered to 42 to 45 mNm⁻¹

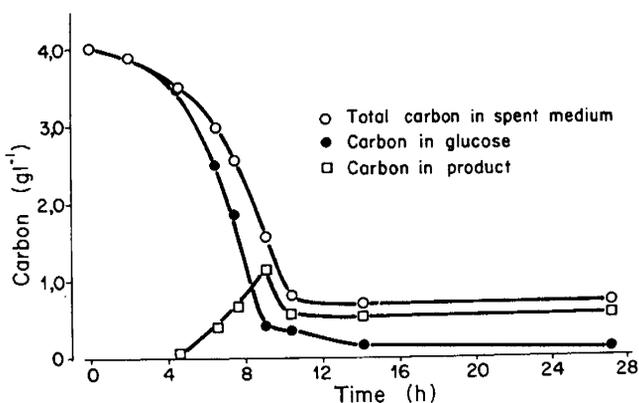


FIG. 4—Distribution of carbon in the spent medium of *Rsan ver* during growth in batch culture.

and 12 to 14 mNm^{-1} respectively with the spent medium of the continuous culture, as compared to approximately 30 mNm^{-1} and 2 mNm^{-1} in the batch culture. The batch results were only approximated when the dilution rate approached the maximum growth rate from the batch culture, which was 0.35 h^{-1} . It is also

noteworthy that only 5 percent of the carbon of the substrate was converted to the product, which is again significantly lower than the batch culture (Fig. 4). These results form the basis for subsequent work in which a maximal productivity of biosurfactant is the aim.

The Effect of HLB on the Surface Activity and Bitumen Extraction Capability of *Corynebacterium Fascians*

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ABSTRACT

Synthetic nonionic surfactants (Pluronic) were added to the growth media of *Corynebacterium fascians*. The effect of surfactant hydrophile-lipophile balance (HLB) on bitumen recovery and surface activity of *C. fascians* was examined. The highest total bitumen recovery by hexadecane grown bacteria occurred at HLB values of 5.5 and 16. Hydrophobic values (HLB 0.5 to 11) stimulated the growth and surface activity of *C. fascians* grown on water soluble substrates such as sucrose. HLB values of 0.5, 16, and 29 improved the emulsification properties of the sucrose grown bacteria.

Membrane filtrates of culture broths to which the surfactant L92 had been added showed that the bacterial cells per se contribute highly to the total surface activity of the culture. Combination of substrates such as sucrose and glycerol did not appear to affect the growth or surface activity of bacteria, although increasing the glycerol concentration improved bacterial emulsification of kerosene and water.

INTRODUCTION

Surface active agents, or surfactants, added to injection waters in oil bearing formations facilitate several enhanced oil recovery methods. Surfactants assist in the release of oil from the rock pores by lowering the oil-water interfacial tension with respect to the oil-mineral interfacial tension. Thus, the surface activity displayed by a microbial culture is a primary consideration when assessing a microorganism for use in enhanced oil recovery. The surface activity observed in cultures may be caused by biosurfactants synthesized by microorganisms¹ or by the surface behavior of the microbial cell.^{2,3}

Biosurfactants have several distinct advantages over synthetic surfactants in the context of enhanced oil recovery. Biosurfactants have been shown to resist the adverse effects of divalent cations (eg: Ca^{2+} and Mg^{2+}) and sodium chloride which are present in reservoir waters.^{4,5} In addition, biosurfactants are potentially less toxic and are generally biodegradable.^{4,6} These are important considerations since they limit ground water pollution.

Cultures of *Corynebacterium fascians* have been shown to display considerable surface activity and emulsification and demulsification ability when grown on hydrocarbons.⁷ Low surface and interfacial tensions and high reciprocal critical micelle concentra-

tion⁷ (a measure of surfactant concentration) indicate that *C. fascians* is a good candidate for use in enhanced oil recovery.

It is also known that the presence of nonionic surfactants influences the ability of microorganisms to utilize hydrophobic substrates such as paraffinic hydrocarbons⁸ and even the insecticide 2,4-D.⁹ Studies of surfactant properties have indicated that the influence is dependent on the hydrophile-lipophile balance (HLB) of the added surfactants.^{8,9} HLB is an empirical index running from 0.1-40 characterizing the relative hydrophilicity and hydrophobicity of a surfactant.¹⁰ Low numbers reflect hydrophobic surfactants while high numbers reflect hydrophilic surfactants. The HLB of nonionic surfactants can also influence the physical deemulsification of heavy oil, water, and clay mixtures.¹¹

The experiments described in this paper examine the effect of surfactant HLB on the surface activity and bitumen recovery capability of *C. fascians*. Bacteria were grown in the presence of nonionic polyol (Pluronic) surfactants ranging in HLB from 0.5 (most hydrophobic) to 29 (most hydrophilic). The effect of HLB on bitumen extraction, growth and surface activity was studied using hexadecane as the sole carbon and energy source for the bacteria. Surface activity, growth, and emulsion characteristics were also studied using sucrose and the sole carbon and energy source.

Hexadecane was replaced by sucrose in this case in order to investigate the effects of HLB on *C. fascians*. The presence of the water insoluble substrate, hexadecane, adds a mass transfer challenge (i.e.: hexadecane emulsification)¹² to the fermentation system which may obscure cellular HLB effects. Also included are experiments examining the effect of varying two hydrophilic substrates (a carbohydrate and a polyalcohol), sucrose and glycerol, on the growth, surface activity and emulsion properties of *C. fascians*.

MATERIALS AND METHODS

The microorganism chosen for these studies, *C. fascians* ICPB CF 15, was obtained from the International Collection of Phytopathogenic Bacteria. The tests described herein were performed with both *C. fascians* cultures and uninoculated media.

Inocula of 2 percent (v/v) of *C. fascians* broth cul-

tures were added to 100 ml of a mineral salts basal media using 4 percent (w/v) of hexadecane, sucrose, or glycerol as carbon sources. The mineral salts basal media consisted of NH₄NO₃, 4 g/l; KH₂PO₄, 4 g/l; Na₂HPO₄, 6 g/l; MgSO₄, 0.2 g/l; CaCl₂ 2H₂O, 0.001 g/l; FeSO₄ 7H₂O, 0.001 g/l; Na₂EDTA, 0.0014 g/l; yeast extract (Difco), 1 g/l; and one liter of deionized water (pH 7.0). In the bitumen recovery experiments, hexadecane was the sole carbon source.

In further HLB experiments, sucrose was used. In substrate variation experiments, sucrose, glycerol, and mixtures of both were used. Pluronic polyol nonionic surfactants (Wyandotte) were added to hexadecane containing media at concentrations of 0.1 percent (w/v). In sucrose containing media, the same surfactants were added at 0.05 percent (w/v) concentrations. The surfactants used were the Pluronics L121, L92, L63, L62, L44, and F68, having respectively HLB values of 0.5, 5.5, 7, 11, 16 and 29.

All cultures of *C. fascians* and control media were incubated in 500 ml shake flasks at 25°C for 48 h, shaken at 200 rpm. Biomass was determined by centrifuging 10 ml of the 48 hour culture at 5°C and 15,000 rpm for 10 min. Sedimented cells were resuspended in 10 ml of 0.85 percent (w/v) saline and recentrifuged to wash the cells. The washed cells were resuspended in 5 ml of water, transferred to pre-weighed pans, and dried in an oven (100°C) for 18–24 h. Biomass was then determined as dry weight. pH of media was determined at the termination of each experiment.

Bitumen extraction tests were performed in a low shear extraction model.⁶ The extraction system consisted of 4 g of Utah tar sands (Triangle Area) and 50 ml of deionized water in a 500 ml shake flask. To this flask was added 8 ml of hexadecane grown *C. fascians* culture or 8 ml of control medium. Incubation proceeded, as described above, and resulted in the separation of bitumen into four fractions. Fraction one was the surface oil, collected by skimming the liquid surface with silicone coated glass fiber (Prosil).

Fraction 2 was reduced tar sand, also called enriched tar balls, collected by straining the liquid through a 40 mesh brass screen. Fraction 3 was the remaining sand and clay, collected on Whatman No. 41 filter paper. Fraction 4, the emulsified bitumen remaining in the filtrate, was extracted with toluene. The bitumen content of each fraction was determined by extracting with toluene, evaporating the toluene at 92°C, and measuring the remaining bitumen as dry weight.

The surface activity of the cultures and control media was determined by use of a duNuoy type tensiometer (Fisher). The surface activity measured includes surface tension, interfacial tension against hexadecane, and CMC⁻¹. The critical micelle concentrations (CMC) were determined by monitoring the decrease in surface tension as a function of increasing culture or control medium concentration. The point at which no further decrease in surface tension can be induced by increasing concentration is the CMC. The reciprocal form, CMC⁻¹, is reported and is used to represent the relative concentration of surfactant.

Emulsion formation and the property of demulsification by *C. fascians* cultures and control media were measured by determining emulsion decay half lives

(t_{1/2}). Two kinds of kinetics, so-called fast and slow kinetics, are observed in emulsion breakage. Only the slow kinetic half lives are reported herein; i.e., only that demulsification taking place after 30 min. The expression:

$$t_{1/2} = \frac{-\log 2}{d(\log \text{ percent Emulsion}) / dt} \quad (\text{hrs})$$

is used to determine half lives (t_{1/2}) assuming first order decay kinetics.

Emulsification tests were prepared by adding 1 ml of culture or control medium to a test tube containing 4 ml of water and 5 ml of kerosine. The contents were then vortexed at speed 6 for 60 sec. Demulsification tests were conducted using 4.5 ml of water, 0.5 ml of 1 percent (w/v) Pluronic P104 surfactant and 5 ml of kerosine in a test tube. Aliquots of 1 ml of culture or control medium were tested as demulsificants and the contents are vortexed as described above.

Cells were removed and filtrates prepared using 0.45μ membrane filters and Millipore apparatus coupled to 500 ml suction flasks. The viscosity of cultures and control media was measured with a Brookfield viscometer at 16°C and 100 rpm using a No. 2 spindle.

RESULTS

The total bitumen separation from 4 g samples of Utah tar sands by cultures of *C. fascians* peaked at HLB 5.5 and 16, respectively 166 mg and 149 mg (Table 1). The amounts of bitumen extracted should be compared to the amount of bitumen present in 4 g of Utah tar sands as determined by pentane and heptane extractions. This amounted to 200 mg of bitumen. The peak oil recovery observed at HLB 5.5 and 16 correlates well with maximum biomass production at HLB 5.5 and 16. Maximum biosurfactant production as reflected by CMC⁻¹ values for bacterial cultures were also observed at HLB 5.5 and 16 (Table 2). One should note that the uninoculated control of HLB 11 gave excellent extraction values of bitumen.

Growth of *C. fascians* on hexadecane gave fairly uniform reductions in surface tension regardless of HLB. In most instances the surface tension was approximately 30 mN/n. The interfacial tension against

TABLE 1—Extraction of Bitumen from Utah Tar Sands by *C. fascians* Cultures and Control Media as a Function of HLB.

HLB	Wt Bitumen Recovered by							
	<i>C. fascians</i> Cultures				Uninoculated Control Medium			
	Surface Oil	Reduced Tar Sand	Emulsified Bitumen	Total	Surface Oil	Reduced Tar Sand	Emulsified Bitumen	Total
mg	mg	mg	mg	mg	mg	mg	mg	mg
0.5	79	15	4	98	66	24	0	90
5.5	78	72	16	166	96	8	2	106
11.0	56	42	19	117	145	21	4	170
16.0	74	74	1	149	42	25	1	68
29.0	52	20	1	73	44	26	2	72

TABLE 2—The Effect of HLB on the Surface Activity of *C. fascians* Cultures and Control Media

HLB	Surface Activity of					
	<i>C. fascians</i> Cultures			Control Media		
	γ_s (mN/m)	γ_i (mN/m)	CMC ⁻¹	γ_s (mN/m)	γ_i (mN/m)	CMC ⁻¹
0.5	30.0	1.8	1	28.5	1.7	1
5.5	30.6	1.2	15	33.5	5.1	20
11.0	36.2	8.7	1	37.0	9.0	1
16.0	29.6	2.2	55	44.6	15.5	5
29.0	30.4	1.9	5	46.4	16.5	5

Surface tension is γ_s and interfacial tension is γ_i (measured against hexadecane).

TABLE 3—The Effect of L92 Surfactant on the Surface Activity of Whole Cultures

	Sample	γ_s (mN/m)	γ_i (mN/m)	CMC ⁻¹	pH	Biomass
						(g/100 ml)
Culture	Whole	38.8	4.2	157.0	5.5	0.5713
Broth+	Filtrate	40.4	5.4	1.9	5.4	—
	Control	39.8	5.3	17.8	6.4	—
Culture	Whole	61.1	20.2	1	5.7	0.4683
Broth, no	Filtrate	66.9	26.5	1	5.7	—
	Control	65.9	30.1	1	6.5	—

Cultures (Whole), culture filtrates (Filtrate), and uninoculated control media (Control). Surfactant containing cultures are compared to surfactant free cultures.

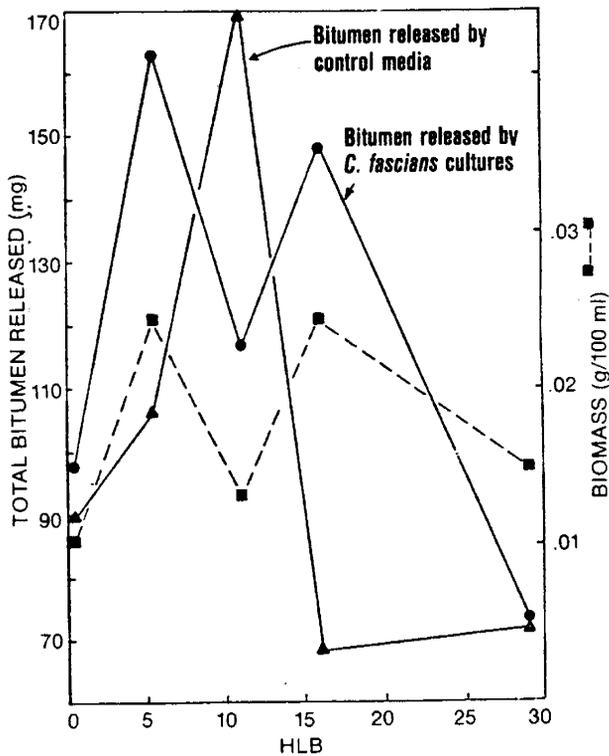


FIG. 1—The effect of HLB of the medium on the recovery of bitumen from Utah tar sands by *Corynebacterium fascians*.

hexadecane ranged from 1.2–2.2 mN/m with a slightly higher value observed at an HLB 11. It is important to compare the uninoculated medium controls with the production flasks in Table 2. In general *C. fascians* tends to reduce γ_s and γ_i ; however, increased growth does not always show increased levels of surfactant as reflected by CMC⁻¹.

The formation of biosurfactants by microbes is in general believed to be induced by the addition of an insoluble hydrocarbon substrate. Most microbes producing biosurfactants grow on both hydrocarbon and carbohydrate substrates. Very few studies report on biosurfactant production from carbohydrates. Thus a comparison was made of the surface properties of broths of *C. fascians* which had been grown upon either hexadecane or sucrose and/or glycerol. This study included examining the effect of HLB upon cells grown upon carbohydrate containing media.

Culture broths prepared by growing *C. fascians* on sucrose in the low HLB ranges of .5–10 were far better in giving systems which produced the lowest surface and interfacial tensions (Fig. 1). The lowest surface and interfacial tensions were observed at HLB's of 0.5 and 5.5. Microbial growth of *C. fascians* on sucrose at these low HLB values tended to increase the γ_s and γ_i over the controls. This was not true for culture broths receiving Pluronic at HLB 7 or greater. At the higher HLB's the surfactants induce cells to produce components which reduced surface and interfacial tensions below control values. Thus in *C. fascians* the biosurfactant property appears to be induced both by hydrocarbons and surfactants (Fig. 1).

Several other growth parameters were also measured. Biomass production was far greater for *C. fascians* at low HLB's (.77 g/100 at HLB of 0.5). In general the higher the HLB, the poorer the growth (Fig. 2). The terminal pH of the broths tended to increase with HLB.

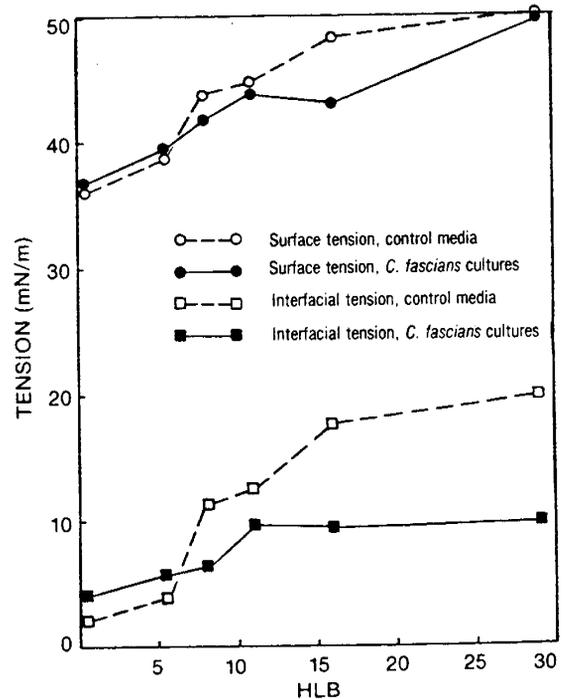


FIG. 2—The effect of HLB of the medium on the surface and interfacial tensions of cultures of *C. fascians* and medium controls.

At an HLB of 5.5 the terminal pH of the broth was 5.3, whereas at HLB of 29 the terminal pH was 5.8. At HLB values of the media of 0.5-11 the amount of biosurfactant produced was far greater than at HLB's > 15 (Fig. 3). This is reflected in the reciprocal CMC values shown in Fig. 3. Emulsification and demulsification were determined at each HLB (Fig. 4). Both were evaluated by examining emulsion stability which was expressed in terms of $t_{1/2}$ (half-life, h).

Demulsification was greatly improved by *C. fascians* (Fig. 4) at all HLB's. The best demulsification was achieved at HLB 11 which gave a $t_{1/2}$ of 8 h. Emulsion stability was improved by *C. fascians* over media controls, with or without added surfactant. Culture emulsification was especially improved at high HLB's (>15) while a decrease was especially noted at HLB of 5.5. As one would expect if the system is a good demulsificant, its properties of emulsification are limited.

C. fascians was cultivated on mixtures of sucrose and glycerol as shown in Fig. 5. As the weight to volume ratio of sucrose and glycerol shift from a predominance of sucrose to a predominance of glycerol, the γ_s and γ_i values tend to pass through a minima at 3 percent sucrose and 1 percent glycerol to a slight maxima at 1 percent sucrose and 3 percent glycerol. In general, mixtures of these substrates do not contribute to the production of surface active properties by *C. fascians*. Cells of *C. fascians* were cultured on sucrose both in the presence and absence of L92 surfactant.

Under these growth conditions cells cultivated in the presence of L92 are far more surface active. This is reflected by a CMC⁻¹ of 197 and a low γ_i of 4.2 mN/m (Table 3). When these cells are removed by filtration the γ_i increase to 5.4 mN/m and the CMC⁻¹ decreased to 1.9. In culture broths to which no L92 was added the γ_i of the whole broth was 20.2 mN/m. Other surface properties of cells and broth in these systems were uninteresting.

DISCUSSION AND SUMMARY

In evaluating broth of *C. fascians* in the cold water extraction of bitumen from Utah tar sands, the best extractions were achieved at HLB 5.5 and 16. These results may be related to the required HLB number for emulsification of the oil phase. For kerosine the required HLB number to form stable w/o emulsions is 6, and for o/w emulsions the required HLB number is 12 (reference 14). For mineral oil (paraffinic) the numbers are 6 and 10 respectively. The best extraction at HLB values of 5.5 and 16 are thus typical of the required HLB for emulsifying an oil phase.

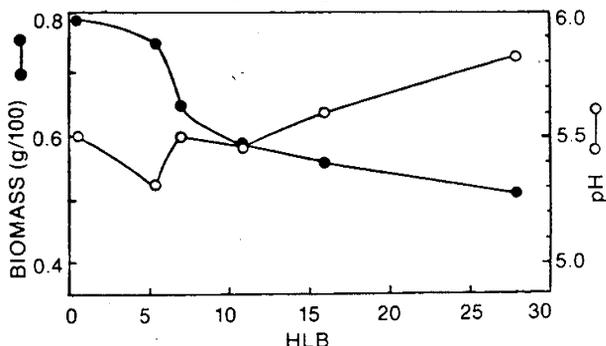


FIG. 3—The effect of HLB of the medium on biomass and change in pH in a sucrose medium inoculated with *C. fascians*.

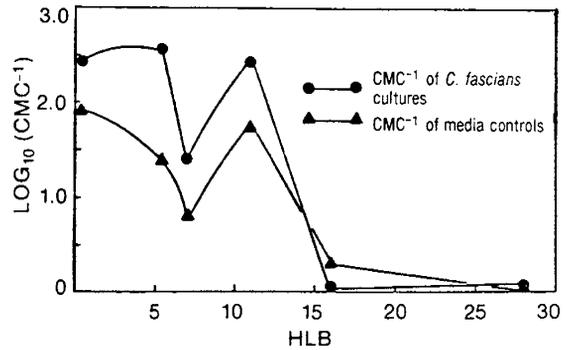


FIG. 4—The effect of HLB of the medium on the reciprocal critical micelle concentration of *C. fascians* cultivated on a sucrose medium.

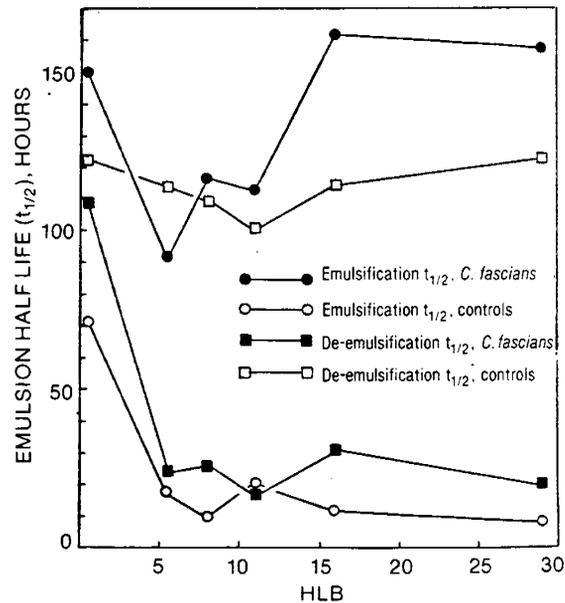


FIG. 5—The effect of HLB on the emulsification and de-emulsification properties of *C. fascians* cultivated on a sucrose medium.

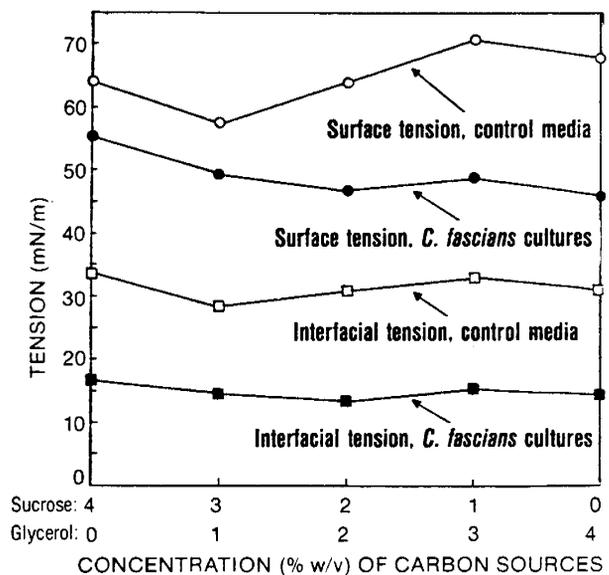


FIG. 6—The effect of various weight percent concentrations of sucrose and glycerol upon surface and interfacial tension changes in growth media inoculated with *C. fascians*.

At HLB 11, the extraction of bitumen was much higher with the growth medium alone than with culture broth. *C. fascians* cultured in the presence of Pluronic surfactants with HLB's from 0.5 to 29 showed that surfactants did in general reduce surface tension except at HLB's of 0.5 and 5.5. However, at the low HLB's more surfactant was synthesized than at higher HLB's. Biomass production was favorable over an HLB range of 0.5 to 11 with the lowest biomass produced at an HLB of 29. The property of demulsification was favored at HLB's of 5.5, 7.0 and 11.

Cells of *C. fascians* did not have to be cultivated on hexadecane to produce cells with surface active properties. It was shown that cells cultivated in sucrose in the presence of Pluronic surfactants were very surface active. Thus it would appear that surface active agents can have a profound effect upon both the ability of cells to produce biosurfactants as well as the HLB properties of the cell surface per se.

ACKNOWLEDGMENT

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NOMENCLATURE

- CMC = Critical micelle concentration
 CMC⁻¹ = Reciprocal critical micelle concentration
 γ_s = Surface tension (liquid-vapor interface)
 γ_i = Interfacial tension (aqueous-hexadecane interface)
 HLB = Hydrophile-lipophile balance
 μ = Viscosity
 t = Time (hours)
 $t_{1/2}$ = Emulsion decay half-life

$$= \frac{\log 2}{d(\log \text{ percent Emulsion}) / dt}$$

REFERENCES

1. Cooper, D. B., Zajic, J. E., "Surface Active Compounds from Microorganisms," *Adv. App. Mic.* 26, 229-253 (1980).

2. Marshall, K. C., Cruickshank, R. H., "Cell Surface Hydrophobicity and Orientation of Certain Bacteria at Interfaces," *Arch. Mikrobiol.* 19, 29-40 (1973).

3. Neufeld, R. J., Zajic, J. E., Gerson, D. F., "Cell Surface Measurements in Hydrocarbon and Carbohydrate Fermentations," *Appl. Env. Mic.* 39, 511-517 (1980).

4. Zajic, J. E., Guignard, H., Gerson, D. F., "Emulsifying and Surface Active Agents from *Corynebacterium hydrocarboclastus*," *Biotech. Bioeng.* 19, 1303-1321 (1977).

5. Bubela, B., "Role of Geomicrobiology in Enhanced Recovery of Oil, Status Quo," *APEA J.* 18 (1), 161-166 (1978).

6. Zajic, J. E., Gerson, D. F., "Microbial Extraction of Bitumen from Athabasca Oil Sand," *ACS Preprints* 22(3), 195-203 (1978).

7. Akit, J., "Investigation of Potential Biosurfactant Production Among Phytopathogenic *Corynebacteria* and Related Soil Microbes," *Curr. Mic.* 6, 145-150 (1981).

8. Lupton, F. S., Marshall, K. C., "Effectiveness of Surfactants in the Microbial Degradation of Oil," *Geomic. J.* 1(3), 235-247 (1979).

9. Amonette, J., O'Connor, G. A., "Nonionic Surfactant Effects on Absorption and Degradation of 2,4-D," *Soil Sci. Soc. Am. J.* 44(3), 540-544 (1980).

10. Osipow, L. I., *Surface Chemistry*, ACS Monograph Series, Reinhold, New York, pp. 311-314 (1962).

11. Cooper, D. G., Zajic, J. E., Cannel, E. J., Wood, J. W., "The Relevance of 'HLB' to De-Emulsification of a Mixture of Heavy Oil, Water and Clay," *Can. J. Chem. Eng.* 58, 576-579 (1980).

12. Gutierrez, J. R., Erickson, L. E., "Hydrocarbon Uptake in Hydrocarbon Fermentations," *Biotech. Bioeng.* 19, 1331-1349 (1977).

13. "The Wonderful World of Pluronic Polyols," publ. of BASF, Wyandotte Corp. (1973).

14. "The HLB System," publ. of ICI Americas, Inc. (1980).

Adherence of Bacteria to Hydrocarbons

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ABSTRACT

Two general types of interaction between microorganisms and hydrocarbons have been postulated: (A) adherence of cells directly to large oil droplets and (B) release of extracellular surfactants or emulsifiers that greatly increase the hydrocarbon:aqueous interfacial area. In the case of the hydrocarbon-degrading *Acinetobacter calcoaceticus* RAG-1 both types of interaction are operative; cells adhere avidly to test hydrocarbons (xylene, octane, hexadecane and crude oil) and also produce a potent polyanionic emulsifier referred to as emulsan. Mutants of *A. calcoaceticus* RAG-1 deficient in emulsan synthesis are still able to adhere to hydrocarbons and grow on hexadecane or crude oil as the sole source of carbon and energy. However, mutants of *A. calcoaceticus* RAG-1 unable to adhere to hydrocarbons also failed to grow on hydrocarbon substrates.

It was demonstrated that adherence is a prerequisite for growth on hexadecane under two conditions: low initial cell density and limited emulsification of the substrate. Such conditions prevail in most natural environments. On the other hand, bioemulsification is a cell density dependent phenomenon. Relatively high cell densities are required to produce enough extracellular emulsifying agent to markedly affect the hydrocarbon substrate.

Adherence of microorganisms to hydrocarbons is neither an exclusive property of hydrocarbon-degrading microorganisms nor restricted to those hydrocarbons that the microorganism can metabolize. For example, *Staphylococcus aureus*, *Serratia marcescens* and *Streptococcus pyogenes* adhered avidly to test hydrocarbons as a result of their high cell surface hydrophobicity, but were unable to metabolize any of the hydrocarbon substrates tested. *A. calcoaceticus* RAG-1 can grow on alkanes, but not aromatics; however, it adhered equally well to both substances. Further, certain bacteria that have the genetic potential to degrade hydrocarbons, e.g., *Pseudomonas aeruginosa*, adhere poorly to hydrocarbons. It follows that introduction of hydrocarbon-degrading plasmids into microorganisms with low cell surface hydrophobicity may not lead to cells that interact well with hydrocarbons in open systems.

INTRODUCTION

The importance of cell adherence in growth physiology of many bacterial species is well documented. For example, *Caulobacter* cells divide only after they attach to solid surfaces.³⁶ The initial step in the life cycle of *Bdellovibrio* is attachment to Gram-negative bacteria.^{39,41} Specific cell-to-cell interactions pervade

the entire life cycle of the myxobacteria.^{8,38} Adhesion is a prerequisite to the successful colonization by microorganisms of animals²⁴ and plants.¹⁸ Microbial adhesion to surfaces has been the subject of several recent reviews.^{6,17,18,19,24}

In cases where the carbon or energy source is a water-insoluble material, such as cellulose, or chitin, bacterial adhesion can facilitate growth,^{17,38,40} but cell contact is not an absolute requirement because extracellular enzymes can degrade these polymers into water-soluble substrates. However, growth of microorganisms on hydrocarbons presents a special problem since not only are hydrocarbons immiscible with water, but also their breakdown cannot occur extracellularly. The first step in aromatic¹² or aliphatic²⁷ hydrocarbon degradation is introduction of molecular oxygen into the molecules by cell-associated enzymes.¹⁰

Two general types of hydrocarbon-cell interactions have been postulated, depending upon the state and size of the oil droplets relative to the size of the microbial cells:¹⁵ adherence of cells to large oil droplets and pseudosolubilization involving the cellular assimilation of emulsified small hydrocarbon droplets. The relative contribution of these two types of interactions to the growth of bacteria on hydrocarbon has been difficult to ascertain experimentally. The frequently observed relationship between hydrocarbon: aqueous interfacial area and growth rate can be used to support either hypothesis.²²

Adherence and growth of microorganisms on hydrocarbons is one example of a wide spectrum of phenomena in which bacterial hydrophobicity and adherence to hydrophobic surfaces are involved. The hydrophobic nature of the outermost bacterial surface has been cited as a factor in the partitioning of microorganisms at the air:water interface,^{4,19} in adherence of bacteria to non-wettable plastic surfaces,^{7,9,11,31} and in the attachment of bacteria to phagocytes⁴² and other mammalian cells.^{2,25,33,37}

We previously described a rapid and quantitative method for the measurement of bacterial adherence to hydrocarbons.^{32,34} The present report deals with the use of this technique in (1) examining the cell surface hydrophobicity of various bacterial species, some of which do not metabolize hydrocarbons, and (2) selecting and isolating non-adherent mutants. The isolation of a non-adherent mutant of the hydrocarbon-degrading bacterium *Acinetobacter calcoaceticus* RAG-1 has enabled us to examine the role of adherence in bacterial growth on hexadecane.

MATERIALS AND METHODS

Bacterial strains—Tables 1 and 2 contain a list of bacteria used in this study. *Acinetobacter calcoaceticus* MR-481 is a non-adhering derivative of strain RAG-1.

Media and growth conditions—For measuring adherence to test hydrocarbons or preparing inocula for growth experiments, 0.1 ml bacteria of an overnight culture were inoculated into 50 ml growth medium and incubated in a New Brunswick Model G-53 gyrotory shaker at 150 rpm at 30°C. Cells were harvested at the end of exponential phase, washed twice and resuspended in PUM buffer (22.2 g $K_2HPO_4 \cdot 3H_2O$; 7.26 g KH_2PO_4 ; 1.8 g urea, 0.2 g $MgSO_4 \cdot 7H_2O$, and distilled water to 1000 ml, pH 7.1). Growth media were: BHI—brain heart infusion broth (Difco); hexadecane medium—PUM buffer supplemented with 0.2 percent (v/v) hexadecane (olefin-free, 99 percent purity, Fluka, Switzerland). P-hexadecane medium—5 g NH_4NO_3 , 2.5 g K_2HPO_4 , 1 g $MgSO_4 \cdot 7H_2O$, 2 ml hexadecane, 50 mg threonine and tap-water to 1000 ml; NB—nutrient broth (Difco) supplemented with 0.5 percent NaCl. CT medium contained 1 percent casitone (Difco) and 0.2 percent $MgSO_4 \cdot 7H_2O$.²⁹

In the growth experiments, washed cells of RAG-1 and MR-481 were inoculated into acid-washed Klett tubes (14 mm diameter) containing 4.2 ml aqueous medium to give an initial turbidity of 5 Klett units. Acetate growth medium consisted of PUM buffer supplemented with 0.2 percent sodium acetate. Hexadecane growth medium consisted of 0.5 ml hexadecane (olefin-free, 99 percent purity, Fluka, Switzerland) layered onto 4.2 ml of PUM buffer. The test tubes were incubated upright in a New Brunswick gyrotory water bath shaker (Model G-76) at 330 rpm at 30°C. Turbidity was monitored directly in the growth tubes by means of a Klett-Summerson colorimeter fitted with a green filter.

Adherence to hydrocarbons—The technique for measuring adherence of bacteria to hydrocarbons has been described previously.²⁴ Cells were washed twice and resuspended in PUM buffer to an initial A_{400} of approximately 1.5. Various volumes of the test hydrocarbons (n-hexadecane, n-octane, or p-xylene) were added to round-bottom acid-washed test tubes (10 mm diameter) containing 1.2 ml of washed cell suspension and the phases were vortexed uniformly for 120 sec. After allowing 15 min for the phases to separate, the absorbance of the lower aqueous phase of each tube was measured at 400 nm (Gilford model 240 spectrophotometer, 1 cm light path). Adherence was calculated as the percentage loss in absorbance relative to that of the initial cell suspension.

Isolation of the non-adherent mutant MR-481—A spontaneous mutant of RAG-1 which is deficient in its ability to adhere to hydrocarbons was isolated by the following technique: RAG-1 cells were inoculated into 50 ml nutrient broth medium (NB) and incubated in a New Brunswick model G-53 gyrotory shaker at 150 rpm at 30°C. Cells were harvested in stationary phase following 18 hr growth, washed twice and resuspended in PUM buffer to an A_{400} of approximately 1.5. To 5 ml of the cell suspension were added 2 ml of n-octane, and the phases vortexed for 5 min. Following phase separation, the lower aqueous phase was transferred to a second test tube. Octane was again added and the phases

were mixed as described previously. This extraction process was continued until cells in the lower aqueous phase were too few to be visible under microscopic examination. This final aqueous phase served as the inoculum for a second nutrient broth culture which was grown to stationary phase as previously described. Cells were again harvested, washed and extracted with octane; the final aqueous phase was then inoculated into a third nutrient broth culture. After four such growth and extraction cycles, considerable turbidity remained in the aqueous phase, even following several extraction attempts with octane. The final aqueous phase was plated on nutrient agar, and three individual colony types were isolated and tested for adherence to hydrocarbons. One clone of cells, termed MR-481, exhibited no significant affinity towards the test hydrocarbons, and was chosen for further study.

Emulsan preparation—The general method for preparing the extracellular emulsifying agent of *A. calcoaceticus* RAG-1, referred to as emulsan, was described previously.³⁰ The particular preparation used in this study was further purified by precipitating an aqueous solution of emulsan with cetyltrimethylammonium bromide (BDH Chem. Ltd.), redissolving the precipitate in 0.5 M Na_2SO_4 , and dialyzing extensively against distilled water. The material was then deproteinized by the phenol method⁴⁵ and dialyzed. The final product had a reduced viscosity of 490 cc per g, an emulsifying activity of 150 units per mg, an ester content of 0.5 μ moles per mg and contained less than 0.5 percent protein. This emulsan preparation was dissolved in PUM buffer (2 mg/ml) and sterilized by autoclave prior to its addition in the growth experiments.

RESULTS

Bacterial adherence to hydrocarbons—The basic assay for bacterial adherence to hydrocarbons is illustrated in Fig. 1.

The assay was performed with varying volumes of 3 test hydrocarbons, n-hexadecane, n-octane and p-xylene. Following phase separation the turbidity of the lower aqueous phase was compared to that of the initial suspension. When the assay was performed with cells having no affinity for the test hydrocarbons (e.g. *E. coli*), the hydrocarbon droplets rose and coalesced soon after mixing and no significant changes were observed in the turbidity of the lower aqueous phase. However, when adherent cells were similarly tested (e.g., *A. calcoaceticus* RAG-1), a 'creamy' upper layer was obtained following mixing.

Microscopic observation of this layer revealed an oil-in-water emulsion consisting of hydrocarbon droplets, each covered by a layer of bacteria. The extraction of cells into this emulsion layer was accompanied by a corresponding decrease in turbidity of the lower aqueous phase. The fraction of adherent cells was determined by comparing the optical densities of the lower aqueous phase before and after mixing. Bacterial adherence was found to be a function of volume of hydrocarbon added. The cell-coated hydrocarbon droplets in the upper layer remained stable for a period of days. Addition of 5 percent isopropanol resulted in immediate droplet coalescence and the concomitant release of extracted cells into the aqueous phase, thereby demonstrating that the bacteria had partitioned to the hydrocarbon:water interface.

Tables 1 and 2 summarize a number of experiments in which cell surface hydrophobicity of a variety of bacteria was measured by adherence to hexadecane. The majority of laboratory strains examined exhibited low affinity towards the test hydrocarbon. Among the bacterial strains exhibiting high affinity for hexadecane were hydrocarbon-degrading *Acinetobacter calcoaceticus* strains RAG-1, HO1-N, B5W str 71 and CDC 7828, and bacteria which do not metabolize hydrocarbons: *Serratia marcescens*, *Staphylococcus aureus* and *Streptococcus pyogenes*.

Pseudomonas aeruginosa (which can utilize hexadecane as sole carbon and energy source) exhibited no significant affinity towards any of the test hydrocarbons when grown to late exponential phase in nutrient broth (Fig. 2). Since adherence in this organism might have required the presence of hydrocarbon during growth, *P. aeruginosa* cells were also assayed after growth on hexadecane (Fig. 2). The affinity of alkane-grown *P. aeruginosa* towards hexadecane did increase somewhat (e.g., 32 percent of such cells were removed from the aqueous phase in the presence of 0.2 ml hexadecane). In contrast, *A. calcoaceticus* RAG-1 exhibited

much higher affinity when grown on hexadecane (93 percent of the cells could be removed by as little as 0.05 ml hexadecane).

Role of adherence in the growth of *A. calcoaceticus* RAG-1 on hexadecane

Acinetobacter calcoaceticus RAG-1 adheres avidly to hydrocarbons.^{32,34} A spontaneous mutant of RAG-1, deficient in its ability to adhere to hydrocarbons, was selected by enriching for cells which did not adhere to octane. This mutant, referred to as MR-481, was unable to adhere to any of the three test hydrocarbons, even when grown under conditions for which RAG-1 adherence was optimal (Fig. 3).

Mutant MR-481 resembled the wild type in the following characteristics: (a) colonial and cell morphology, (b) sensitivity to two bacteriophages, ap2 and ap3²⁶ which are specific for *A. calcoaceticus* RAG-1 strains, (c) production of extracellular emulsifying activity during growth on ethanol as sole carbon and energy source,²² (d) doubling time on brain heart infusion broth (38 min at 30°C with aeration) and (e) agglutinability by antibodies raised against RAG-1 cells.¹

TABLE 1—Adherence of Bacteria to Hexadecane

Strain	Source	Growth Medium ^a	Adherence to Hexadecane, pct ^b
<i>Acinetobacter calcoaceticus</i> RAG-1	This laboratory	BHI	97
<i>Bacillus cereus</i>	Local isolate	NB	1
<i>Bacillus subtilis</i> 168	E. Z. Ron	NB	0
<i>Enterobacter aerogenes</i> CDC 659/66	E. Z. Ron	NB	9
<i>Escherichia coli</i> B	E. Z. Ron	NB	0
<i>Escherichia coli</i> K12	E. Z. Ron	NB	2
<i>Escherichia coli</i> J5 (rough)	E. Z. Ron	NB	11
<i>Micrococcus lysodeikticus</i> (ATCC 4698)	I. Friedberg	NB	0
<i>Myxococcus xanthus</i>	This laboratory	CT	35
<i>Proteus mirabilis</i> S1959	S. Rottem	NB	0
<i>Pseudomonas aeruginosa</i> PAS279	J. Shapiro	NB	0
<i>Serratia marcescens</i>	R. Zack	NB	96
<i>Staphylococcus albus</i>	R. Zack	NB	0
<i>Staphylococcus aureus</i>	R. Zack	NB	91
<i>Streptococcus pyogenes</i> M5	I. Ofek	BHI	84

^a Cells were harvested at the end of the exponential phase, washed twice and suspended in PUM buffer to an initial absorbance at 400 nm of 1.1-1.6.

^b Adherence to 0.2 ml hexadecane was performed as described in Materials and Methods.

TABLE 2—Adherence of *Acinetobacter* Strains to Hexadecane

Strain Designation	Source	Growth on Hexadecane ^a	Adherence to Hexadecane, pct ^b
RAG-1 (ATCC 31012)	This laboratory	+	82
HO1-N	W. R. Finnerty	+	99
BD4	E. Juni	+	4
BD413	E. Juni	+	0
B5W str 71 (ATCC 15149)	K. Bryn	+	84
CDC 7827 (ATCC 17959)	K. Bryn	+	86
B5W (ATCC 17903)	K. Bryn	-	0
ATCC 17906	K. Bryn	-	52
ATCC 17977	K. Bryn	-	7
ATCC 17985	K. Bryn	-	41

^a Growth was examined on PUM agar in the presence of hexadecane vapors.

^b Adherence was measured on cells grown to stationary phase on BHI broth; the standard adherence assay was performed with 0.05 ml hexadecane.

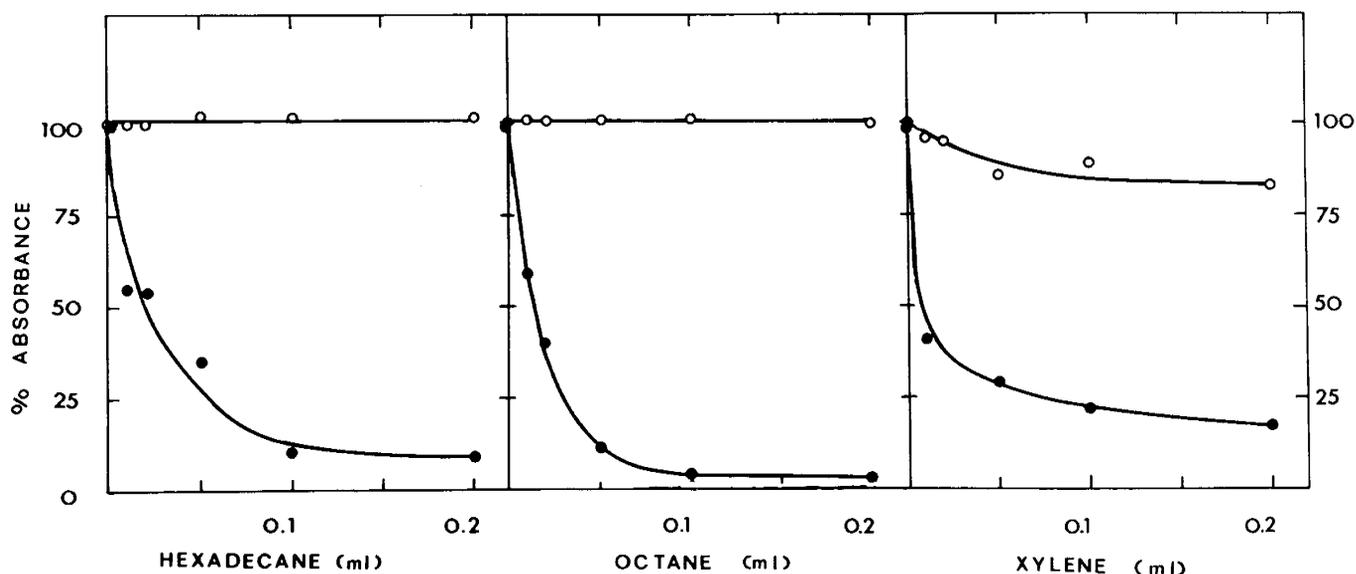


FIG. 1—Adherence of *Escherichia coli* B (○) and *Acinetobacter calcoaceticus* RAG-1 (●) to hydrocarbons. Cells were harvested at the end of the exponential growth phase, washed twice in PUM buffer and then resuspended to an initial A_{400} of 1.2-1.4. The

adherence assay was performed as described in Materials and Methods. The ordinate is the percentage of initial absorbance of the cell suspension.

It should be emphasized that in all growth experiments inocula were prepared in the same manner as was described for measuring adherence in Fig. 3. In defined medium with sodium acetate as the soluble carbon and energy source, RAG-1 and MR-481 showed similar growth kinetics (Fig. 4). Following a 2 hr lag, both strains grew with a doubling time of 48 min.

Using hexadecane as the sole source of carbon and energy (and under limited dispersion conditions), RAG-1 grew whereas the non-adherent mutant MR-481 failed to grow for at least 54 hr (Fig. 5). Growth of RAG-1 was accompanied by breakage of the upper hydrocarbon layer into droplets. Microscopic examination of the upper phase indicated that these droplets were covered by patches of adhering cells.

Hydrocarbon droplets were not observed in the aqueous phase under these conditions; thus the observed turbidity of the lower aqueous phase was due to

unbound cells. The values presented for growth of RAG-1 on hexadecane should be considered minimal since attached bacteria were not measured. In the corresponding tubes inoculated with mutant MR-481, neither growth nor substantial breakage of the hexadecane layer into droplets was observed (Fig. 6).

Effect of emulsan on the growth of MR-481 on hexadecane

A. calcoaceticus RAG-1 produces a potent extracellular emulsifying agent, referred to as emulsan.^{3,28,30,45} It was of interest to examine the effect of a highly purified preparation of emulsan (see Materials and Methods) on the growth of RAG-1 and the non-adherent mutant MR-481 on hexadecane (Fig. 7). In the absence of hexadecane, emulsan did not serve as a carbon and energy source for either RAG-1 or MR-481.

However, emulsan did enable growth of MR-481 on hexadecane. Growth of MR-481 in the presence of both

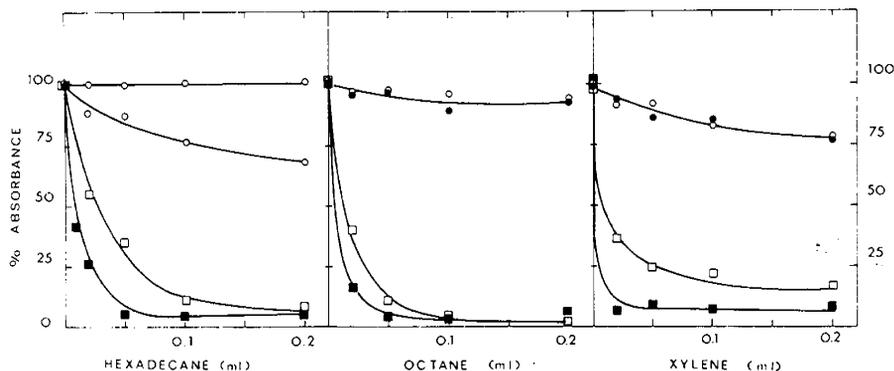


FIG. 2—Adherence of *Pseudomonas aeruginosa* PAS 279 and *Acinetobacter calcoaceticus* RAG-1 to hydrocarbons. The experiment was performed as described in Fig. 1 with cells harvested at the end of the exponential growth phase: *P. aeruginosa*, NB medium (○), P-hexadecane medium (●); *A. calcoaceticus*, NB medium (□), hexadecane medium (■).

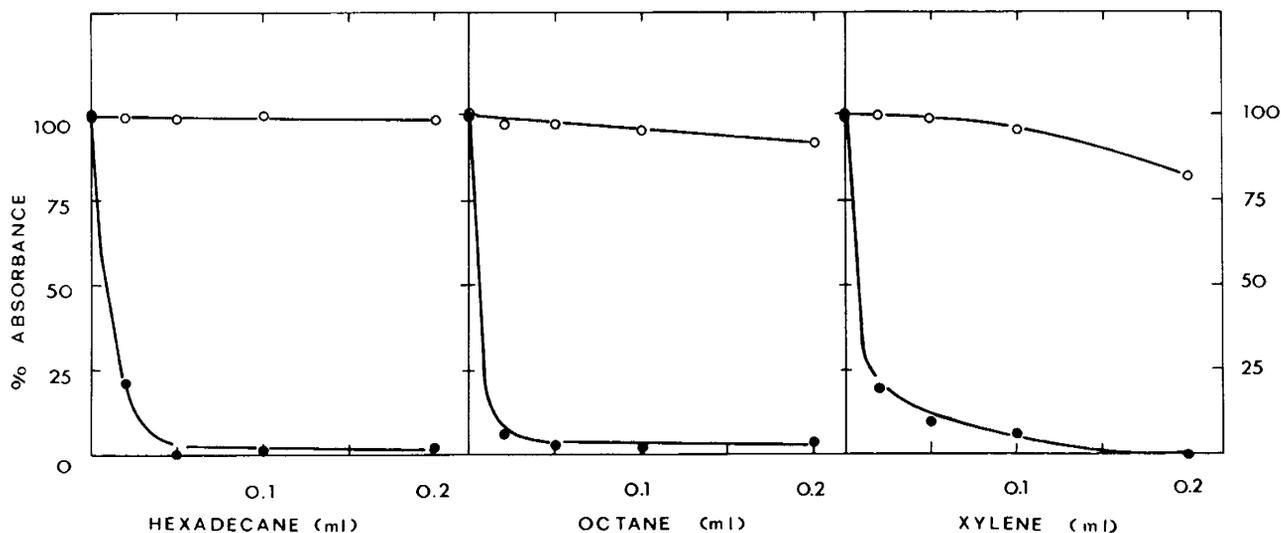


FIG. 3—Adherence of RAG-1 and MR-481 to hydrocarbons. Stationary phase cells were harvested from brain heart infusion broth washed twice and resuspended in PUM buffer to an $A_{400} = 1.45$ to 1.50 . To 1.2 ml of suspended RAG-1 (●) or MR-481 (○)

cells were added to various volumes of test hydrocarbon and the mixtures vortexed for 120 sec. Following phase separation the turbidity of the lower aqueous phase was measured. The ordinate is the percent of initial absorbance of the cell suspension.

emulsan and hexadecane began after about 6 hr, whereas neither component alone supported growth for 54 hr. At the weight ratio of hexadecane: emulsan used, $1,925:1$, the hexadecane droplets formed were too large to disperse into the aqueous phase and thus did not interfere with turbidity measurements. Emulsan had no significant effect on the growth of RAG-1 on hexadecane under these conditions.

Growth of MR-481 on hexadecane was also induced if the emulsan was added after several hours of incubation (Fig. 8). In each case of emulsan addition, the re-

sultant breakage of the hexadecane layer into droplets was followed by subsequent growth of the mutant cells. The time required for the commencement of growth following emulsan addition appeared to increase with the length of incubation prior to addition of the bioemulsifier.

Growth of MR-481 on hexadecane in the presence of emulsan was not the result of genotypic or phenotypic

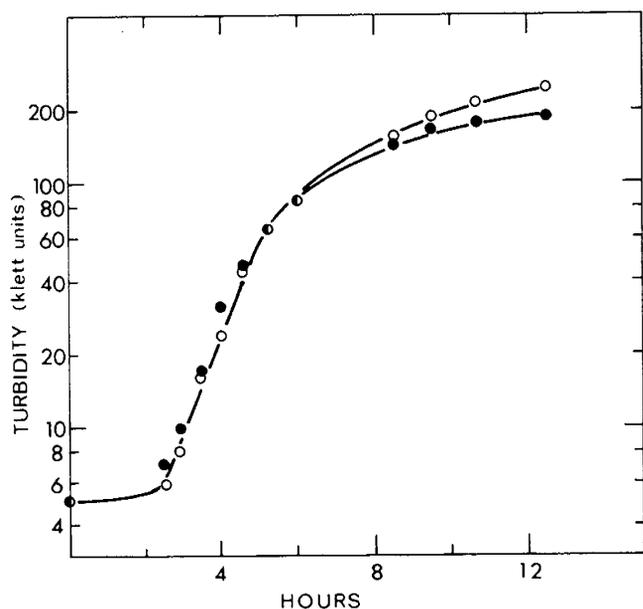


FIG. 4—Growth of RAG-1 and MR-481 in acetate medium. Stationary phase cells were harvested from brain heart infusion broth, washed twice and resuspended in PUM buffer supplemented with 0.2 percent sodium acetate. RAG-1 (●) and MR-481 (○) cells were inoculated into 4.2 ml acetate medium in Klett tubes to an initial turbidity of 5 Klett units.

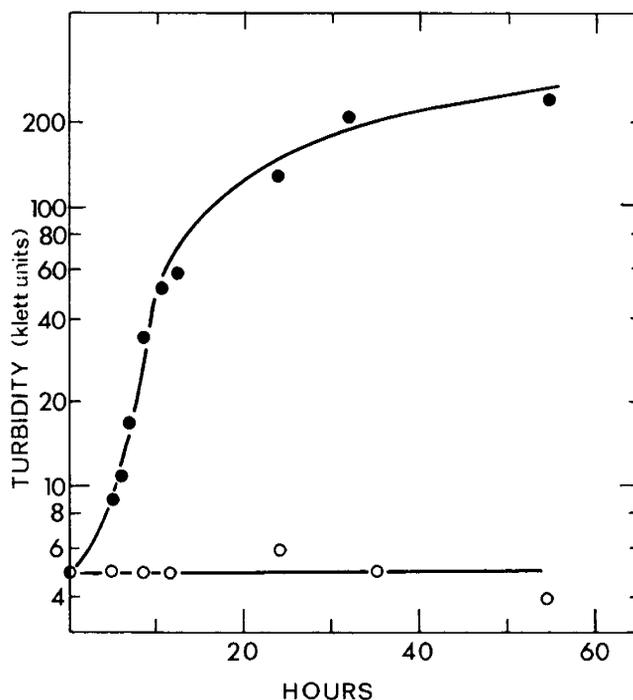


FIG. 5—Growth of RAG-1 and MR-481 in hexadecane medium. Klett tubes containing 4.2 ml PUM buffer were inoculated with either RAG-1 (●) or MR-481 (○) to an initial turbidity of 5 Klett units. Tubes were overlaid with 0.5 ml hexadecane and incubated as described in Materials and Methods.

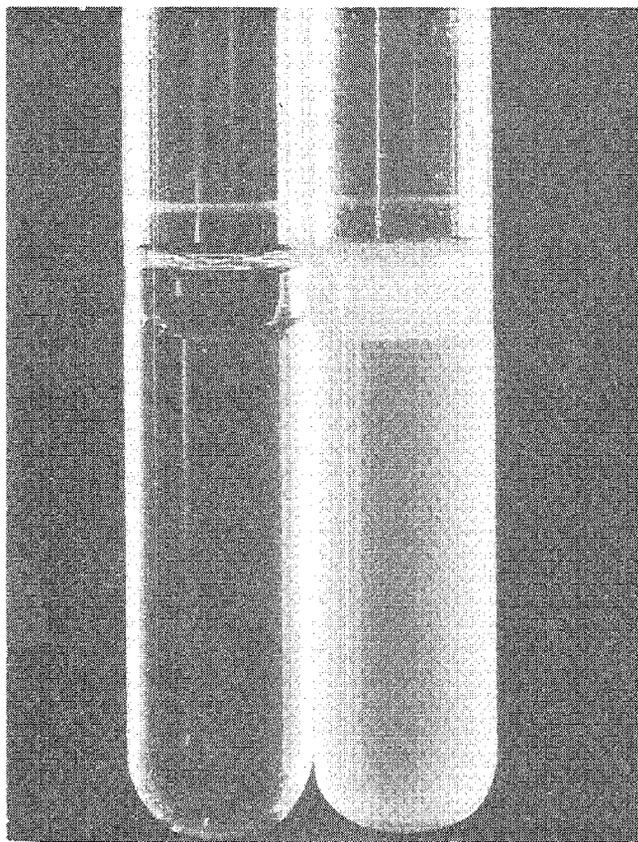


FIG. 6—Growth of MR-481 (left) and RAG-1 (right) on hexadecane medium. Tubes were photographed following 48 hr incubation under the conditions described in Fig. 3.

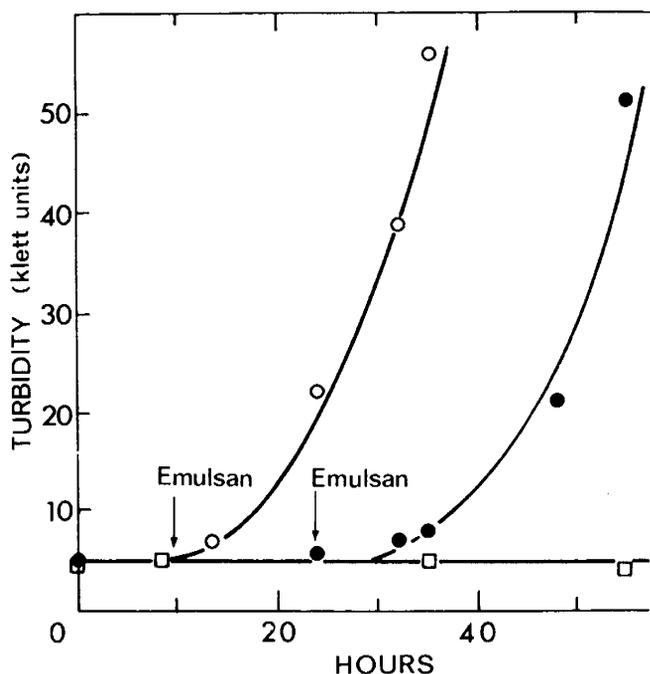


FIG. 8—Induction of growth of MR-481 on hexadecane by emulsan addition. Inocula preparation and incubation conditions were as described in Fig. 3. Emulsan was added to a final concentration of 50 $\mu\text{g}/\text{ml}$ at 10 hr (○) or 24 hr (●). Tubes containing no emulsan served as controls (□).

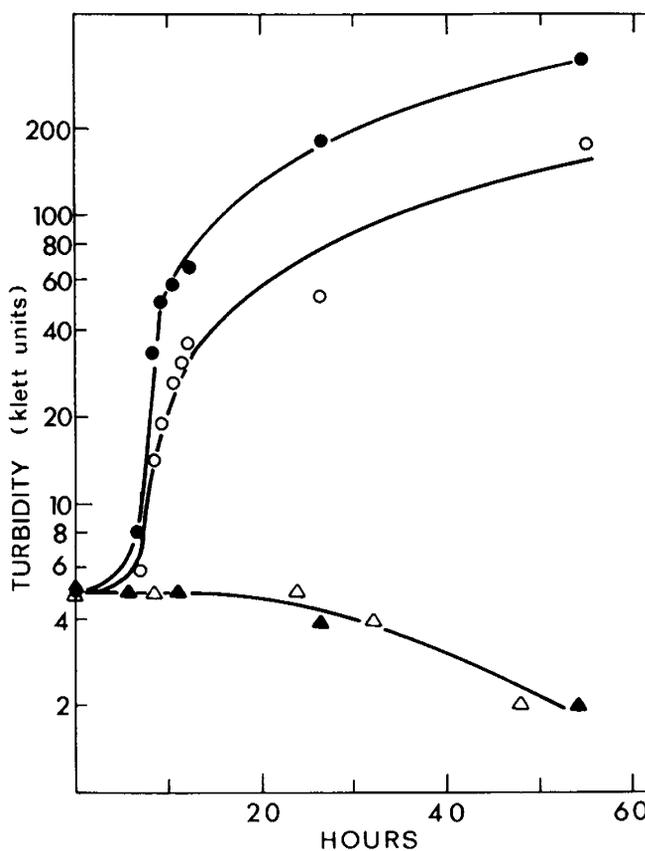


FIG. 7—Effect of emulsan on the growth of MR-481 on hexadecane. Inocula preparation and incubation conditions were as described in Fig. 3. RAG-1 (●) and MR-481 (○) were grown on hexadecane medium in the presence of 50 $\mu\text{g}/\text{ml}$ emulsan. RAG-1 (▲) and MR-481 (△) controls were grown in PUM buffer supplemented with 50 $\mu\text{g}/\text{ml}$ emulsan, in the absence of hexadecane.

reversion of MR-481 from non-adherent to adherent cells. As shown in Table 3, there was no significant increase in the adherence of MR-481 grown on hexadecane in the presence of emulsan as compared to the initial inoculum grown in rich medium (Fig. 3).

RAG-1 cells which were harvested and washed following growth on hexadecane both in the presence and absence of emulsan, exhibited high affinity towards hexadecane (Table 3). Thus, the appearance of free RAG-1 cells in the lower phase during growth on hexadecane did not result from a loss in their adherent properties.

DISCUSSION

Mudd and Mudd²¹ reported over 50 years ago that certain microorganisms tend to concentrate at oil:water interfaces. Although this observation has been frequently corroborated during studies on the growth of microorganisms on liquid hydrocarbons,^{13,15,19,20,22,23} there has been no direct evidence that adherence is an essential aspect of microbial growth on hydrocarbons. Certain investigators have argued that microbial growth on hydrocarbons is due to emulsification of the water-insoluble substrate rather than by direct contact between cells and hydrocarbon droplets.^{5,43,44} *A. calcoaceticus* RAG-1 is an interesting microorganism in

TABLE 3—Adherence of RAG-1 and MR-481 Following Growth on Hexadecane

Strain	Turbidity (A ₄₀₀) ^a		Percent Adherence
	Initial	Final	
RAG-1 ^b	1.42	0.09	94
RAG-1 ^c	1.40	0.05	96
MR-481 ^c	1.43	1.36	4.9

^a Cells were washed twice and resuspended in PUM buffer to initial turbidities as indicated. Hexadecane (0.2 ml) was added to 1.2 ml of cell suspension and the mixture vortexed for 120 sec. Following phase separation, the turbidity of the aqueous phase was measured.

^b RAG-1 cells were harvested following 54 hr growth on hexadecane medium, washed twice and resuspended in PUM buffer.

^c RAG-1 and MR-481 were harvested following 54 hr growth on hexadecane medium in the presence of 50 µg/ml emulsan, washed twice and resuspended in PUM buffer.

this regard because it both adheres avidly to hydrocarbons^{32,33,34} and produces an extracellular emulsifying agent, emulsan, which has been extensively characterized.^{3,28,30,45}

Isolation of a mutant strain of *A. calcoaceticus* RAG-1 deficient in its ability to adhere to hydrocarbon enabled an examination of the role of adherence in the growth of *A. calcoaceticus* RAG-1 on hexadecane. The non-adherent mutant strain, MR-481, failed to grow on hexadecane under the moderate agitation conditions which supported growth of wild type cells. Two trivial explanations for the inability of MR-481 to grow on hexadecane, insufficient oxygen and a faulty hydrocarbon-metabolizing enzyme system, were excluded by the following data: (1) MR-481 and RAG-1 cells grew with similar kinetics on acetate medium under conditions identical to those employed for growth on hexadecane; (2) MR-481 was able to grow on hexadecane medium following addition of an emulsifier; and (3) MR-481 cells were able to grow on solid medium supplemented with hexadecane vapors as sole carbon and energy source. We conclude from these data that adherence is a prerequisite for growth of RAG-1 on liquid hexadecane in the absence of strong mechanical agitation or emulsification of substrate.

Both RAG-1 and MR-481 produce a potent extracellular emulsifying agent which enables growth of non-adhering MR-481 cells on hexadecane. The observation that MR-481 does not grow on hexadecane even though it produces normal amounts of emulsan can readily be explained by considering emulsification as a cell-density dependent phenomenon.^{16,29,35} At the low inoculum employed (5×10^7 cells/ml) the concentration of emulsan which the cells produce is too low to emulsify the hydrocarbon and permit growth.

These studies suggest that adherence is a prerequisite for growth on liquid hydrocarbon under two conditions: low cell density and limited emulsification. The ability of MR-481 to grow on hexadecane following emulsan addition demonstrates that the presence of extracellular emulsifying agents can enable growth of non-adherent bacteria on hydrocarbons. The poor adherence of *P. aeruginosa* PAS 279 to hydrocarbon (Fig. 2) suggests that its growth, similar to that of MR-481,

depends on dispersion of the hydrocarbon phase by extracellular emulsification or vigorous mechanical agitation. In most natural environments, conditions of low cell density and limited emulsification prevail; thus, the growth of poorly-adhering *pseudomonas* strains in shake flasks and fermentors carried out in the presence of extraneous emulsifiers or vigorous agitation^{5,14,43,44} probably does not reflect growth in the open environment, where emulsifier and dispersed hydrocarbon would tend to diffuse away from the cell.

The adherence to hydrocarbon of bacteria which do not metabolize hydrocarbons (Tables 1 and 2) can be explained in terms of general cell surface hydrophobicity.^{21,32} *S. pyogenes*, for example, has been reported to adhere to buccal epithelial cells through hydrophobic interactions.² Its adherence to hydrocarbons may thus be mediated by such interactions. Similarly, hydrophobic cell surface components which enable *Serratia* to partition at the air:water interface⁴ may also promote their adherence to hydrocarbons as observed in this study.

Bacterial hydrophobicity has been cited as an important factor in several biological phenomena, including bacterial pathogenicity,⁴² and in adherence and growth of bacteria on various hydrophobic surfaces.^{2,19,24,35} Adherence to hydrocarbons provides a simple quantitative technique for measuring cell surface hydrophobicity and can be used for the selection and isolation of non-adhering mutants. Adherence of RAG-1 to hydrocarbons may reflect a general phenomenon in microbiology: A biological advantage is conferred on microbial cells which can escape by means of hydrophobic surface components from the bulk aqueous phase to an interface which provides nutrition and enables growth.

REFERENCES

1. Bayer, E. A., E. Rosenberg and D. Gutnick. 1981. The isolation of cell surface mutants of *Acinetobacter calcoaceticus* RAG-1. *J. Gen Microbiol.* 127: 295-300.
2. Beachey, E. H., W. A. Simpson and I. Ofek. 1980. Interaction of surface polymers of *Streptococcus pyogenes* with animal cells, p. 389-405. In R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter and B. Vincent (Eds.). *Microbial Adhesion to Surfaces*. Society of Chemical Industry, London.
3. Belsky, I., D. L. Gutnick and E. Rosenberg. 1979. Emulsifier of *Arthrobacter* RAG-1: Determination of emulsifier-bound fatty acids. *FEBS Lett.* 101: 175-178.
4. Blanchard, D. C. and L. D. Syzdek. 1978. Seven problems in bubble and jet drop researches. *Limnol. Oceanogr.* 23: 389-400.
5. Chakravarty, M., H. D. Singh and J. N. Baruah. 1975. A kinetic model for microbial growth on liquid hydrocarbons. *Biotech. Bioeng.* 17: 399-412.
6. Daniels, S. L. 1980. Mechanisms involved in sorption of microorganisms to solid surfaces, pp. 7-58. In G. Bitton and K. C. Marshall (Eds.). *Adsorption of Microorganisms to Surfaces*. John Wiley & Sons, New York.
7. Dexter, S. C., J. D. Sullivan, Jr., J. Williams, III, and S. W. Watson. 1975. Influence of substrate wettabil-

ity on the attachment of marine bacteria to various surfaces. *Appl. Microbiol.* 30: 298-308.

8. Dworkin, M. 1973. Cell-to-cell interactions in the myxobacteria. *Symp. Soc. Gen. Microbiol.* 23: 125-142.

9. Fletcher, M. and G. I. Loeb. 1979. The influence of substratum characteristics on the attachment of a marine pseudomonad to solid surfaces. *Appl. Environ. Microbiol.* 37: 67-72.

10. Foster, J. W. 1962. Bacterial oxidation of hydrocarbons, pp. 241-261. *In: Oxygenases.* O. Hayaishi (Ed.), Academic Press, New York.

11. Gersen, D. F. and D. Sheer. 1980. Cell surface energy, contact angles and phase partition. III Adhesion of bacterial cells to hydrophobic surfaces. *Biochem. Biophys. Acta* 602: 506-510.

12. Gibson, D. T. 1971. The microbial oxidation of aromatic hydrocarbons. *Crit. Rev. Microbiol.* 1: 199-223.

13. Gutnick, D. L. and E. Rosenberg. 1977. Oil tankers and pollution: A microbiological approach. *Ann. Rev. Microbiol.* 31: 379-396.

14. Hisatsuka, K., T. Nakahara, Y. Minoda and K. Yamada. 1975. Capacity to oxidize n-alkane in EDTA-treated cells of *Pseudomonas aeruginosa* S₇B₁. *Agr. Biol. Chem.* 39: 999-1005.

15. Kirschner Zilber, I., E. Rosenberg and D. Gutnick. 1980. Incorporation of ³²P and growth of pseudomonad UP-2 on n-tetracosane. *Appl. Environ. Microbiol.* 40: 1086-1093.

16. Lankford, C. E., J. R. Walker, J. B. Reeves, N. H. Nabbut, B. R. Byers and R. J. Jones. 1966. Inoculum-dependent division lag of *Bacillus* cultures and its relation to an endogenous factor(s). ("Schizokinen"). *J. Bacteriol.* 91: 1070-1079.

17. Latham, M. J. 1980. Adhesion of rumen bacteria to plant cell walls, p. 339-350. *In: R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter, and B. Vincent (Eds.), Microbial Adhesion to Surfaces.* Society of Chemical Industry.

18. Lippincott, J. A. and B. Lippincott. 1980. Microbial adherence in plants, p. 375-398. *In: E. H. Beachey, (Ed.), Bacterial Adherence.* Chapman and Hall, New York.

19. Marshall, K. C. 1976. *Interfaces in Microbial Ecology.* Harvard University Press. Cambridge, Mass.

20. McLee, A. G. and S. L. Davies. 1972. Linear growth of a *Torulopsis* sp. on n-alkanes. *Can. J. Microbiol.* 18: 315-319.

21. Mudd, S. and E. B. H. Mudd. 1924. The penetration of bacteria through capillary spaces. IV. A kinetic mechanism in interfaces. *J. Exp. Med.* 40: 633-645.

22. Nakahara, T., L. E. Erickson and J. R. Gutierrez. 1977. Characteristics of hydrocarbon uptake in cultures with two liquid phases. *Biotech. Bioeng.* 19: 9-25.

23. Neufeld, R. J., J. E. Zajic and D. F. Gerson. 1980. Cell surface measurements in hydrocarbon and carbohydrate fermentations. *Appl. Environ. Microbiol.* 39: 511-517.

24. Ofek, I. and E. H. Beachey. 1980. General concepts and principles of bacterial adherence in animals

and man. p. 1-31. *In: E. H. Beachey (Ed.), Bacterial Adherence,* Chapman and Hall, London and New York.

25. Perers, L., L. Andaker, L. Edebo, O. Stendahl and C. Tagesson. 1977. Association of some enterobacteria with the intestinal mucosa of mouse in relation to their partition in aqueous polymer two-phase systems. *Acta. Path. Microbiol. Scand. Sect. B* 85: 308-316.

26. Pines, O. and D. L. Gutnick. 1981. Relationship between phage resistance—emulsan production and interaction of phages with the cell surface of *Acinetobacter calcoaceticus* RAG-1. *Arch. Microbiol.* 130: 129-133.

27. Ratledge, C. 1978. Degradation of aliphatic hydrocarbons, p. 1-46. *In: R. J. Watkinson (Ed.), Developments in Biodegradation of Hydrocarbons—I.* Applied Science Publishers, London.

28. Reisfeld, A., E. Rosenberg and D. Gutnick. 1972. Microbial degradation of crude oil: factors affecting the dispersion in sea water by mixed and pure cultures. *Appl. Environ. Microbiol.* 24: 363-368.

29. Rosenberg, E., K. H. Keller and M. Dworkin. 1977. Cell density-dependent growth of *Myxococcus xanthus* on casein. *J. Bacteriol.* 129: 770-777.

30. Rosenberg, E., A. Zuckerberg, C. Rubinowitz and D. L. Gutnick. 1979. Emulsifier of *Arthrobacter* RAG-1: isolation and emulsifying properties. *Appl. Environ. Microbiol.* 37: 402-408.

31. Rosenberg, M. 1981. Bacterial adherence to polystyrene: a replica method of screening for bacterial hydrophobicity. *Appl. Environ. Microbiol.* 42: 375-377.

32. Rosenberg, M., D. Gutnick and E. Rosenberg. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol. Lett.* 9: 29-33.

33. Rosenberg, M., A. Perry, E. A. Bayer, D. L. Gutnick, E. Rosenberg and I. Ofek. 1981. Adherence of *Acinetobacter calcoaceticus* RAG-1 to human epithelial cells and to hexadecane. *Infect. Immun.* 33: 29-33.

34. Rosenberg, M., E. Rosenberg and D. Gutnick. 1980. Bacterial adherence to hydrocarbons, p. 541-542. *In: R. C. W. Berkeley, J. M. Lynch, J. Melling, P. R. Rutter and B. Vincent (Eds.), Microbial Adhesion to Surfaces.* Society of Chemical Industry, London.

35. Rosenberg, M. and E. Rosenberg. 1981. Role of adherence in growth of *Acinetobacter calcoaceticus* RAG-1 on hexadecane. *J. Bacteriol.* 148: 51-57.

36. Shapiro, L. 1976. Differentiation in the caulobacter cell cycle. *Ann. Rev. Microbiol.* 30: 377-407.

37. Smyth, C. J., P. Jonsson, E. Olsson, O. Söderlind, J. Rosengren, S. Hjertén and T. Wadström. 1978. Differences in hydrophobic surface characteristics of porcine enteropathogenic *Escherichia coli* with or without K88 antigen as revealed by hydrophobic interaction chromatography. *Infect. Immun.* 22: 462-472.

38. Stanier, R. Y. 1942. The cytophaga group: a contribution to the biology of myxobacteria. *Bacteriol. Rev.* 6: 143-196.

39. Starr, M. P. and J. C. C. Huang. 1972. Physiology of the *Bdellovibrios*. *In: Advances of Microbiological Physiology.* 8: 21-261. A. H. Rose and D. W. Tempest (Eds.). Academic Press, London.

40. Sundarrj, C. P and J. V. Bhat. 1972. Breakdown of chitin by *Cytophaga johnsoni*. Arch. fur Mikrobiologie 85: 159-167.
41. Varon, M. and M. Shilo. 1968. Interaction of *Bdellovibrio bacteriovorus* and host bacteria. I. Kinetic studies of attachment and invasion of *Escherichia coli* B by *B. Bacteriovorus*. J. Bacteriol. 95: 744-753.
42. van Oss, C. J. 1978. Phagocytosis as a surface phenomenon. Ann. Rev. Microbiol. 32: 19-39.
43. Velankar, S. K., S. M. Barnett, C. W. Houston and A. R. Thompson. 1975. Microbial growth on hydrocarbons—some experimental results. Biotech. Bioeng. 17: 241-251.
44. Wang, D. I. C. and A. Ochoa. 1972. Measurements on the interfacial areas of hydrocarbon in yeast fermentation and relationships to specific growth rates. Biotech. Bioeng. 14: 345-360.
45. Zuckerberg, A., A. Diver, Z. Peeri, D. L. Gutnick and E. Rosenberg. 1979. Emulsifier of *Arthrobacter* RAG-1: chemical and physical properties. Appl. Environ. Microbiol. 37: 414-420.

A Microbial Polysaccharide Produced from Crude Oil or Liquid Paraffin and Its Application in Petroleum Industry*

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ABSTRACT

A strain of bacterium 74-230 producing extracellular polysaccharide from crude oil or heavy liquid paraffin as C-source was isolated and named *Brevibacterium viscogenes* n. sp. 74-230. The yields of polysaccharide were 8 g/l from 12 percent (W/V) crude oil and 12 g/l from 4 percent (V/V) heavy liquid paraffin. The conversion rate of added paraffin was over 40 percent.

This polysaccharide is composed of D-glucose, D-mannose, D-galactose, D-rhamnose and D-arabinose with molar ratio of 4:3:2:1:1/4, and contains 0.50-0.7 percent pyruvic acid.

Its rheological properties, shear thinning effect, shearing resistance, sensitivity to salts etc. were determined and compared with xanthan, partially hydrolyzed polyacrylamide, and carboxymethylcellulose. The properties of this polysaccharide are well comparable to those of xanthan, and far better than the two others. It is noteworthy that this polysaccharide is produced from crude oil or its fraction, while xanthan is produced from carbohydrate.

The diluted fermentation fluid was used as driving fluid in laboratory scale model experiments. When the injection volume corresponded to 20 percent of the pore volume, the oil recovery was enhanced to about 9 percent of the original reserves.

Laboratory evaluation of the fermented gummy fluid from heavy liquid paraffin indicated that the polysaccharide is a suitable drilling fluid additive. Preliminary drilling experiments showed a promising result.

There are about 70 genera of microorganisms capable of producing extracellular polysaccharide.¹⁻³ Xanthan and dextran produced from carbohydrates have been applied in the petroleum industry.⁴⁻⁹ Some bacterium species of the genera *Alcaligenes*,⁹ *Arthrobacter*,^{10,11} *Brevibacterium*,^{11,12} *Corynebacterium*,^{11,13} and *Mycobacterium*,¹⁴ etc., have been reported to be able to produce polysaccharides from n-alkanes and liquid paraffin. However, information concerning their application and the production of polysaccharide directly from crude oil by microorganisms are unavailable. We have carried out a series of experiments in this area.¹⁵⁻¹⁹

This report describes the production of polysaccha-

ride from crude oil or heavy liquid paraffin by a strain of bacterium, 74-230, and preliminary laboratory evaluation of its usages as a driving fluid for enhancement of oil recovery or as a drilling fluid additive.

MATERIALS AND METHODS

The bacterial strain 74-230 was isolated from an oil-containing soil sample.

A suitable medium for strain 74-230¹⁸ consists of (g/l): NaNO₃ 1.5-2.0, NaHPO₄·12H₂O 2.0, KH₂PO₄ 0.6, MgSO₄·7H₂O 0.25, yeast extract 1.5-2.0, CaCl₂·2H₂O 0.06, crude oil 12 percent (W/V) or heavy liquid paraffin 4 percent (V/V), tap water 1,000 ml, pH 7.5, and sterilized at 8 lb for 30 min, unless otherwise stated.

Cultivation, assays

Seed cultures grown on broth peptone agar slant for 3 to 5 days were washed and suspended in water for inoculation. Cultures in 250 or 500 ml Erlenmeyer flasks containing 50 or 100 ml medium were incubated on a rotary shaker with 50 mm amplitude and 200 rpm for 4 to 5 days at 30 to 31°C.

Assays

Viscosity was measured by Ostward viscometer at 45°C after dilution of the sample 2 to 3 fold with tap water. Total sugar was determined by the phenol-sulfuric acid method of Dubois et al.²⁰ or the anthrone method of Morris, cell nitrogen by Kjeldahl's method, NO₃⁻ by reduction method,²¹ and inorganic phosphate by Fiske's method.²²

Estimations of the properties of polysaccharide gummy fluid were carried out by instruments and methods of API procedures. Rheological property was measured by rotary viscometer Rheotest 2 (East Germany).

Isolation and purification of polysaccharides

After extraction of the residual oil by chloroform and removal of cells by centrifugation at 12,000 rpm for 30 min, ethanol was added with stirring to a concentration of 60 percent (V/V) and NaCl 1.0 g/l. Insoluble material was obtained by centrifugation. This process was repeated 2 to 3 times. The final product was vacuum dried. Before analysis of its components the product was further purified with Sepharose 2B gel column (60-120 mesh) and dialyzed. Sedimentation analysis was performed with a HITACHI UCA-1 ultracentrifuge

*Cooperation with: The Scientific Research Institute of Petroleum Exploration and Development, and The Scientific Research and Design Institute of Daqing Oilfield, Ministry of Petroleum Industry, China.



FIG. 1—Electron micrograph of *Brevibacterium viscogenes* n. sp. 74-230. Nutrient agar slant: A. 24 h. B. 48 h. C. 96 h. $\times 9000$.

at 55,000 rpm.

Analysis of the compositions of crude oil

After fermentation the residual crude oil from cultural flasks or control (sterile) flasks was extracted with chloroform and the extracts were evaporated to dryness. The residues were treated with petroleum ether over night and then fractionated with Cilite column using petroleum ether and benzene to elute the deasphalted oil (fraction I) and benzene soluble asphaltenes (fraction II). The weight of the benzene insoluble asphaltenes was calculated by subtraction. Fraction I was further separated on a column with a bed of two phases of silica gel and alumina, by successive eluting with petroleum ether, benzene and benzene-ethanol (1:1) to obtain the saturated, aromatic, and soluble polar compounds, while the weight of the insoluble polar compounds was calculated by subtraction. n-Alkane was separated by the method of urea complexing. Saturated hydrocarbon, n-alkanes, and branched chain as well as cyclic hydrocarbons were analyzed by gas-liquid chromatography.

Analysis of the constituents of polysaccharide

The polysaccharide was hydrolyzed in 2N HCl for 5 h, acetylated, and analyzed with a gas chromatography Model 103 (Shanghai).

The organic acid components were determined by paper chromatography: developed with (1) n-propanol: ammonia water: water (6:2:2), (2) n-butanol: formic acid (95:5, water-saturated), detected by 0.1 percent bromocresol green. The content of pyruvic acid was determined by the method of Friedmann.²³

RESULTS AND DISCUSSION

Identification of strain 74-230

This organism is Gram-positive, nonmotile short rods, $0.5 \times 1.3-4.5 \mu\text{m}$ (Fig. 1). The cells are unbranched, non-sporulating, not acid fast, and non-pleomorphic



FIG. 2—Viscous, gummy fluid produced from heavy liquid paraffin.

during the development process. Colonies on nutrient agar are circular, raised, edge entire, slightly orange red, smooth, and moist-glistening. Nitrate is reduced slightly. Gelatine is not liquefied. Litmus milk is reduced. Glucose is oxidized into acid, but lactose not. The G-C-content of DNA determined in SSC-system is 63.12 ± 2.02 mol. percent.

According to its morphological and physiological properties, the strain belongs to the genus *Brevibacterium*, but it differs from all known 40 species in this genus. On the ground of its ability to produce exopolysaccharide the strain is considered to be a new species, and named as *Brevibacterium viscogenes* n. sp. 74-230.¹⁵

Polysaccharide production

After 4 to 5 days growing in shake flask containing crude oil or liquid paraffin as C-source, the organisms produced a viscous gummy fluid (Fig. 2). After 3-fold dilution of the gummy liquid, its viscosity was over

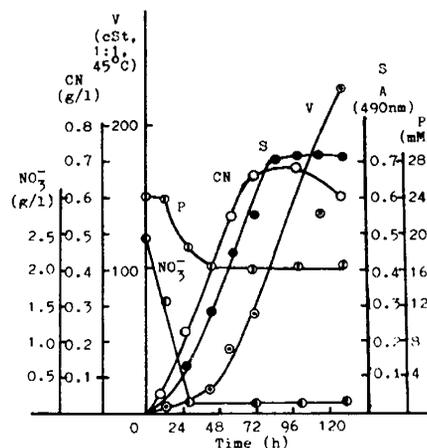


FIG. 3—Time course of polysaccharide production in 50 l fermenter. Culture medium (g/l): NaNO_3 2.5, $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 2.0, KH_2PO_4 0.6, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.03, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.06, corn steep liquor 0.5, crude oil 120, pH 7.5, inoculum: 4 percent. Aeration: 1:0.75. Agitation: 180 rpm. V = viscosity; S = total sugar; CN = cell nitrogen; P = inorganic phosphate.

TABLE 1—Composition of the Crude Oil
(obtained from control flasks)

Composition	Content, Percent
Saturated hydrocarbon	80.1
Aromatic hydrocarbon	7.8
Benzene soluble asphaltenes	1.1
Benzene insoluble asphaltenes	2.8
Soluble polar compounds	0.9
Insoluble polar compounds	7.3

TABLE 2—Composition of the Residual Oil After Fermentation

Composition	Control Percent. (W/V)	Residual Oil, Pct. (W/V)	Difference, (g)	Relative Consumption, Percent*
Crude oil	8.40	6.00	2.40	100
Saturated hydrocarbon..	6.73	4.43	2.30	95.83
Aromatic hydrocarbon...	0.66	0.61	0.05	2.06
Benzene soluble and insoluble asphaltenes..	0.33	0.32	0.01	0.42
Soluble and insoluble polar compounds	0.69	0.65	0.04	1.68

*Take the crude oil consumption as 100.

500-700 cSt measured by Ostward viscometer at 45°C.

In 50 or 240 l batch fermentation using crude oil as C-source, the time course (Fig. 3) showed that nitrate was almost run out in 24 h, phosphate decreased initially, and remained constant after 36 h. The amounts of cell nitrogen and polysaccharide increased in parallel after a lag of 12 h, and achieved to maxima after 72 h, while the viscosity kept on increasing. These are similar to those of other polysaccharide formations.³ About 8.0 g/l of polysaccharide was obtained and the conversion rate of added crude oil was 6.7 percent.

The crude oil used in these experiments mainly contained over 80 percent saturated hydrocarbon (Table 1). Composition of the residual oil after fermentation is presented in Table 2. Comparing with control experiments, the bacterium mainly utilized saturated hydrocarbon by about 95 percent of the consumed crude oil (Table 2.).

The gas chromatographic analyses of saturated hydrocarbon, n-alkanes, branched chain and cyclic hydrocarbons (Fig. 4) indicated that the bacterium substantially converted n-alkanes lower than C₃₀ to polysaccharide, which is consistent with the following experiments.

When individual pure C₁₀₋₂₂ n-alkanes and solid paraffin were used as C-source, results (Fig. 5) indicated that C_{12-C19} n-alkanes, especially C_{16-C18} were converted to polysaccharide, and even-C n-alkanes were better than their adjacent odd-C ones. Thus the heavy liquid paraffin consisted of C_{12-C19} n-alkanes was chosen as C-source for batch fermentations in 5t. fermentors. The time course of polysaccharide production (Fig. 6) is consistent with that of the above mentioned fermentation of crude oil. The polysaccharide yield was about 12.0 g/l. The conversion rate of added heavy liquid paraffin was over 40 percent.

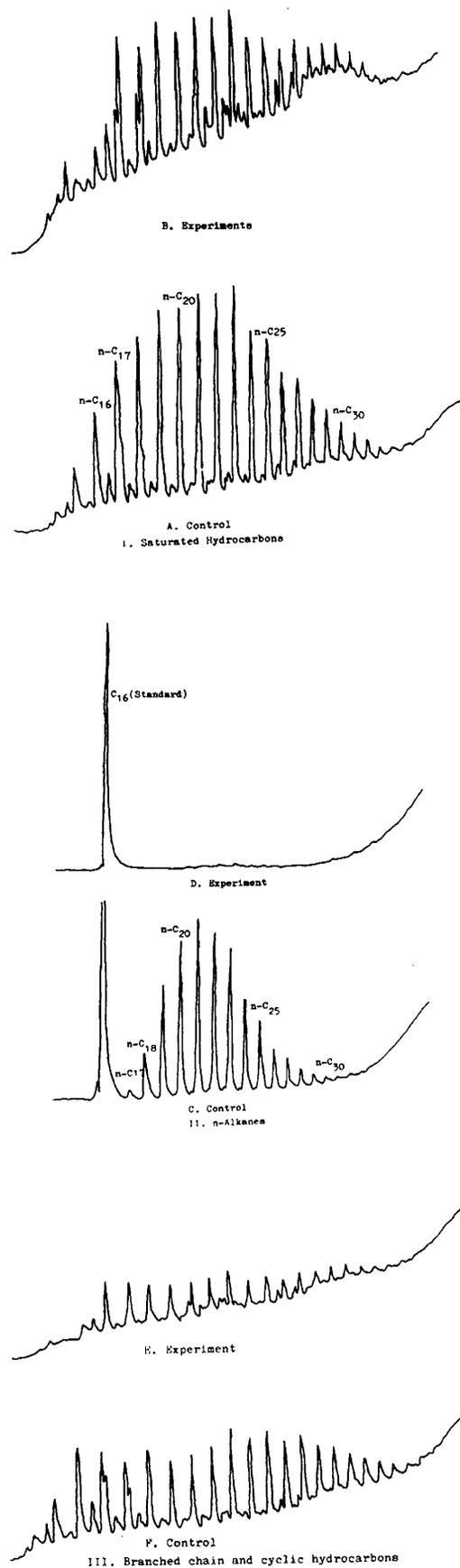


FIG. 4—Gas chromatographic analysis of original oil.

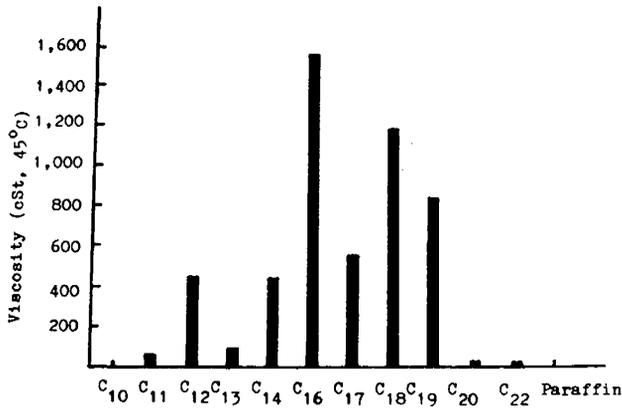


FIG. 5—Effects of chain length of n-alkanes (2 percent W/V) on the polysaccharide synthesis.

Polysaccharide component analysis

The polysaccharide purified by gel filtration (Fig. 7) seems to be homogeneous by ultracentrifugal analysis (Fig. 8).

Gas chromatographic analysis showed this polysaccharide is composed of D-glucose, D-mannose, D-galactose, D-rhamnose, and D-arabinose with molar ratio of 4:3:2:1:1/4 (Fig. 9).

Paper chromatography of organic acids indicated that the polysaccharide contained pyruvic acid (Fig. 10), which was further confirmed by the absorption spectrum of phenylhydrazone derivative (Fig. 11). Its content was 0.5-0.7 percent. So this polysaccharide differs in composition from the reported polysaccharides^{1,2} and may be a new one.

Some properties of the polysaccharide fluid

Its viscosity and yield point were determined and compared with those of xanthan (XC), partially hydrolyzed polyacrylamide (PHP), and carboxymethylcellulose (CMC). Among them the plastic viscosity (PV, Fig.

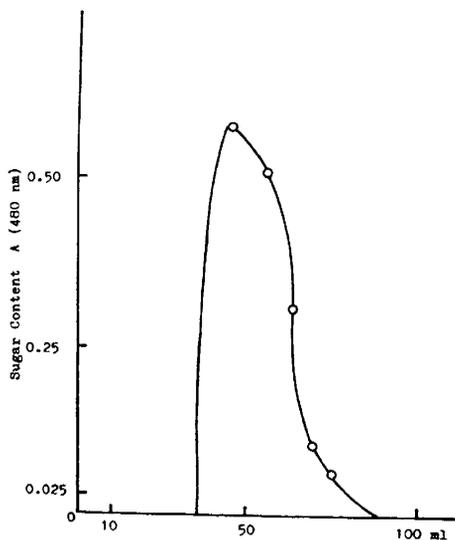


FIG. 7—Sepharose 2B gel filtration of polysaccharide. Column: 19.5 × 1.8 cm. Sample: 4 ml of 0.1 percent polysaccharide. 0.1 M, pH 7.0. Phosphate buffer was used both for equilibrium and elution.

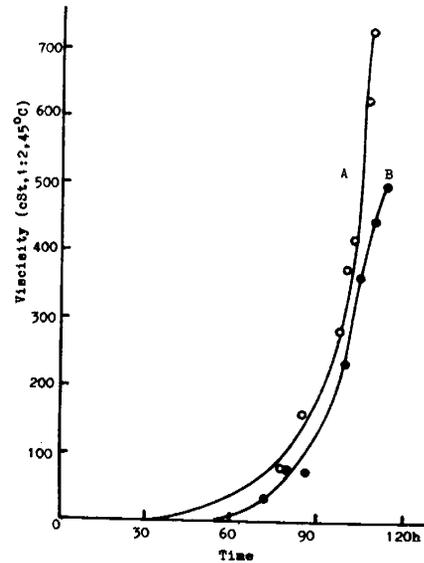


FIG. 6—Time course of polysaccharide production in 5 fermenters. The heavy liquid paraffin used as C-source consists (percent) of: C₁₂, 1.7; C₁₃, 8.6; C₁₄, 18.7; C₁₅, 21.7; C₁₆, 21.4; C₁₇, 17.7; C₁₈, 8.1; C₁₉, 2.8.

12) and yield point (YP, Fig. 13) of polysaccharide (BV) 74-230 were the highest, but its YP/PV was lower than that of XC (Fig. 14), which may be due to its highest plastic viscosity and the interference of other factors in the fermented fluid.

Relationship between plastic viscosity and shear rate

Viscosity changes of the four kinds of polymers with the shear rate (Fig. 15) showed that the curve of BV 74-230 was almost parallel to that of XC at the same concentration (0.4 percent). If their viscosities

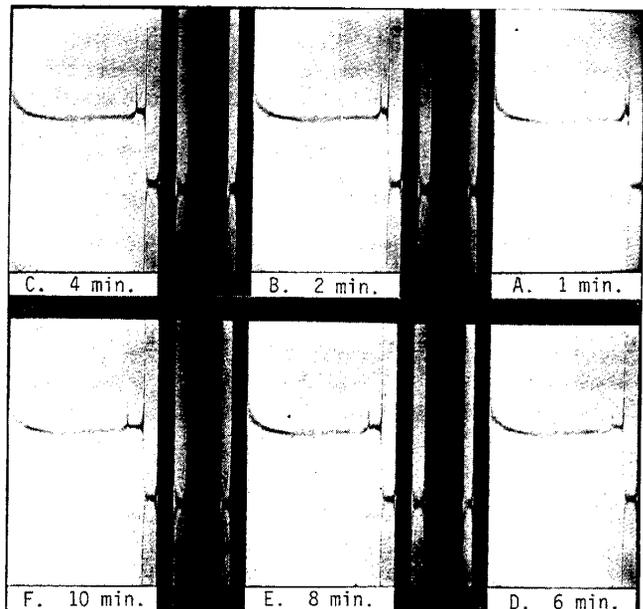


FIG. 8—Sedimentation patterns of polysaccharide 74-230 after Sepharose 4B gel filtration. 0.05 percent polysaccharide in 0.2 M NaCl, centrifugized at 55,000 rpm. S_{20,w} 7.9.

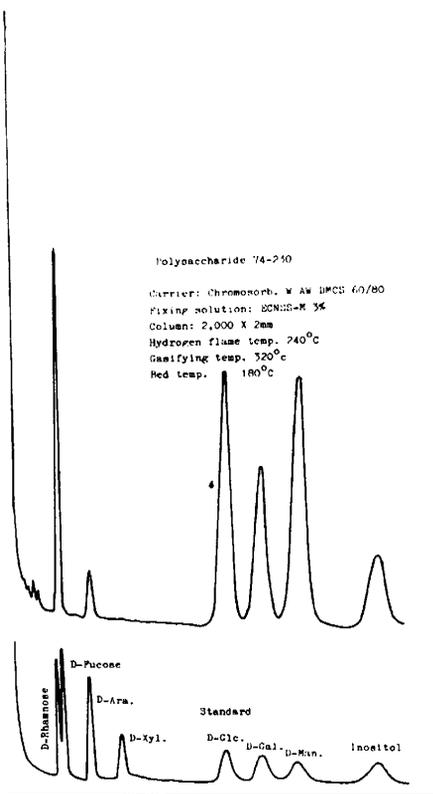


FIG. 9—Gas chromatograms of the monosaccharide constituents of polysaccharide 74-230.

were the same, both curves may coincide. PHP (0.5 percent) and CMC (1.0 percent) were much worse.

Viscosity changes and shear resistance at high shear rate

Viscosity was measured by pressuring capillary rheologic viscometer. The viscosity of BV 74-230 was as low as that of XC, which is beneficial to spraying drilling (Table 3); its shearing resistance at the shear rate of

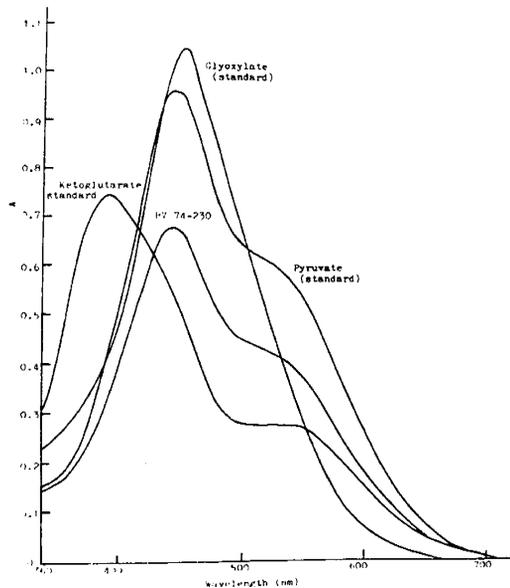


FIG. 11—Identification of pyruvic acid in polysaccharide 74-230.

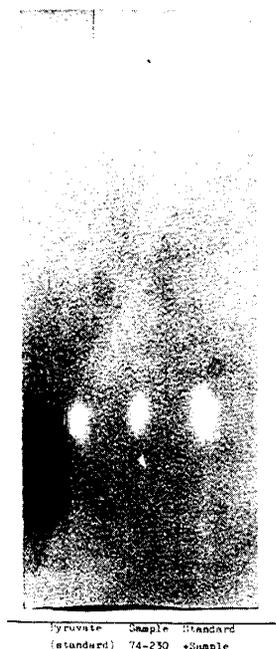


FIG. 10—Paper chromatogram of the organic acid in polysaccharide 74-230. Developed with no-propanol. Ammonia water: Water (6:2:2). Detected by 0.1 percent bromocresol green.

100,000s⁻¹ was slightly worse than that of XC (Table 4).

Sensitivity to salts—Rheological properties of various polymer fluids were shown in Fig. 16-19. The polymer concentrations were: BV 74-230, 0.4 percent; XC, 0.5 percent; PHP, 0.5 percent; CMC, 1.0 percent. The BV 74-230 was not so good as XC, but much better than CMC and PHP. Since the fermented polysaccharide fluid contained considerable amount of residual inorganic salts, it is expected that the contamination resistance of BV 74-230 can be improved by purification.

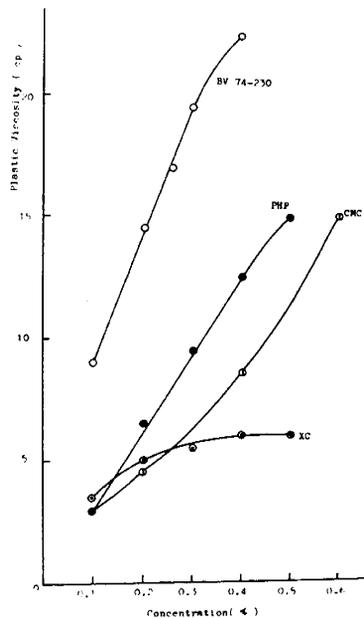


FIG. 12—The relationship between polysaccharide concentration and its viscosity.

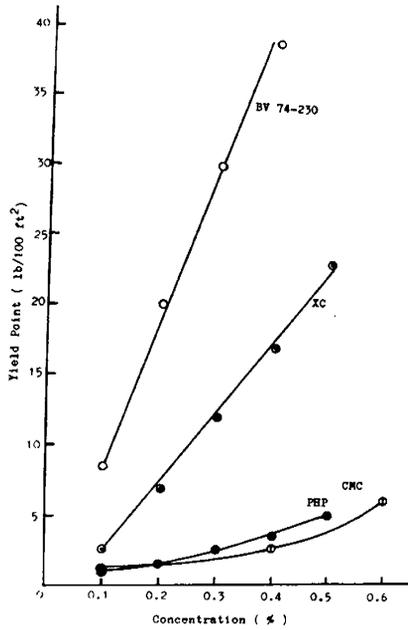


FIG. 13—The relationship between polysaccharide concentration and yield point.

Applications of polysaccharide 74-230

As mentioned above, the properties of BV 74-230 was almost comparable to those of XC, and far better than the two others. So this polysaccharide may be not only used as a drilling fluid additive, but also as a driving fluid for enhancement of oil recovery.

Drilling fluid additive—For further evaluation of the property of BV 74-230 as a drilling fluid additive, we added it into a base mud. The results indicated that BV 74-230 was a suitable additive.¹⁹ There are many advantages over the other polymers tested: the lowest concentration needed, better shear-thinning property, rapid enhancement of viscosity, the highest yield point, promising lubricity, contamination resistance, heat resistance, compatibility, and filtration property. Preliminary drilling experiments gave promising results.

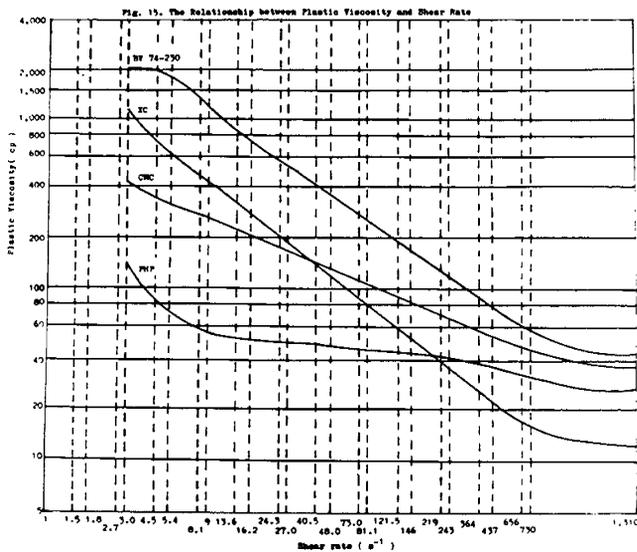


FIG. 15—The relationship between plastic viscosity and shear rate.

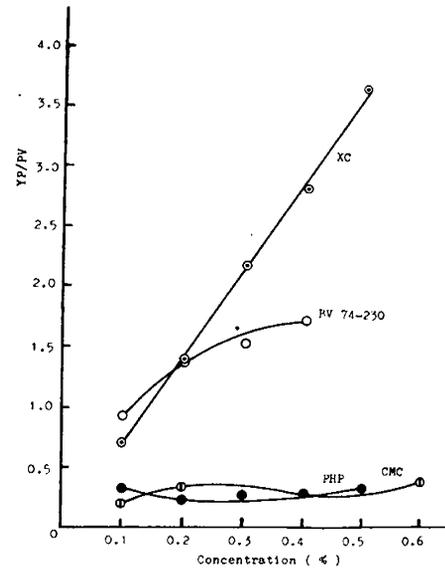


FIG. 14—The relationship between polysaccharide concentration and UP/PV.

Displacement efficiency of polysaccharide 74-230

Using double pipes filled with heterogeneous oil-sand (75 × 3.8 cm, one of them with lower permeability than the other), we carried out the enhancement oil recovery test under normal pressure and 45°C. The fermented polysaccharide fluid was diluted to about 5 cSt, and used as driving fluid. After the water-oil ratio had been reached to 98 percent during the water injection process for oil recovery, the polysaccharide fluid was injected up to 10 or 20 percent pore volume, followed by water injection again.

The performance curves (Fig. 20) showed the increase of flowing pressure difference, the decrease of water factor, and the enhancement of oil recovery during the injection process. It is obviously due to improvement of the mobility of injected fluid, thereby the sweep

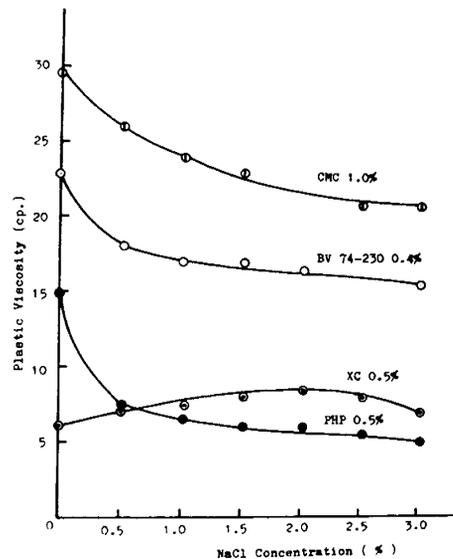


FIG. 16—Effect of NaCl on the viscosity of BV 74-230.

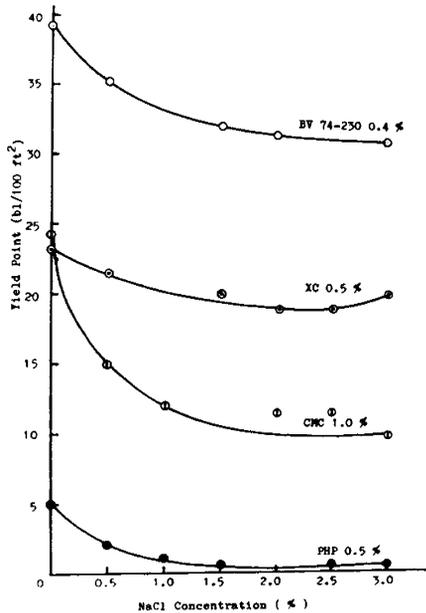


FIG. 17—Effect on NaCl on the yield point of BV 74-230.

area was enlarged. The average oil recovery was increased about 9 percent of the original reserves (Table 5).

It is well known that only one third of the oil reserves can be recovered by the existing technologies. Considerable progresses have been made in this area, since ZoBell²⁴ first patented a microbial oil recovery method. But almost all of the efficient processes²⁵⁻²⁷ in field experiments demanded the addition of carbohydrates. However, they are difficult to apply in large scale. Therefore, we must attach importance to the research on direct microbial conversion of crude oil and its fractions for petroleum industry, thus what is taken

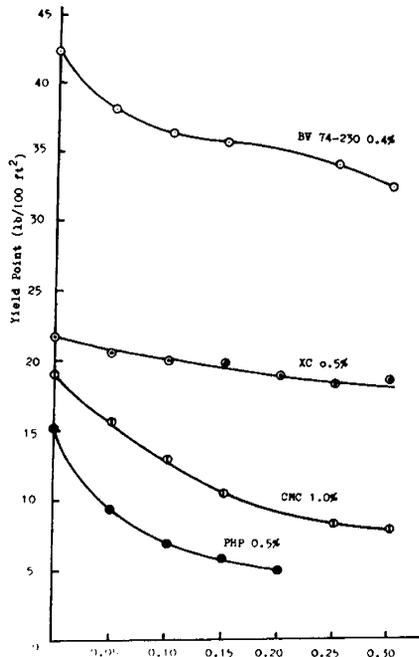


FIG. 19—Effect of CaCl₂ on the yield point of BV 74-230.

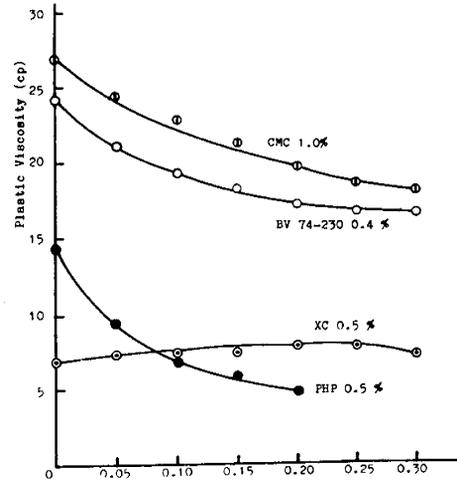


FIG. 18—Effect of CaCl₂ on the viscosity of BV 74-230.

from the oil is used in the interests of oil industry. Lately, Wagner²⁸ patented the application of microbial surfactants from hydrocarbon fermentation to oil recovery. Our work confirmed the usage of polysaccharide 74-230 in petroleum industry, which suggested that the microbial conversion of crude oil may be a more interesting aspect.

REFERENCES

1. Eveleigh, O. E., 1973. In "Handbook of Microbiology" ed. by Laskin, A. I. et al., Vol. II, CRC, p. 89-96.
2. Sandford, P. A., 1979. Adv. Carbohydr. Chem. Biochem., 36: 265-313.
3. Slodki, M. E. et al., 1978. Adv. in Appl. Microbiol., 23: 19-54.
4. Cabriel, A., 1979. In "Microbial Polysaccharides and Polysaccharases" ed. by R. C. W. Berkeley, et al., Academic Press, London, p. 191-204.
5. Deily, F. H., Lindblom, G. P., Patton, J. T., and Holman, W. E., 1976. Oil and Gas J., 65 (26): 62-70.
6. Jeanes, A., 1977. In "Extracellular Microbial Polysaccharides" ed. by Sandford, P. A., et al. ACS, Washington, p. 284-298.

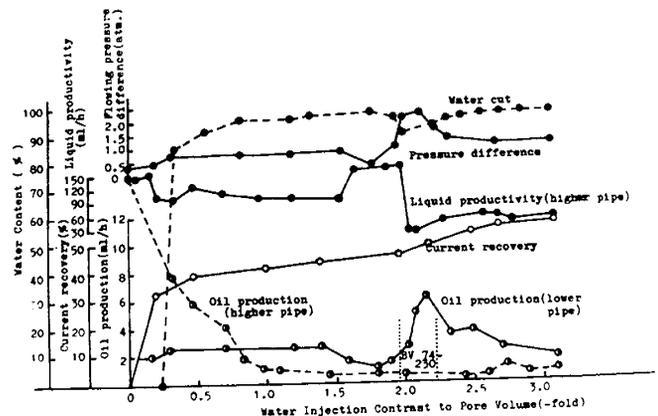


FIG. 20—Performance curves.

TABLE 3—Viscosity Changes of BV 74-230 at High Shearing Rate

Rate (s ⁻¹), Viscosity (cp), Sample	5.4	9.0	16.2	27.0	48.6	81.0	145.8	243	437.4	729	1312	19,320	38,790	56,630	113,700
74-230 (0.25)	770	626	444	322	222	159	106	75.3	75.6	38.7	28.6	8.97		6.12	4.42
XC (0.40)	420	296	181	121	75.7	51.4	32.9	23.5	15.9	11.5	8.31		3.24		

TABLE 4—Shearing Resistance of BV 74-230 at Shear Rate 10⁵ s⁻¹

Shear Time Viscosity (cp) Sample	0				Change After Shear Times, (Percent)
	0	3	6	9	
74-230 (0.25 percent)	600	514	511	507	15.5
XC (0.4 percent)	134	128	126	128	4.5

7. Owen, W. L., Sugar, 45 (3): 42-43, 1950; 46 (7): 28-30, 1951; 47 (7): 50-51, 1952; 50 (5): 47-48, 1955.

8. Sandrick, E. I., and Maerker, J. M., 1977. In "Extracellular Microbial Polysaccharides" ed. by Sandford, P. A. et al., p. 242-264.

9. Yamaguchi, M. and Sato, A., 1977. Report of the Fermentation Research Institute, 49: 115-120.

10. Suzuki, T. et al., 1969. Agr. Biol. Chem., 33 (1): 190-195.

11. Kayowa Hakko Kogyo Co. Ltd., Fr. Brevet D'Invention, No. 1,530,165.

12. Kanamaru, T. and Yamamodani, S., 1969. Agr. Biol. Chem., 33 (10): 1521-1522.

13. Research Group of Systematic Bacteriology, et al., 1979.

14. Grechushkina, N. N., and Rozanova, L. I., 1971. Microbiol., 40 (5): 820-824.

15. Wang Xiuyuan, Liu Xiufang, Wang Xianji, Tian Xinyu, 1980. Acta Microbiologica Sinica, 20 (4): 345-350.

16. Wang Xiuyuan, Wang Chuanzhu, 1980. Acta Petrolei Sinica, 1 (4): 77-85.

17. Wang Xiuyuan, Yu Nanxiong, Liu Xiufang, Tian Xinyu, Cui Wenhua, 1982. Acta Microbiologica Sinica, 22 (1): 71-78.

18. Wang Xiuyuan, Liu Xiufang, Shi Zhijing, Wu Chenghua, 1982. Acta Microbiologica Sinica, 22 (4) (In press).

19. Wang Xiuyuan, Zhang Kaiqin, Cui Wenhua, Liu Yuqing, Liu Xiufang, Li Lianho Shi Zhinjing, Hou Yueting, Wu Peiyi, 1982. Acta Petrolei Sinica, 3 (4) (In press).

20. Dubois, M., Gilles, K. A., Hamilton, J. K., Robers, P. A., and Smith, F., 1965. Analytical Chem., 28 (3): 315.

21. Institute of Hygiene, China Academy of Medical Sciences, 1974. Analytic Methods of Water Quality, People's Hygiene Publishing House, Beijing, p. 148-153.

22. Fiske, C. H., and Subborow, Y., 1925. J. Biol. Chem., 66: 375.

23. Friedemann, T. E., and Haugen, G. E., 1943. J. Biol. Chem., 147: 415-422.

24. ZoBell, C. E., 1946. U.S. Patent, 2,413,278.

TABLE 5—Displacement Efficiency of Polysaccharide 74-230

Test No.	Property of Model			Driving Fluid	Primary Oil Recovery			Pore Volume Injected with Water (-fold)	Driving Fluid	Secondary Oil Recovery			Oil Production Increased in Avg. (Percent)	
	Permeability (md)	Porosity (Pct.)	Oil Saturation (Pct.)		Efficiency of Oil Displacement, Percent		Efficiency of Oil Displacement, Percent			At the End	Enhanced	Pore Volume Injected with Water (-fold)		
					Water-Free Period	At the End	At the End							Enhanced
8	106	31.6	60.1	Water	1.84	20.52	2.02	20 pct P.V. injected with the bacterial gummy solution 5.27 cSt, 45°C	42.5	9.35	1.35			
	2,960	37.3	70.1		31.2	45.0			54.2				63.53	
9	115	34.4	61.5		2.77	20.97	1.76		42.17	10.35	1.27	9.12		
	3,000	37.2	71.0		30.8	45.3			55.85				66.64	
10	112	33.2	56.4		4.4	29.2	2.11		47.4	8.2	1.4			
	3,100	36.1	64.9		33.7	51.4			59.6				67.9	
11	107	35.3	56.6		3.23	20.9	1.91	As above, 5.5 cSt, 45°C	37.6	8.3	1.3			
	3,080	37.5	67.6		32.6	45.0			53.3				64.1	
12	98.5	32.6	64.2		1.68	14.98	2.90		35.48	9.38	1.27			
	3,080	35.3	66.8		29.4	41.8			52.18				63.75	
		38.0	68.0		51.05	63.25								

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25. Jaranyi, I., Kiss, L. and Szalancy, Gy., 1966. Vortrage des Internationalen Symposiums "Erdolmikrobiologie." Akademie-Verlage, Berlin, p. 69-72.

26. Karaskevich, E., 1977. Proceedings of the USSR Academy of Sciences, Biolog. Ser. No. 5: 790-794.

27. Lonsane, B. K. et al., 1976. J. Scient. Ind. Res., 35 (5): 316-324.

28. Wagner, F., Lindorfer, W., und Schulz, W., 1976. Deutsches Patent, 2,410,267.

Physiological Types of Microorganisms Useful for Enhanced Oil Recovery

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INTRODUCTION

Microorganisms are often viewed in a negative fashion by many people associated with oil recovery. This stems from the detrimental effects of microorganisms on oil recovery processes such as pipe corrosion or facial plugging of the well bore (see Davis¹ for more detailed discussion). However, microorganisms produce a variety of compounds such as biopolymers, biosurfactants, solvents, gases and fermentation acids that could potentially enhance oil recovery.

The *in situ* production of these compounds would eliminate many of the steps involved in the surface production of these compounds and avoids the problems associated with the injection and absorption of the compound. In essence, these compounds would be produced at the location where they would do the most good making a microbial enhanced oil recovery process more economically feasible.

The success of an *in situ* microbial process depends on the ability of the microorganisms to grow and produce the desired product in the reservoir. For this to occur, viable microbial cells and the nutrients required for growth must be transported throughout the reservoir rock stratum. Laboratory studies using sandstone cores^{2,3,4} and field trials (see Davis¹) show that these conditions are met with varying degrees of success.

The ability of the microorganism to grow and produce the desired product depends on the selection of the appropriate organism that can function in the reservoir environment as well as the ability to regulate its metabolism *in situ*. The remainder of the paper will examine the reservoir parameters that will affect microbial growth and present some ideas on how to regulate the growth and metabolism of the microorganisms *in situ*.

THE RESERVOIR ENVIRONMENT

Generalizations about the environment of a typical oil reservoir are difficult since wide variations in parameters often occur from reservoir to reservoir. Thus, actual microbial enhanced oil recovery (MEOR) processes will clearly involve the selection of the particular microbe most suited for the environment of a particular reservoir. However, some general guidelines can be used in the selection of the appropriate microbial strains.

Clark et al⁵ surveyed various environmental conditions that exist in petroleum reservoirs in the top 10

oil-producing states in this country. Average reservoir temperatures in these states ranged from 49° to 90°C. The pH of the reservoirs varied from 3.0 to 9.9 with 66 percent of the reservoirs having pH values between 6.0 and 8.0. Large variations in brine concentrations were observed with average percent total solids ranging from 1.3 to 15.6. About 88 to 98 percent of the brine was potassium or sodium chloride.

The concentration of macronutrients such as sulfur, magnesium, iron, phosphate, nitrate and oxygen required for microbial growth were also studied.⁵ Magnesium and iron were present in concentrations sufficient to support microbial growth, but sulfur, nitrate and phosphate were not. Oxygen concentration was also low or undetectable indicating anaerobic conditions.

ENVIRONMENTAL PARAMETERS AFFECTING MICROBIAL GROWTH

From the above data, petroleum reservoirs have environmental conditions which would severely limit the growth of most known microbial species. Since few of the environmental conditions of the reservoir can be changed, suitable microbes must be selected which can grow in the environment of the reservoir.

The effects of temperature,^{6,7} salinity,⁸ pH⁹ and pressure^{10,11} in microbial growth and metabolism have been recently reviewed. In general, microorganisms grow and reproduce at wide ranges of temperature (0° to 100°C), pH (1 to 10), salinity (0 to 35 percent) and pressure (up to 1000 atm). However, this does not mean that a particular microbial species can tolerate these wide variations in environmental parameters. Most species have a restricted range of parameters in which they can grow. In the case of temperature, this rarely exceeds a span of 30°C.

Microbial species are classified based on their tolerance to a particular environmental parameter. Temperaturewise, microbial species can be classified as psychrophiles with growth spans from -5 to 22°C, mesophiles with growth spans from 10 to 45°C and thermophiles with growth spans between 40 to 80°C or above for the extreme thermophiles.¹² Similar classifications have been developed for the growth response of microbial species in relation to salt concentrations,⁸ pH,⁹ and pressure.^{10,11} The reader is referred to the above cited articles for a more detailed discussion of the physiology of growth in these environments.

The depth of the reservoir will be an important con-

sideration for MEOR processes since it directly relates to two important parameters that affect microbial growth, temperature and pressure. Pressure increases with depth in the earth at an average rate of about 0.1 atm per m.¹⁰ Temperature increases with depth at an average rate of 0.027°C per m⁵ but major discontinuities in the temperature-depth profile occur. The use of mesophilic microorganisms will be restricted to shallow reservoirs.

Thermophilic microbes will be required for deeper reservoirs but the temperature-depth profile will restrict their use to reservoirs less than about 3500 m and in general, limit their application to reservoirs of depth less than 2500 m. Pressures of 100 to 200 atm generally do not affect bacterial growth¹⁰ and truly barophilic bacteria can grow at pressures up to 1035 atm.¹³ The tolerance to pressure varies from species to species and pressure effects become critical when the bacteria grow under suboptimal conditions of temperature, pH, etc.^{10,11} Thus, temperature would be a more important factor than pressure in restricting the application of MEOR processes. However, since pressure inhibits biochemical reactions involving volume increases,^{10,11} pressure may be an important consideration for *in situ* gas production by microorganisms.

The salt concentrations above 2 percent would inhibit the growth of most microbial species. Thus, marine bacteria which can grow in salt concentrations of 0.1 to 5 percent or moderate halophiles which can grow in salt concentrations of 2 to 20 percent would be required. Since most of the reservoir brines had pH range of 6 to 8, the pH of an oil reservoir would not be a serious limitation to MEOR processes.

The lack of certain essential nutrients such as sulfur, phosphate, and nitrate will pose severe limitations to MEOR processes unless these nutrients are added to the injection waters. A suitable carbon and energy source also will be required. Some microorganisms use various components of crude oil as a carbon and energy source; however, significant oil degradation does not occur unless molecular oxygen is present.¹⁴ The petroleum reservoirs are anaerobic with little if any available oxygen and it would be difficult to supply oxygen at the required amounts for hydrocarbon metabolism.

Based on the above information a thermophilic anaerobic bacterium that grows in 5 percent sodium chloride at neutral pH would be most suitable for MEOR processes. *In situ* growth could be achieved by supplying the required nutrients along with a cheap fermentable carbon source such as molasses. A large variety of thermophilic anaerobes has been characterized (Table 1) and Dr. E. Grula (this book) has recently isolated a variety of *Clostridium* species useful in MEOR processes. The environmental impact of *in situ* MEOR processes on the groundwaters and other drinking water sources needs further study. The requirement for nutrient additions may actually be a benefit to the operator and process control can be achieved by the appropriate medium formulation.

ENERGY METABOLISM IN SACCHAROLYTIC CLOSTRIDIA

The metabolism of all living cells is an open system with a continuous input and output of matter and energy. Every cell has the enzymatic capability to

TABLE 1—Thermophilic Anaerobic Bacteria^a

Group, Genus, Species	Temperature Limit, °C
Saccharolytic <i>Clostridium</i> species	
<i>C. thermoaceticum</i>	65
<i>C. thermosaccharolyticum</i>	65
<i>C. tartarivorum</i>	67
<i>C. thermohydrosulfuricum</i>	76
<i>C. thermocellum</i>	65
<i>C. thermocellulaseum</i>	70
Saccharolytic non-spore formers	
<i>Thermoanaerobium brockii</i>	85
<i>Thermobacterioides acetoethylicus</i> ..	85
<i>Thermoanaerobacter ethanolicus</i> ...	78
Sulfate-reducing bacteria	
<i>Desulfotomaculum nigrificans</i>	70
<i>Desulfovibrio thermophilus</i>	85
Sulfur-reducing bacteria	
<i>Thermoproteus tenax</i>	95
Methanogen	
<i>Methanobacterium thermoautotrophicum</i>	75

^a See (6,7,12) for a more complete list.

transform chemical or physical energy into biologically useful energy which is then used to perform work such as active transport of nutrients, active motility or biosyntheses. The universal carrier of biological energy is adenosine-5'-triphosphate (ATP). The synthesis of ATP from adenosine-5'-diphosphate (ADP) and inorganic phosphate (Pi) requires about +43.9 kJ/mol at physiological and reversible conditions (equation 1):¹⁵



Since life proceeds irreversibly the synthesis of ATP is not reversible. Part of the energy taken up by the cell is always dissipated as heat. Thus, the thermodynamic efficiency (η) (equation 2) of ATP synthesis is usually not higher than 60 percent.¹⁵

$$\eta = \frac{n \times \Delta G (ADP + Pi \rightarrow ATP)}{\Delta G (S \rightarrow P)} \times 100 \text{ percent} \quad (2)$$

where n = moles of ATP formed in the fermentation of substrate (S) to products (P).

The saccharolytic *clostridia* derive their energy mainly from the fermentation of sugars to acids, solvents, CO₂ and H₂ (Fig. 1).¹⁵ Glucose is fermented to

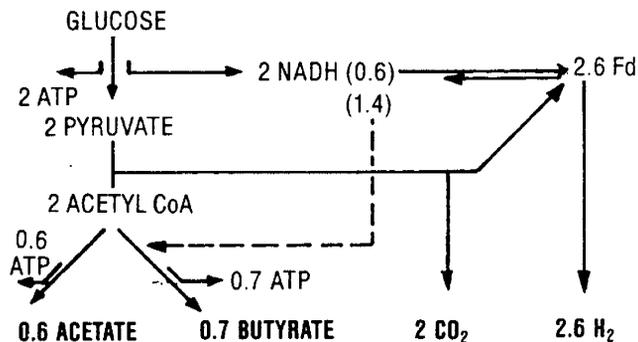


FIG. 1—Glucose fermentation products by saccharolytic clostridia. Products are underlined.

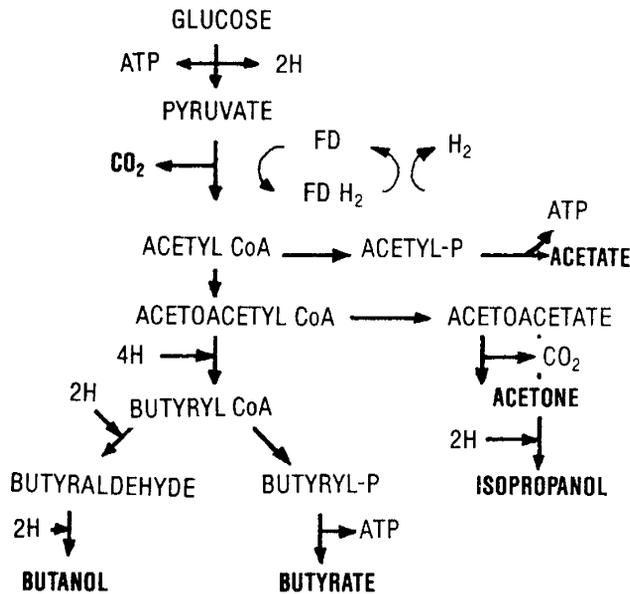


FIG. 2—Glucose fermentation by *Clostridium pasteurianicum*.¹⁵ Products are underlined.

pyruvate via the Embden-Meyerhof pathway with the production of ATP and reduced nicotinamide adenine denucleotide (NADH) designated at 2H in the figure. Pyruvate is further metabolized to acetyl coenzyme A (CoA) by pyruvate:ferridoxin (Fd) oxidoreductase. The reduced ferridoxin is oxidized by hydrogenase to yield H₂. Acetyl CoA can be further metabolized to the underlined products. The carbon flux in the different branches are adjusted to have optimal ATP and thermodynamic efficiency for the respective growth condition. Thus, the ATP/entropy quotient, i.e., the thermodynamic efficiency, is the term that is regulated.¹⁵

In the fermentation of glucose to butyrate, acetate, CO₂ and H₂ by *Clostridium pasteurianum* or *Clostridium butyricum* (Fig. 2), acetyl CoA occupies the branch point position in the overall fermentation and is stoichiometrically coupled to the NADH branch point. Both NADH and acetyl CoA are regulatory effectors controlling ATP and entropy generation by this branched metabolic pathway.¹⁵ The conversion of glucose to butyrate, CO₂ and H₂ yields 3 moles of ATP per mole of glucose with a thermodynamic efficiency of 52 percent.¹⁵

The conversion of glucose to acetate, CO₂ and H₂ yields 4 moles of ATP per mole of glucose with H₂ production from NADH: ferridoxin oxidoreductase plus ferridoxin hydrogenase.

The thermodynamic efficiency of ATP synthesis is 85 percent in this reaction sequence.¹⁵ Since glucose fermentation solely to acetate, CO₂ and H₂ has never been observed in *C. pasteurianum* and *C. butyricum*, it appears that such high efficiency is incompatible with the entropy requirements of clostridial metabolism. Thus, butyrate is always formed. The acetyl CoA/CoA ratio regulates the H₂ formed from NADH,¹⁵ adjusting the fraction of acetyl CoA converted to acetate and butyrate so that 3.3 moles of ATP per mole of glucose are obtained with a thermodynamic efficiency of 62 percent.¹⁵

The production of butanol instead of butyrate yields less ATP lowering growth yields. This shift towards

solvent production may be a response to an unfavorable environment or connected to a growth stage where maximum growth is no longer necessary such as the sporulation stage.¹⁶ Gottshalk and Bahl¹⁶ studied butanol production by *Clostridium acetobutylicum* grown in continuous culture at different pH values. Below pH 4.7, butanol appeared as a product and butanol concentration in the effluent was maximum at pH 4.3. Thus, butanol production in the reservoir may be controlled by alterations in pH. Further studies on the effects of medium components on butanol formation may lead to solvent production that is not preceded by acid production.

EFFECT OF NUTRIENT LIMITATION

Bacteria grow at an exponential rate (equation 3):

$$\frac{dx}{dt} = \mu x \quad (3)$$

where \bar{x} is the cell concentration (dry weight per unit volume) at time t , μ is the specific growth rate in units of reciprocal time. In equation 3, μ is constant only when all substrates required for growth are present in excess.

Monod¹⁷ showed that μ is proportioned to the substrate concentration according to equation 4:

$$\mu = \mu_{\max} \left(\frac{S}{K_s + S} \right) \quad (4)$$

where s is the substrate concentration, μ_{\max} is the maximum specific growth rate constant (i.e., the maximum value of μ at saturating substrate concentrations) and K_s is the saturation constant numerically equal to the substrate concentration at which $\mu = \frac{1}{2} \mu_{\max}$. From equation 4, it follows that exponential growth can occur at specific growth rates between 0 and μ_{\max} provided that the substrate concentration can be held constant. This is of great importance to *in situ* MEOR processes since the rate of microbial growth can be controlled by the rate at which substrate is provided. Models of *in situ* MEOR processes can be developed using equations 3 and 4, and fixed-film and substrate diffusion kinetics. For a more detailed discussion of the kinetics of bacterial growth the reader is referred to articles by Tempest¹⁸ and Herbert et al.¹⁹

In natural environments, growth conditions are often unstable and are rarely optimal. Rarely are all the essential nutrients present in sufficient concentrations to allow microbial growth to proceed at its maximum rate. There may be periods of fluctuations in these nutrients as well as in temperature, pH, ionic strength or some other environmental parameter. Microorganisms change their metabolism and physiology so that they are optimally adapted to this "feast or famine" existence.²⁰

Neijssel and Tempest²⁰ studied the effects of different nutrient limitations on the yield of cells (gram of cells produced per gram of substrate used), the rate of substrate utilization and the kinds of metabolites produced by *Klebsiella aerogenes* grown in continuous culture (Table 2). The carbon-limited cultures were characterized by efficient conversion of carbon into cell material with lower substrate utilization rates and minimal diversion to extracellular products. However, when growth was limited by the availability of a nutri-

TABLE 2—Effect of Nutrient Limitation on the Growth, Product Formation and Substrate Utilization of *Klebsiella aerogenes*^a

Growth Limitation	Milliatom Carbon per Hour			Yield (g/g Hexose)
	Glucose Used	Organic Acids Formed	Extracellular Polysaccharide	
Carbon	36.8	0	0	0.45
Sulfate	98.7	35.0	7.0	0.19
Ammonia	107.4	31.7	36.0	0.16
Phosphate	112.8	54.0	15.1	0.15
Potassium	175.0	78.8	0	0.09

^a Data of Neijssel and Tempest²⁰: All cultures were grown with glucose as the energy source at a dilution rate of 0.17 per hr, pH of 6.8 and temperature of 35°C. All values were adjusted to a cell carbon production rate of 20 milliatoms per hr.

ent other than carbon, less efficient conversion of the carbon to cell material was observed with higher carbon utilization rates and more carbon diversion to the production of extracellular products.

The extent and kinds of products produced depended on the nature of the growth limitation. Ammonia-limited cultures produced large amounts of extracellular polysaccharides (Table 2) and result in the overproduction of protease in *Bacillus licheniformis*.²¹ The amount of lactate and ethanol produced by *Clostridium thermohydrosulfuricum* depended on whether the culture was carbon or ammonia-limited.²² These studies have great implications for the control of *in situ* MEOR processes. Biopolymer production can be controlled by varying the amount of ammonia added to the injection waters. It would be interesting to determine if biosurfactant production could be controlled by appropriate nutrient limitation. For a more detailed discussion on the physiology of nutrient limitation the reader is referred to articles by Tempest and Wouters²¹ and Neijssel and Tempest.²⁰

CONCLUSION

The success of an *in situ* MEOR process depends on the selection of useful organisms that can function in the environment of the reservoir. Thermophilic, anaerobic microorganisms that grow in high salt concentrations (2-10 percent) at neutral pH would be suitable for most reservoirs. Knowledge of how nutrient limitation affects microbial growth and metabolism of the organism will be important for process control.

Nitrogen limitation may be effective in controlling biopolymer production. The kind of nutrient limitation as well as the thermodynamic efficiency of the metabolic pathway are important considerations in regulating the proportions of fermentation products produced by anaerobic bacteria. The pH of the reservoir will be important for optimal butanol production. Further work on the effects of nutrient limitation as a means to control *in situ* MEOR processes is needed using populations attached to surfaces as the experimental system.

REFERENCES

1. Davis, J. B. 1967. Petroleum Microbiology. Elsevier Publishing Co., New York.
2. Raleigh, J. T., and D. L. Flock. 1965. A study of formation plugging with bacteria. J. Petrol. Tech. 17:201-206.
3. Kalish, P. J., J. E. Stewart, W. F. Rogers, and E. O. Bennet. 1964. The effect of bacteria on sandstone permeability. J. Pet. Tech. 16:805-814.
4. Jenneman, G. E., R. M. Knapp, M. J. McInerney, D. E. Menzie, and D. E. Revus. 1982. Experimental studies of *in situ* microbial enhanced oil recovery. Proceedings of the Third Joint SPE/DOE Symposium on Enhanced Oil Recovery, pp. 921-932.
5. Clark, J. B., D. M. Munnecke, and G. E. Jenneman. 1981. *In situ* microbial enhancement of oil recovery. Devel. Indust. Microbiol. 22:695-701.
6. Tamsey, M. R., and T. D. Brock. 1978. Microbial life at high temperatures: ecological aspects. In: D. J. Kushner (ed.), Microbial Life in Extreme Environments, pp. 159-216. Academic Press, New York.
7. Brock, T. D. 1978. Thermophilic Microorganisms and Life at High Temperatures. Springer-Verlag, New York.
8. Kushner, D. J. 1978. Life in high salt and solute concentrations: halophilic bacteria. In: D. J. Kushner (ed.), Microbial Life in Extreme Environments, pp. 318-368. Academic Press, New York.
9. Langworthy, T. A. 1978. Microbial life in extreme pH values. In: D. J. Kushner (ed.), Microbial Life in Extreme Environments, pp. 279-317. Academic Press, New York.
10. Marquis, R. E., and P. Matsumura. 1978. Microbial life under pressure. In: D. J. Kushner (ed.), Microbial Life in Extreme Environments, pp. 105-159. Academic Press, New York.
11. Marquis, R. E. 1982. Microbial barobiology. Bioscience 32:267-271.
12. Ljungdahl, L. G. 1979. Physiology of thermophilic bacteria. Adv. Microbiol. Physiol. 19:149-243.
13. Yayanos, A. A., A. S. Dietz and R. Van Boxtel. 1981. Obligately barophilic bacterium from the Mariana Trench. Proc. Natl. Acad. Sci. USA 78:5212-5215.
14. Atlas, R. M. 1981. Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol. Rev. 45:180-209.
15. Thauer, R. K., K. Jungermann, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. 41:100-180.
16. Gottshalk, G., and H. Bahl. 1981. Feasible improvements in the butanol fermentation by *Clostridium acetobutylicum*. In: A. Hollaender (ed.), Trends in the Biology of Fermentation for Fuels and Chemicals, pp. 463-471. Plenum Press, Inc., New York.
17. Monod, J. 1949. The growth of bacterial cultures. Ann. Rev. Microbiol. 3:371-394.
18. Tempest, D. W. 1970. Theory and practice of continuous culture. In: J. R. Norris and D. W. Ribbons (eds.), Methods in Microbiology, vol. 2, pp. 260-327. Academic Press, London.
19. Herbert, D., R. Elsworth, and R. C. Telling. 1956. The continuous culture of bacteria: a theoretical and

experimental study. *J. Gen. Microbiol.* 14:601-622.

20. Neijssel, O. M., and D. W. Tempest. 1979. The physiology of metabolite over-production. *Sym. Soc. Gen. Microbiol.* 29:53-82.

21. Tempest, D. W., and J. T. M. Wouters. 1981. Properties and performance of microorganisms in chemo-

stat culture. *Enzyme Microb. Technol.* 3:283-290.

22. Zeikus, J. G., A. Ben-Bassat, T. K. Ny, and R. J. Lamed. 1981. Thermophilic ethanol fermentations. *In: A. Hollaender (ed.), Trends in the Biology of Fermentations for Fuels and Chemicals*, pp. 441-471. Plenum Press, Inc., New York.

Isolation and Screening of Clostridia for Possible Use in Microbially Enhanced Oil Recovery

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INTRODUCTION

Our research effort in MEOR is primarily directed toward isolation of naturally occurring species and/or strains of *Clostridium* that possess the metabolic characteristics we desire. Choice of *Clostridium* is based on reports of work carried out in eastern Europe (see report by D. Hitzman in these *Proceedings*), and the field studies reported by Johnson² (1979) and The Mobil group (see report by H. F. Yarbrough in these *Proceedings*).

Desired metabolic characteristics include the following: a) Production of large amounts of gas (primarily CO₂ to aid in repressurization of the reservoir and because miscibility of this gas in oil increases with increases in pressure effectively lowering the viscosity of crude oil). b) Production of large amounts of low molecular weight organic acids: Sufficient amounts of such acids should help to dissolve limestone (CaCO₃) rock thus leading to increased movement of bacteria through the reservoir as well as dislodgement of oil that was attached to such rock. c) Production of large amounts of lower molecular weight solvents, primarily alcohols and acetone. Presence of these compounds in sufficient quantities should result in solubilization of crude oil that is particle attached or trapped in rock pores. d) Production of large amounts of low molecular weight non-ionic emulsifiers that would form oil-in-water emulsions and thus result in "solubilization" of the crude oil from surface and rock pores.

Although each of the above metabolic end-products should function individually to bring about release of oil in a reservoir, the combined activities are not understood nor are we knowledgeable about the effects in a microenvironment such as would exist in a rock pore.

An additional result of bacterial growth might involve the physical displacement of crude oil from surfaces by growing bacateria.

It is necessary that all of the above desired metabolic end-products be produced from cheap and plentiful sources of carbon and energy such as molasses with some form of ammonia salt present as the nitrogen source. Our downwell parameters are limited to 5 to 7.5 percent salt concentration, 45°C and depths of less than 2500 ft.

As our studies have progressed, the major problem encountered has been to isolate clostridia capable of

growth and production of the desired metabolic end-products at the higher salt concentrations. It is not difficult to isolate organisms that will grow quite well in NaCl concentrations up to 10 percent (many can be obtained from the salt flats at Jet, Oklahoma); however, such organisms are not clostridia nor will they produce the desired metabolic end-products. Overall morphology of a typical *Clostridium* is shown in Fig. 1.

RESULTS AND DISCUSSION

Gas production—Although several techniques have been tried and utilized, two procedures are now in general use regarding gas production. When rough screening is being done and only a quantitative estimation of total gases is desired, distance of migration of a Vaspar plug is measured. A diagrammatic representation of this system is shown in Fig. 2. By knowing the distance the plug is pushed up a tube, it is fairly easy to

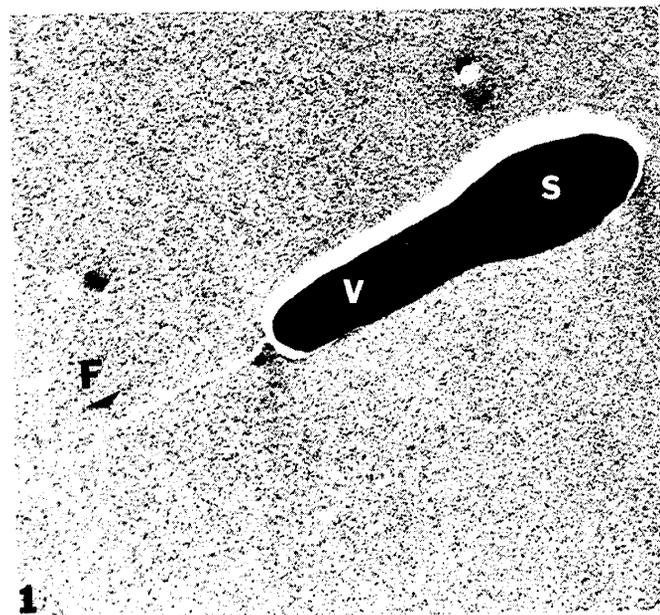


FIG. 1—Electron micrograph of clostridial isolate HR-3. This is a heavily shadowed preparation to show overall morphology. The bulging at one end is the spore (s) inside the vegetative cell (v). A single polar flagellum (f) is at the opposite end of the cell. Flagella are responsible for motility of the cells and should aid movement through a reservoir.

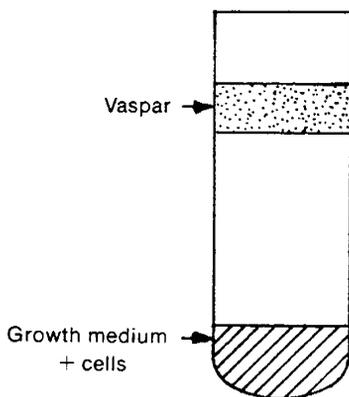


FIG. 2—A diagrammatic representation of the Vaspar plug procedure. Generation of gases by the growing cultures will move the plug up the tube; distance moved is directly proportional to the amount of gas produced.

calculate volume of gas produced at any time period without disturbing the culture.

Comparisons between cultures can then readily be made from the numbers obtained and plottings thereof. Test situations showing Vaspar plug movement in cultures in the presence of 1 and 5 percent NaCl are shown in Fig. 3. As can readily be seen, the higher level of salt can significantly inhibit production of gas in some cultures of clostridia.

The second major analytical procedure utilized for gas production that yields both qualitative and quantitative data is gas chromatography. Representative data from some promising cultures are given in Table 1. Although 5 percent levels of NaCl are generally inhibitory to varying degrees, some of the cultures shown perform better at the higher salt levels at both temperatures. It can also be pointed out that the major gas produced by all cultures thus far tested is CO₂. Small amounts of hydrogen can be detected in the gaseous phase of only a few cultures. Presumably any hydrogen gas produced is utilized as reducing power for formation of metabolic end-products by the bacteria. In addition, hydrogen produced by the cultures will react with any oxygen available in the anaerobic incubator to form water in the presence of the palladium coated alumina pellets that are present.

TABLE 1—CO₂ Production by Different Cultures of Clostridia After Growth for 72 Hours at 37° and 45°C in 1 and 5 Percent NaCl*

Culture Designation	37°C, Percent NaCl		45°C, Percent NaCl	
	1.0	5.0	1.0	5.0
72-B-1	507,927	361,002	360,895	AL
3-C	142,509	293,139	176,448	302,227
MX-1-B	AL	779,781	176,243	628,433
59 II	597,598	458,127	467,160	538,651
66D	366,068	424,065	492,295	437,765
MX-1A	402,485	319,978	325,641	AL
51B	265,540	187,528	258,412	324,827
66I	591,178	466,543	552,826	599,204

*Integrated values given in mm² (injection volume = 0.25 ml); AL = loss of anaerobic conditions.

Although we are not yet certain whether presence of CaCO₃ (limestone) stimulates metabolic production of CO₂ by cells from the sugar present or whether acids produced by the growing clostridia react with limestone yielding CO₂, it is apparent that when cells are permitted to grow in the presence of limestone, cultures produce significantly increased amounts of CO₂. Gaseous CO₂ would be produced from H₂CO₃ (carbonic acid) under saturating conditions of CaCO₃ (limestone).

Acid production—Representative data are incorporated into Table 2. In addition to serving as an extra substrate source for formation of CO₂, limestone can also be used as a buffer in place of phosphate. The latter will usually form insoluble precipitates with calcium or heavy cationic species known to be present in many drilling muds and found downhole; therefore, minimal concentrations of phosphates should be used in reservoir EOR operations.

It has been somewhat surprising to observe that outcroppings of limestone or sandstone that we have tested will supply needed phosphate and other inorganic ions necessary to obtain good growth of several clostridial isolates (Fig. 4). This is a third possible function for limestone in MEOR. Usually, one is told that reservoir rock is severely depleted of phosphates. Based on the data shown in Fig. 4, it appears advisable to determine rock content of phosphate and be guided by what is observed rather than automatically making such an addition to any downwell growth medium. It would appear that if reservoir rock is depleted of phosphates and other ions necessary for growth of bacteria, such depletion is most likely due to continued water flooding and along with toxic muds represents another way in which wells have, because of various treatments, been made less suitable for biologicals such as bacteria.

Solvent production—Representative data are incorporated into Tables 2 and 3. It is apparent that our isolates produce varying amounts of the different sol-

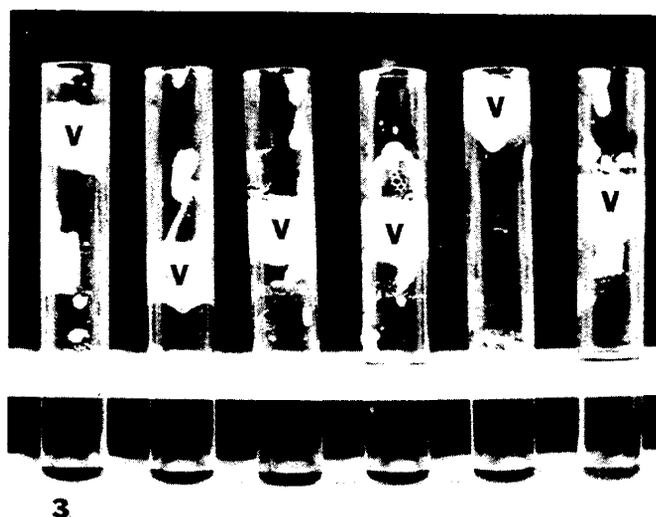


FIG. 3—Paired cultures growing in low (1,3,5) and high (2,4,6) concentrations of NaCl. At the higher salt concentration, growth and metabolic activity (including formation of gas) of cultures in tube 2 (MX-1A) and 6 (HR-14A1) are significantly inhibited. Culture MX-1B (tubes 3,4) was not significantly affected by the higher salt concentration. v = vaspar plug.

TABLE 2—Variations in Production of Solvents and Acids by Culture HR-4 After 1, 7 and 11 Weeks Growth in Different Media at 1 Percent Levels of NaCl*

Medium and Weeks of Growth	Solvents**							Acids**		
	MEOH, HCHO	ETOH	Acetone	Prop.	Isobut.	n-But.	Isoamyl.	Butyric	Isoval.	Val.
Sucrose										
1	570	—	—	—	—	—	—	—	—	—
7	582	461	328	508	492	1080	306	1881	11,712	—
11	1500	400	1100	250	1120	—	—	—	—	6500
TK4										
1	190	110	—	—	—	—	—	—	—	—
7	411	618	231	41	—	—	88	—	—	1321
11	2800	780	810	—	600	—	—	—	—	—
TSB										
1	888	1569	—	252	399	—	1426	—	—	—
7	2003	850	386	1284	—	4986	—	—	—	19,180
11	2800	3118	—	1110	—	3000	—	—	—	15,253
Grandma's Yellow										
1	41	—	—	—	—	—	—	—	—	—
7	504	173	—	484	—	—	—	996	—	88
11	1400	600	—	—	900	—	—	2500	—	7000
Beet										
1	57	—	—	—	—	—	—	49	—	—
7	1798	—	—	131	—	—	—	—	—	—
11	3400	500	1000	—	800	300	110	2100	—	8800
St. 95										
1	127	342	—	—	—	—	—	—	—	—
7	295	—	—	—	—	—	—	—	—	—
11	1000	500	—	—	800	—	—	—	—	—

*Experiment was started with 30 ml medium per flask. After 1 week, 15 ml were withdrawn and 15 ml fresh medium added. Thereafter 1 ml fresh medium was added each week.

**Integrated values given in mm² (injection volume = 2 μl). All identifications are tentative based only on gas chromatographic retention times. Formaldehyde and methanol were indistinguishable by our analysis. Abbreviations used: HCHO = formaldehyde; MEOH = methanol; ETOH = ethanol; Prop. = propyl; Isobut. = isobutyl; n-But. = n-butanol; Isoval. = isovaleric; Val. = valeric acid; TK-4, Beet, St. 95 and Grandma's Yellow are all different molasses products; TSB = trypticase soy broth.

vents at both temperatures. Higher levels of salt have, in most instances, a deleterious effect on production. Also, solvent production occurs over an extended period of time with only minimal supplementation of the growth medium (Table 2). It is to be emphasized that mass spectrometer data have not yet been obtained to aid in identification of these metabolic end products.

In addition to the diversity of solvents and acids formed, the variation that occurs in different media is interesting (Table 2). Further study needs to be done; however, the need for knowing the relationship between growth medium and metabolic end-products produced is apparent. Excellent possibilities exist that addition of specific nutrients to different organisms growing in various media will significantly aid in increasing yields of desirable end products either downwell, or in fermentation vats and such possibilities are being explored. Yield of the largest amount of end products occurs in trypticase soy broth which is the most complex medium utilized; but greatest variability occurred in the sucrose-salts medium at the 7 week time interval.

Production of emulsifiers—When this work was initiated, no reports existed of anaerobic bacteria that produced emulsifier compounds particularly in a medium lacking hydrocarbons. Since that time, it has been reported that *Clostridium pasteurianum* will produce an emulsifier under anaerobic conditions in the

absence of hydrocarbon precursors (Cooper, et al.³).

Of 53 new isolates that we have studied for emulsifier production in non-hydrocarbon-containing media, four gave a 4+ reaction, 7 a 3+ reaction and 11 a 2+ reaction. It can therefore be concluded that significant numbers of clostridia will produce emulsifier compounds under anaerobic conditions in non-hydrocarbon-containing simple media.

GROWTH OF 4 SELECTED CULTURES IN SUCROSE DEFINED MEDIUM WITH LIMESTONE ROCK AS BUFFER

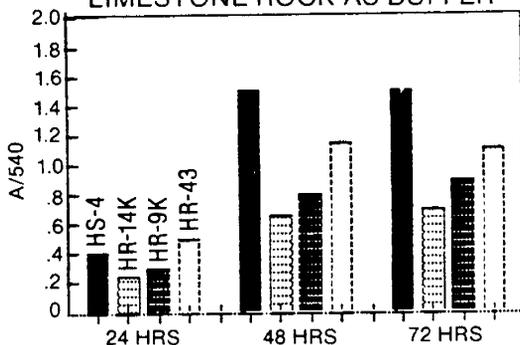


FIG. 4—Growth of selected cultures in limestone rock. Only molasses and ammonia salts were added and significant growth of all 4 cultures (HS-4, HR-14K, HR-9K, and HR-43) occurred.

Production of the emulsifier compound(s) by culture HR-3 is not decreased in 5 percent NaCl-containing media. Further, the emulsifying material is not precipitated or inactivated by Ca⁺⁺ ion and is stable to autoclaving (15 min at 121°C).

In the earlier stages of this study, measurement of "surfactant" production in spent culture media was accomplished using a DuNouy tensiometer and recorded as surface tension in dynes/cm. As other measurements were utilized, it became evident that a decreased surface tension does not necessarily mean that an emulsifier is present.

Data given in Fig. 5 show that alcohol solvents will significantly decrease surface tension. Although data are not shown, such alcohols do not bring about emulsification of hydrocarbons.

Emulsification data are readily obtained by measuring dispersal of a non-miscible hydrocarbon in water. A diagrammatic representation is given in Fig. 6. The technique we have utilized is basically that of Zajic, et al.¹ In place of kerosene, we utilized mineral oil (1.5 ml to 3.5 ml of spent culture supernatant) and left out the Oil-red-O dye. Size and stability of the oil-in-water micelles formed are the critical parameters to observe.

ESTIMATION OF EMULSIFYING ABILITY

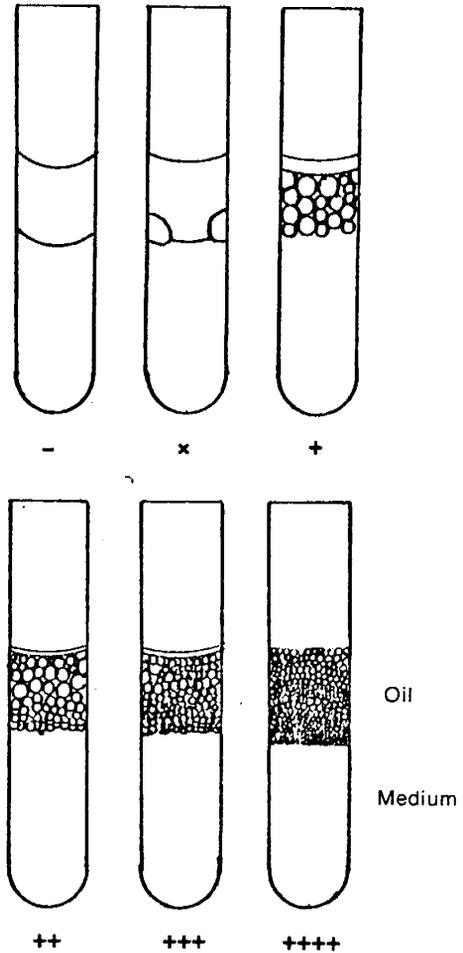
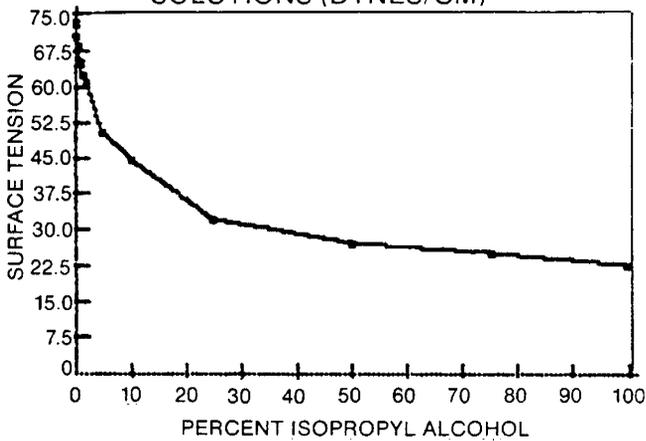
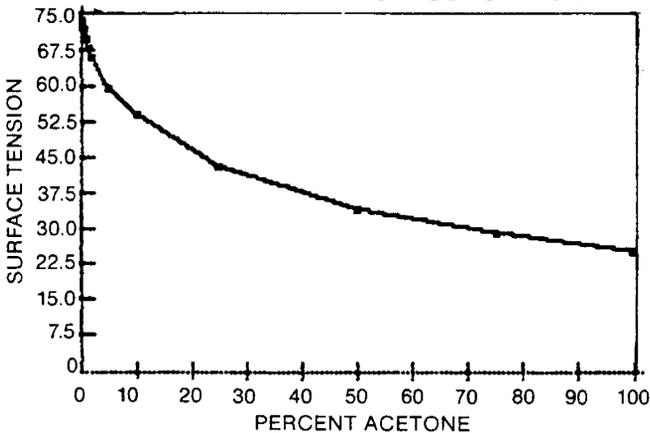


FIG. 6—Diagrammatic representation of various amounts of emulsification of mineral oil. (-) = no emulsification; (x) = questionable reaction; (1 to 4+) = various amounts of emulsification with a 4+ reaction being optimal.

SURFACE TENSION OF ISOPROPYL ALCOHOL SOLUTIONS (DYNES/CM)



SURFACE TENSION VS. ACETONE CONC



tension measurement of various concentrations hol and acetone.

Data given in Fig. 7 are intended to show that no positive correlation exists between surface tension reduction and emulsification ability.

Additional parameters studied—The so-called "Huff and Puff" technology (a single well used as a

SURFACE TENSION REDUCTION VS. EMULSIFYING ABILITY

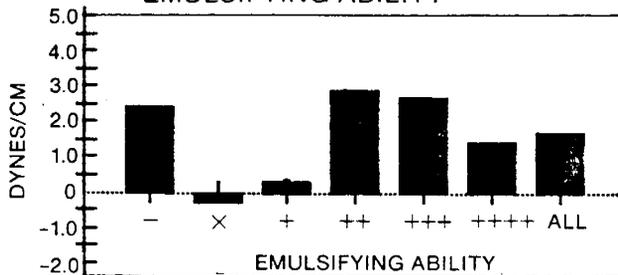


FIG. 7—Data on surface tension and emulsifying ability of cultures averaged out to demonstrate that a direct correlation between surface tension and emulsifying ability does not exist.

fermentation vat and release of oil occurs only in the vicinity of the well bore) will most likely not have to satisfy the more severe constraints that a "line-drive" type recovery imposes wherein live bacteria and their food must travel for significant (several hundred feet) distances underground continually producing their metabolic end-products to bring about release of the crude oil.

To function satisfactorily, particularly in line-drive recovery operations, it appears that, in addition to synthesis of desired metabolic end-products under the physical conditions existing in the reservoir, some additional parameters are important for the overall success of the operation. Some negative factors include the following. a) Production of H₂S downwell by cultures placed into the well. b) Stimulation of undesirable organisms indigenous to the reservoir by the molasses medium. c) Possible plugging in the vicinity of the well bore by vegetative cells or produced capsular type material. d) Antagonism by organisms present in the reservoir waters or water used for compounding the molasses medium. e) Toxic ions or compounds present in drilling muds or waters used in flooding operations.

A positive factor that must be addressed includes ability of the cells to sporulate in the nutrient medium while moving through the reservoir. This can be viewed as an aid to survival of the culture in the event of interrupted nutrient availability. Disregarding surface charge effects, movement of spores should be better

than vegetative cells simply because they are shorter in length; thus sporulating ability is also related to movement of the bacteria through the reservoir.

We've really only begun our studies and much remains to be accomplished. All of the above listed parameters are or will be addressed shortly. Needless to say, field tests will be absolutely essential to determine what happens downwell.

ACKNOWLEDGEMENT

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REFERENCES

1. Zajic, J. E., H. Guignard, and D. F. Gerson. 1977. Emulsifying and surface active agents from *Corynebacterium hydrocarboclastus*. *Biotech. and Bioengr.*, 19: 1285-1301.
2. Johnson, A.C. 1979. Microbial oil release technique for enhanced recovery. Conference on Microbiological Processes Useful in Enhanced Oil Recovery. Final Report. Aug. 29-Sept. 1, San Diego, Calif., U.S. Dept. Energy Publication, contract DE-AT1-78MC08333.
3. Cooper, D. G., J. E. Zajic, D. F. Gerson and K. E. Manninen. 1980. Isolation and identification of biosurfactants produced during anaerobic growth of *Clostridium pasteurianum*. *J. Ferment. Technol.* 58: 83-86.

TABLE 3—Solvent Production by Selected Cultures of Clostridia Grown for 72 Hours at 37° or 45°C in 1 and 5 Percent NaCl*

Culture Designation, Conc. NaCl, and Temp.	MEOH or HCHO	ETOH	Acetone	Isobut.	Isoprop.	n-But.	Isoamyl.
37°C							
MX-1-B, 1	626	13,024	361	396	—	—	175
MX-1-B, 5	669	11,241	115	831	—	—	—
51B, 1	559	2,175	—	5101	123	—	—
51B, 5	707	418	—	1870	206	—	—
66D, 1	578	4,095	—	565	219	—	—
66D, 5	686	4,861	—	313	334	—	—
45°C							
59 II, 1	442	3,959	—	—	1428	—	—
59 II, 5	624	3,881	—	—	878	—	—
72-B-1, 1	181	3,707	—	1188	—	—	—
72-B-1, 5	551	2,260	286	1284	—	—	—
63-A, 1	728	—	358	—	—	—	—
63-A, 5	687	1,924	54	367	—	—	—

*Integrated values given in mm² (injection volume = 2 μl). All identifications are tentative based only on gas chromatography retention times. Formaldehyde and methanol are indistinguishable by our analysis. Abbreviations used: HCHO = formaldehyde; ETOH = ethyl alcohol; Isobut. = isobutyl alcohol; Isoprop. = isopropyl alcohol; n-But. = n-butanol; Isoamyl. = isoamyl alcohol.

Microbial Activity in Waterflooded Oil Fields and Its Possible Regulation

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Various techniques have been reported in literature used to enhance oil recovery from oil-bearing formations: cold and hot water flooding, steam treatment, polymeric flooding, underground combustion, water flooding with surfactants, employment of microbes as oil-release agents, etc. Great hopes are placed on microbiological approaches and solution to this problem.

Most microbiological techniques are based on the introduction of microbial cultures and nutrients into formations,^{5,14,16,19,25} while some others consist in the improvement of oil-washing characteristics of injected water by microbial products: heteropolysaccharides, enzymes, culture liquid.^{7,9,13,18} Andreevsky^{1,2} proposed to activate the natural microflora of oil fields to enhance oil recovery.

Unfortunately, most microbiological recovery processes have not been tested in field conditions or were unsuccessful,⁵ except for some experiments in oil fields of Czechoslovakia, Hungary, USSR and Poland.^{6,12,15,16} Oil recovery was enhanced for some period of time by introduction of gas producing microbes and molasses into formations. Viable bacterial cells were revealed by microbiological tests in stratal water sampled in experimental regions. However, there were no convincing evidences of the microbial activation in stratal conditions.

We think that the practical employment of microbiological techniques should be based first of all on our knowledge of the distribution and activity of microorganisms in formations and their possible regulation during exploitation of oil fields.

Numerous papers are devoted to the studies of the oil field microflora and give primary consideration to sulfate-reducing and hydrocarbon-oxidizing bacteria.^{5,22} Other microbial groups, methanogens in particular, have received inadequate attention.

The purpose of the present work was to study the distribution of various microbial groups, to determine the intensity of bacterial methanogenesis in stratal waters, and to find the possibility of the activation of recent methanogenesis in flooded oil fields.

MATERIALS AND METHODS

The main subject of experiments started in 1977 has been the Bondyuzhskoe oil field in the northwest Tatar ASSR. Oil collectors in this field are sandstones and siltstones of the Upper Devonian (formations D₀ and D₁). Sandstone oil collectors are prevailing. The aver-

age porosity of oil-bearing sandstones was 21.7 percent, and permeability was about 570 millidarcies. The oil-bearing horizons occur at the depth of 1500-1700 m, their temperature is 30-40°C. Oils in the Bondyuzhskoe oil field contain 2.0-2.2 percent S and 4.0-4.1 percent paraffin. Relative oil density is 0.871-0.876 g/cm³.²⁰

Some experiments have been performed in the Romashkinskoe oil field situated in the south apex top of the Tatar anticlinal fold and its slopes. Commercial oil pools occur in the Upper Devonian formations. Oils from Romashkinskoe and Bondyuzhskoe oil fields are quite similar in composition.

Chemical and physico-chemical analyses of stratal waters were made by standard techniques.²¹ Hydrocarbon-oxidizing, sulfate-reducing, methanogenic and other bacteria were counted using a variation of the method of 10-fold dilutions.¹¹ Methanogenic activity was determined by the radioisotope method. Radioactive carbon was used in the form of NaH¹⁴CO₃ and ¹⁴CH₃COONa. Stratal water samples with radioactive isotopes were incubated no more than 2 days at about 30°C. The radioactivity of methane produced was registered by a scintillation counter with the preliminary methane combustion to CO₂.¹⁰

The carbon isotope composition of methane and carbonates was determined using the mass spectrometer CH7 Varian Mat by the double-beam compensation method with CO₂ as an operating gas. The isotope composition of carbon was expressed in δ¹³C values:

$$\delta^{13}\text{C percent} = \left[\frac{(^{13}\text{C}/^{12}\text{C}) \text{ sample}}{(^{13}\text{C}/^{12}\text{C}) \text{ standard}} - 1 \right] \cdot 1000$$

in per mille with reference to the International Standard PDB.^{3,4} Methane was purified from its gaseous homologues on molecular sieves (4A°, 5A° and 13A°); methane purity was controlled by gas chromatography.

RESULTS AND DISCUSSION

Microflora and recent methanogenesis in the Bondyuzhskoe oil field

Stratal waters in the Devonian formations of the region under study belong to the chloride-calcium type with the specific density of 1.18-1.19 g/cm³ and total mineralization of 250-300 g/l; pH of brines is about 5.0.²³

Tables 1 and 2 show that highly mineralized waters of production wells 66, 306 and 316 approximate to the natural ones of the Devonian formations. They are rather acidic, the content of bicarbonate is low, and concentration of organic matter (C_{org}) is high. The lack of viable aerobic and anaerobic bacterial cells is the most important characteristic of these waters which makes it possible to draw a conclusion about the secondary nature of microorganisms occurring in freshened stratal waters of the oil field. These microbes enter the formation together with injected surface water.

Similar results were reported earlier by Kuznetsova et al¹⁷ who studied the sources of contamination of oil-bearing formations D_1 of the Romashkinskoe oil field with sulfate-reducing bacteria.

Fresh waters injected to the Bondyuzhskoe oil field to maintain the intrastratal pressure were weakly alkaline with low concentrations of bicarbonate and acetate. They contained oxygen and appreciable amounts of aerobic and anaerobic microbes (Tables 1 and 2). Contacting the petroliferous strata, the floodwater not

TABLE 1—Distribution of Aerobic Microorganisms in Waters of the Bondyuzhskoe Oil Field

Well, N	Mineralization, g/l	O ₂ , mg/l	pH	Eh, mv	Total Count, Cells·ml ⁻¹ ·10 ⁶	Bacterial Number, Cells·ml ⁻¹		
						Saprophytes	C ₁₄ H ₃₀ Oxidizers	Oil Oxidizers
Injected Surface Water								
428	0.7	6.2	7.3	+405	8.7	—*	2500	600
28	0.8	8.2	7.8	+330	10.0	—	2500	2500
Highly Freshened Oil Field Brine								
348	8.9	0	6.9	+ 80	8.0	750	60	25
265	10.0	0	7.0	+ 65	4.0	250	130	25
295	15.0	0	7.0	+100	4.5	300	25	60
47	30.0	0	6.9	+ 80	2.6	250	60	25
303	45.0	0	6.9	+100	3.8	170	130	25
94	58.0	0	6.8	+ 75	7.0	300	250	60
254	80.0	0	6.7	+ 65	4.0	200	25	60
Weakly Freshened Oil Field Brine								
371	140.0	0	6.6	+130	1.9	50	0	0
370	150.0	0	6.4	+115	2.3	80	25	6
323	170.0	0	6.5	+150	1.5	—	60	6
Highly Mineralized Oil Field Brine								
316	233.2	0	5.8	+140	0.7	0	0	0
306	234.4	0	5.7	+150	—	0	0	0
66	239.6	0	5.9	+150	—	0	0	0

*Note: not analyzed.

TABLE 2—Anaerobic Microorganisms in Waters of the Bondyuzhskoe Oil Field

Well, N	Mineralization, g/l	Eh, mv	Bacterial Number, Cells·l ⁻¹		Methanogenesis Rate, ml CH ₄ ·l ⁻¹ ·10 ⁶ per day	Content, mg·l ⁻¹		
			Sulfate Reducers	Methanogens		C _{org}	CH ₃ COOH	HCO ₃ ⁻
Injected Surface Water								
428	0.7	+405	4000	25000	0	—	0.7	102.0
28	0.8	+330	6000	1300	0	—	0.3	97.6
Highly Freshened Oil Field Brine								
348	8.0	+ 80	9000	6000	145.7	5.0	3.0	348
265	10.0	+ 65	8000	2500	106.0	12.0	2.9	358
295	15.0	+100	250	6000	227.0	16.1	1.0	373
47	30.0	+ 80	1600	2500	195.0	12.0	1.4	361
303	45.0	+100	4000	2500	305.0	5.0	1.4	348
94	58.0	+ 75	250	600	181.0	6.0	0.6	347
254	80.0	+ 65	1500	250	159.6	31.5	1.3	326
Weakly Freshened Oil Field Brine								
371	140.0	+130	3000	—	14.8	38.0	0.3	232
370	150.0	+115	0	250	28.9	124.0	2.4	272
323	170.0	+150	0	0	0	145.4	1.7	159
Highly Mineralized Oil Field Brine								
316	233.2	+140	—	0	0	244.0	12.0	110
306	234.4	+150	0	0	0	315.0	27.3	85
66	239.6	+150	0	0	0	290.5	29.1	85

only brought the viable microflora to stratal liquids, but also changed their composition and characteristics.

Stratal waters in the injection regions may be conditionally divided into two types by the mineralization level. The first are highly freshened waters with mineralization up to 80 g/l; pH 7.0. The values of their redox potential from +65 to +100 mv are the lowest compared to those of other waters of the field (Table 1).

Oxygen was not found even in the most freshened waters sampled in the immediate vicinity to injection wells (348 and 265). This is due to a rapid O₂ consumption by microbes for the aerobic destruction of organic matter. The total bacterial number as well as the numerical strength of specific bacterial groups were rather high but still lower than in the floodwater. This may be explained by the fact that some of the microorganisms entering the formation with injected water are unable to withstand stratal conditions and die.

The second type waters are weakly freshened with mineralization level of 140-170 g/l. They are more acidic and their Eh values are higher (Table 1). Such waters contain less viable cells of aerobic bacteria than highly freshened ones.

Distribution of anaerobic microorganisms in waters of the Bondyuzhskoe oilfield occurs similarly (Table 2). The numbers of sulfate-reducing and methanogenic bacteria in freshened stratal waters were somewhat lower than in the injected ones, the discrepancy however was not so large as in the case of aerobic ones. The number of anaerobic bacteria was higher in strongly freshened water and might be as great as several thousands cells per litre.

Pure cultures of methanogenic bacteria were isolated from the floodwater of injection well 428 and stratal water of production well 47. The study of the morphology and physiologo-biochemical peculiarities of these organisms allowed us to identify them respectively as *Methanobacterium bryantiistr. Omelianski* and *M. formicum strain Kuznetsovii* since they differed from typical strains.²⁴

Using radioactive isotopes it was shown that methanogenic bacteria were active in conditions of stratal waters of the oil field. The total rate of methanogenesis resulting of the sum of CO₂ reduction by hydrogen and reduction of methyl groups of acetate, was from 14.8 to 305.0·10⁻⁶ ml CH₄·l⁻¹·24 h⁻¹ (Table 2). The bulk of biogenic methane was formed by CO₂ reduction.

As seen from Table 2 and Fig. 1, the highest rate of methane production was observed in highly freshened waters with mineralization of 15-45 g/l. The intensity of methanogenesis dropped in stratal waters with higher mineralization level. Methanogens were not found in the water with the total salinity of 170 g/l and higher. High concentrations of dissolved organic matter including acetate and an appreciable decrease of bicarbonate content in highly mineralized brines point to a subsidence of all the microbiological processes in this zone.

The study on the carbon isotope composition of methane and bicarbonate carbon in stratal waters in the Bondyuzhskoe oil field yielded additional information on recent methanogenesis. Fig. 2 shows that in regions of recent methanogenesis methane is significantly enriched with ¹²C compared to methane from highly mineralized waters. On the other hand, carbon

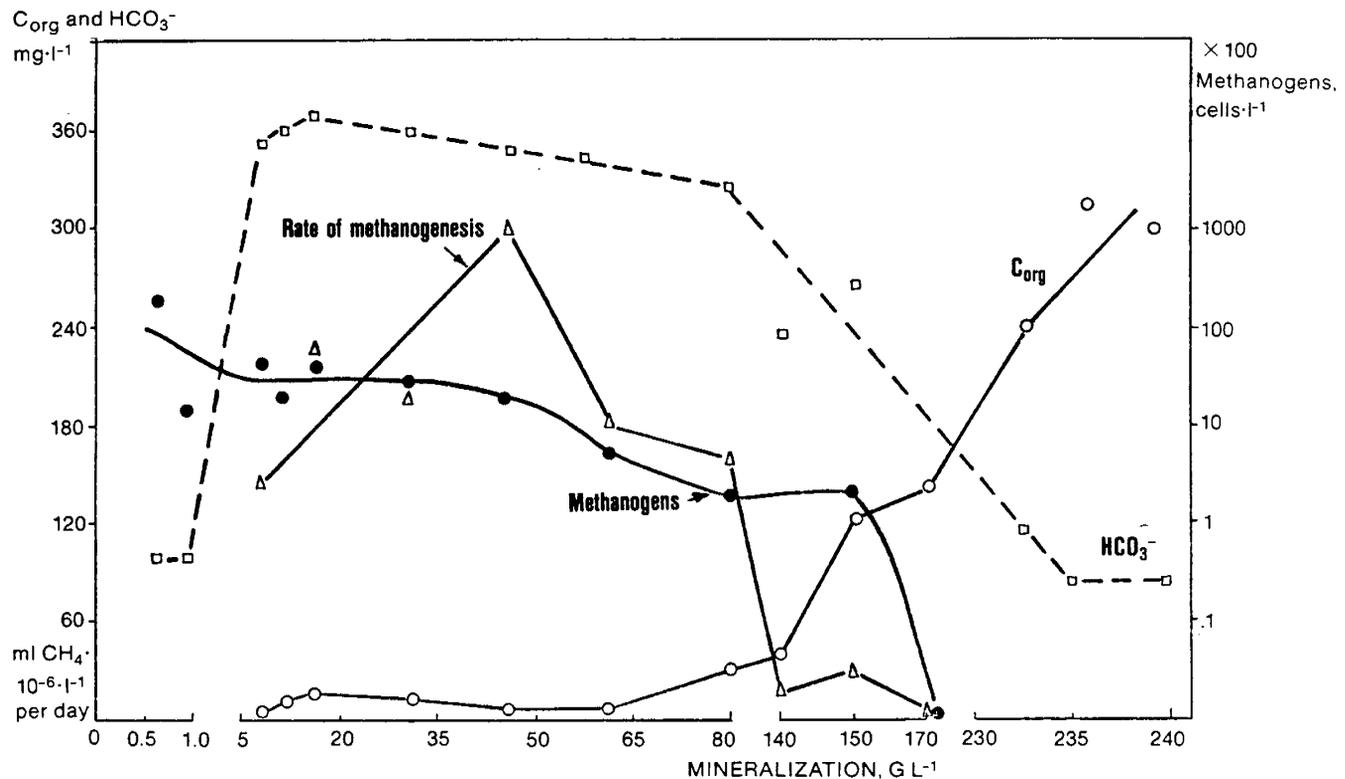


FIG. 1—Physico-chemical and microbiological characteristics of the Bondyuzhskoe oil field waters.

Microbes and Their Metabolites

of bicarbonates in freshened waters is heavier by the isotope composition than carbon of highly mineralized ones.

The major source of mineral carbon in stratal waters is microbial oxidation of oil hydrocarbons with the carbon isotope composition over the range from -25 to -28 percent. The accumulation of heavy isotopes in carbonate carbon may occur only during microbial CO_2 reduction to methane. A light isotope composition of methane carbon in freshened stratal waters is additional evidence of an active microbial methanogenesis in this part of the oil field.

Therefore, microbiological and isotopic investigations demonstrated a significant contribution of recent biogenic methane to the gas composition of stratal waters of the oil field under study and indicated that CO_2 reduction by hydrogen is the main pathway of bacterial methanogenesis.

Microbiological processes in the zone of contact between injected and stratal waters

The microflora and microbial processes occurring in the zone of contact between injected and stratal waters which differ by their composition and properties are of peculiar interest.

To determine the distribution of microorganisms and the methanogenesis rate in the zone of contact, the injection of surface water was stopped and the injection well was placed into the condition of reverse self-discharge. Water samples were taken successively during self-discharge in order to characterize chemical and microbiological processes in the zone of contact.

Table 3, Figs. 3 and 4 show that during flooding of the Bondyuzhskoe and Romashkinskoe oil fields, some

quantity of oxygen is introduced in the petroliferous stratum together with the injected fresh water (samples 1, 2, 8, 9 in Table 3). However, oxygen in the zone of contact is rapidly consumed and redox potential drops after the discharge of 5-7 m^3 of water. Simultaneously, the total number of bacteria and the number of oil-oxidizing bacteria increase significantly. The content of dissolved organic carbon and bicarbonate also increases.

The emergence of anaerobic conditions in the zone of contact and the enrichment of water with soluble organic matter and CO_2 activate microbiological processes, in particular, methanogenesis (samples 3 and 10 in Table 3). The rate may be as great as $(10.7-17.8) \times 10^{-4} \text{ ml CH}_4 \cdot \text{l}^{-1} \cdot 24 \text{ h}^{-1}$ (Figs. 3, 4).

The total number of bacteria and the number of oil-oxidizers diminished as water was further discharged. The content of C_{org} and the rate of bacterial methanogenesis also decreased (Table 3). They approximated gradually to analogous values characteristic of freshened stratal waters of the oil fields under study.

From the experimental data we infer that aerobic bacterial oxidation of the residual oil occurs in the zones of contact when fresh, oxygen-containing water is introduced into oil pools. This results in an increase of concentrations of soluble organic compounds and bicarbonate which after entering the anaerobic zone stimulate bacterial methanogenesis.

The last conclusion was confirmed experimentally when determining the methanogenesis rate by the radioisotope method. For this purpose the enrichment culture of oil-oxidizing bacteria isolated from the stratal water of the Bondyuzhskoe oil field was grown on a mineral medium with NH_4Cl and crude sterile oil (5 percent

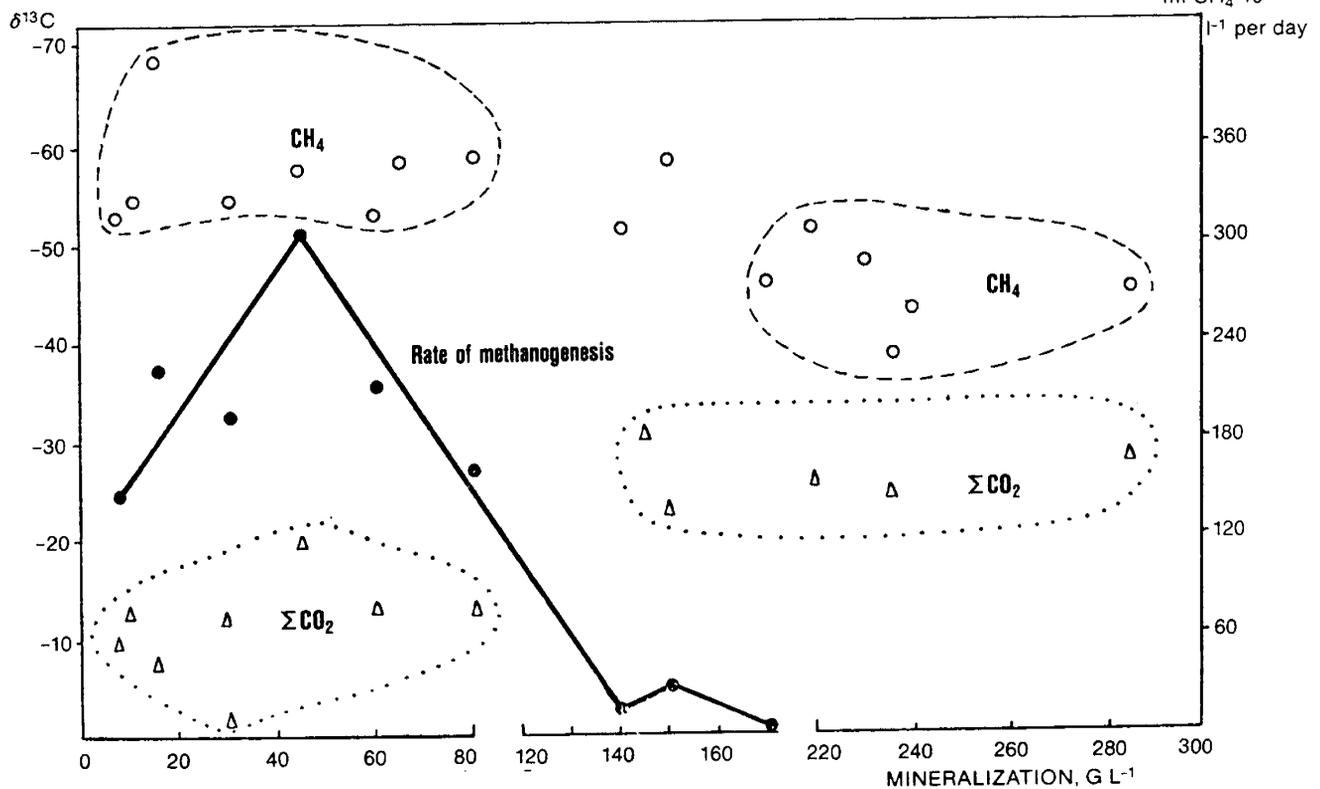


FIG. 2—Modern geochemical activity of methanogens in the Bondyuzhskoe oil field.

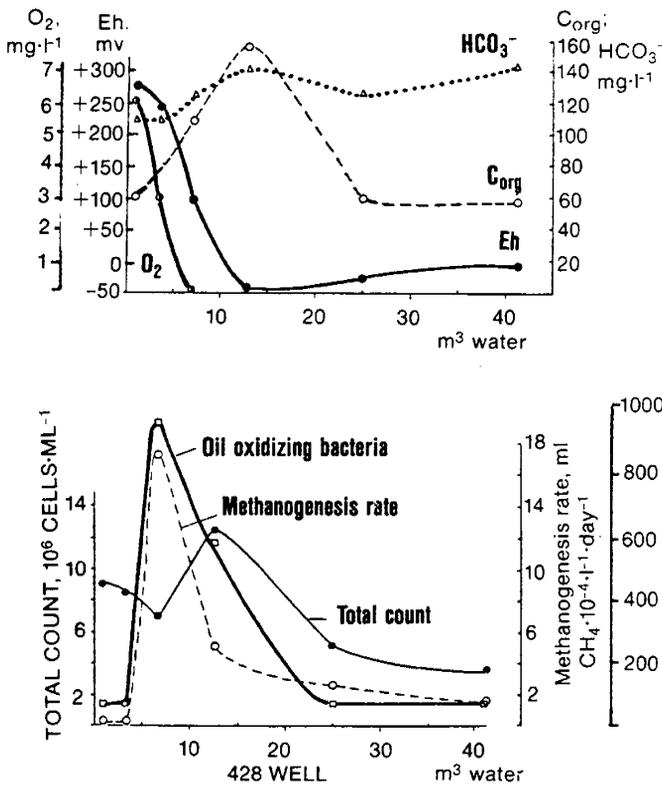


FIG. 3—Microbiological processes in the zone of contact between injected and stratal waters of the oil field.

by volume) at 28°C for 10 days. Bacterial growth was accompanied by the decomposition of oil. The culture liquid consisted of (mg·l⁻¹):11.5 methanol, 40.0 ethanol, 127.0 acetic acid, 5.0 proprionic acid, 2.0 butyric acid and other unidentified organic compounds. Sterile cultural liquid was added to flasks containing stratal water. Since the cultural liquid contained ammonium and phosphate the additional experiments were per-

formed when only these mineral compounds were introduced into the stratal water.

Table 4 and Fig. 5 present data on influence of additives on the methanogenesis rates. Probably there is no need in a detailed analysis of the results obtained for each well. It should be noted however that in highly-mineralized water of well 316, where total mineralization was 233.3 g·l⁻¹, methanogenesis was not observed in all the variants of the experiment. The addition of ammonium activated appreciably methanogenesis in four wells of six, the addition of phosphate had the same effect in five wells of six. The addition of cultural liquid to the stratal water stimulated methanogenesis in all the cases, and its rate increased 6-60 fold.

Further experiments involved the studies of bacterial methanogenesis on mineral media with products of aerobic oil destruction as the sole energy source. Oil was destroyed by the binary bacterial culture: *Pseudomonas sp.* and *Micrococcus rubrum* isolated from the same oil field. After incubation, the cultural liquid, which was a brown homogenous suspension, was used as a substrate for methanogenic bacteria in two variants of the experiment: 1) in the first case the culture liquid was used per se; 2) while in the second it was supplemented with an equal amount of mineral medium.

The control was mineral medium with crude sterile oil (5 percent by volume) just inoculated with the binary culture of aerobic oil-oxidizers. All the three media were placed into anaerobic conditions and inoculated with active enrichment culture of methanogenes isolated from the Bondyuzhskoe oil field. Anaerobic incubation was performed on a shaker for 10 days at 37°C.

Table 5 shows that the culture liquid contains significant amounts of acetate, ethanol and methanol. Besides, the presence of formiate and other organic compounds was shown qualitatively. Trace amounts of low-molecular weight compounds were present also in the control medium and conditioned evidently the formation of some methane.

TABLE 3—Microbial Distribution and Methanogenesis Rate in the Zone of Contact Between Injected and Stratal Waters of the Oil Fields

Sample, N	Volume of Ejected Water, m ³	Eh, mv	Content, mg·l ⁻¹			Microbial Number		Methanogenesis Rate, ml CH ₄ ·10 ⁻⁴ ·l ⁻¹ per day
			O ₂	HCO ₃ ⁻	C _{org}	Total, Cells·ml ⁻¹ ·10 ⁶	Oil Oxidizers, Cells·ml ⁻¹	
Well N 428 (Bondyuzhskoe Oil Field)								
1.	0.5	+280	6.2	109.8	—	8.7	60	0
2.	3.5	+240	2.7	109.8	60	8.3	60	0
3.	7.0	+ 80	0	128.1	110	6.9	1100	17.8
4.	12.5	- 40	0	146.4	160	13.1	600	4.16
5.	25.0	- 20	0	128.1	60	5.3	60	2.8
6.	42.5	- 10	0	146.4	—	3.5	60	1.6
7.	166.0	- 10	0	146.4	60	2.6	10	2.1
Well N 6334 (Romashkinskoe Oil Field)								
8.	0.5	+320	7.1	225.7	60	2.4	60	0
9.	2.5	+235	5.4	219.6	—	3.4	250	0
10.	5.0	+ 80	0	292.8	170	9.5	250	10.7
11.	10.0	+ 40	0	317.2	220	3.0	600	2.3
12.	15.0	+ 30	0	329.4	110	2.3	60	1.5
13.	30.0	+ 20	0	317.2	110	1.4	60	2.8

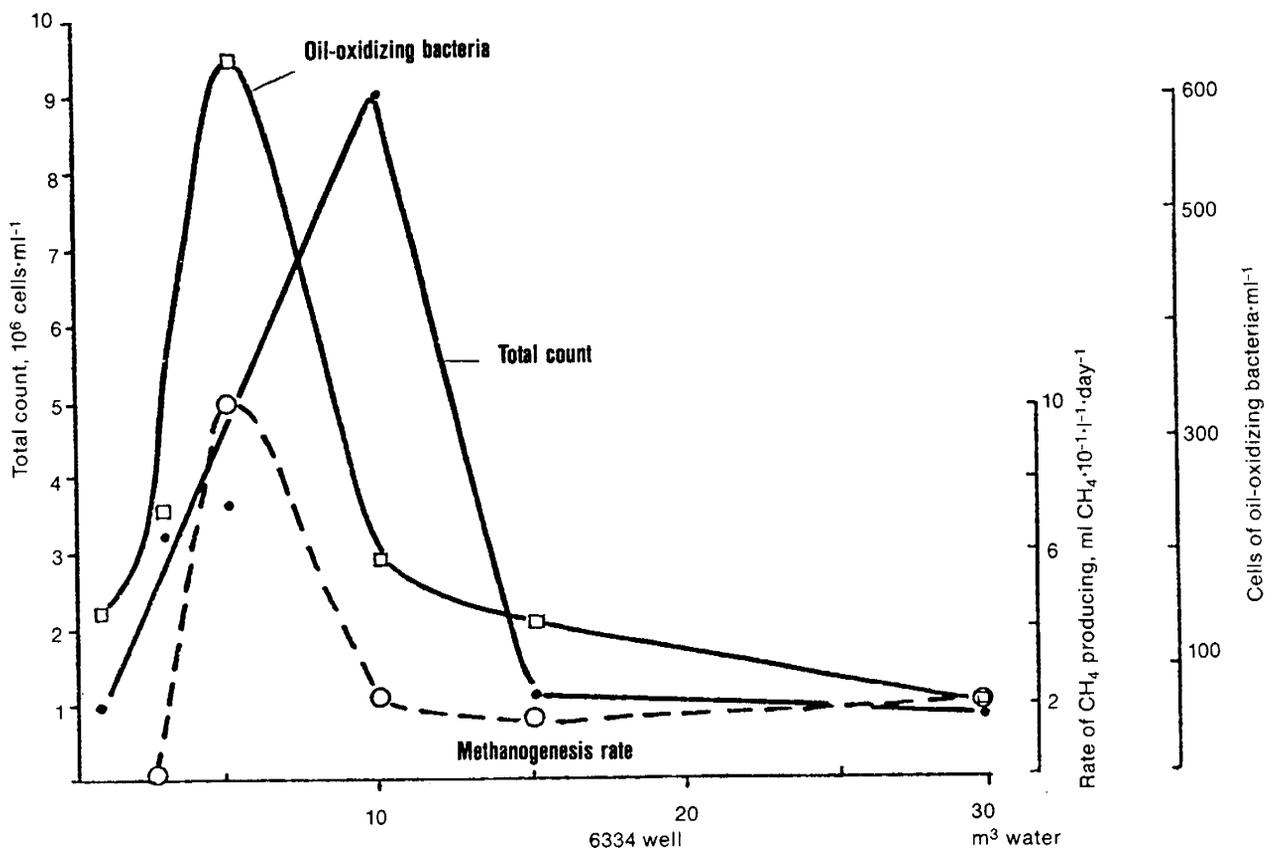
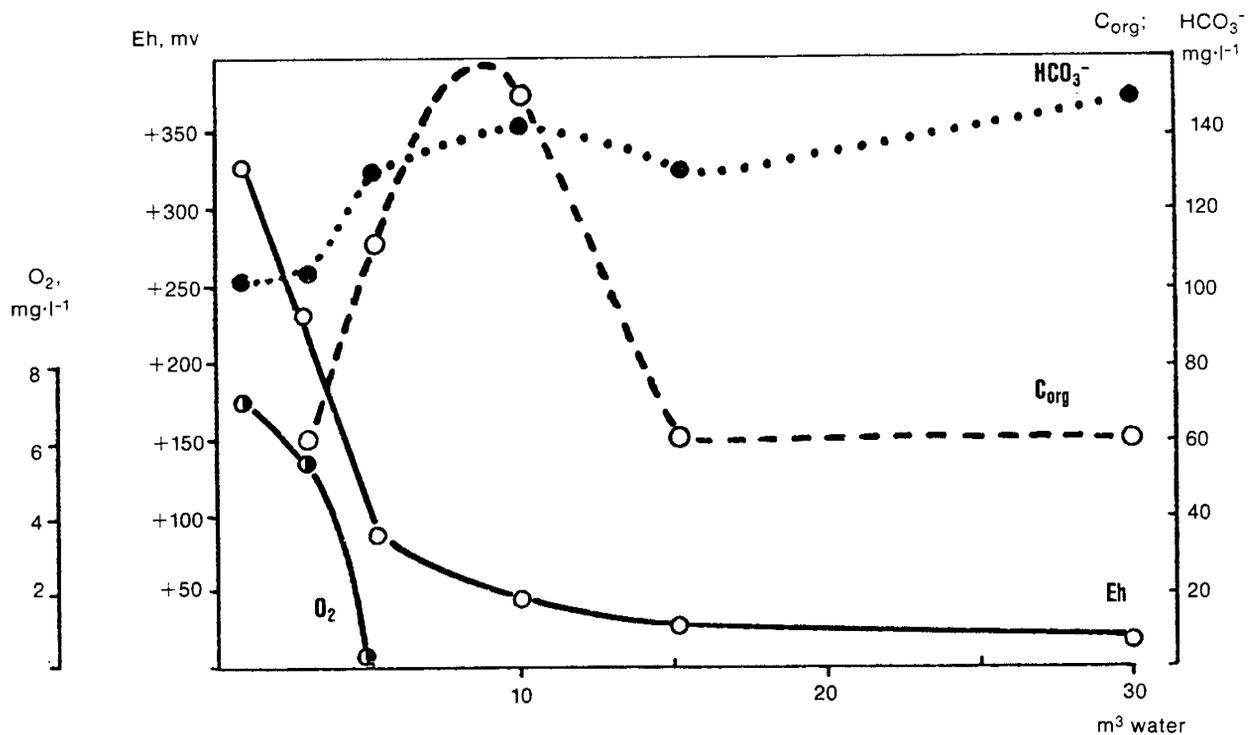


FIG. 4—Microbiological processes in the zone of contact between injected and stratal waters of the oil field.

TABLE 4—Effect of Additives on Methanogenesis Rate in Stratal Waters of Bondyuzhskoe Oil Field

Well, N	Mineralization, g/l	Content in Stratal Water, mg·l ⁻¹		Methanogenesis Rate, ml CH ₄ ·10 ⁻⁶ ·l ⁻¹ per day			
		NH ₄ ⁺	PO ₄ ³⁻	In Stratal Water (no additives)	Addition of NH ₄ ⁺ **	Addition of PO ₄ ³⁻ **	Addition of Culture Liquid***
254	20.0	26.5	0.36	52.8	54.4	252.6	301.6
210	24.0	16.0	0.36	81.4	156.3	102.7	675.3
47	30.0	14.0	0.36	35.3	29.8	118.9	386.2
296	134.0	31.5	0.12	3.1	20.5	26.6	181.4
269	150.4	59.8	0.24	7.2	67.4	16.7	310.2
251	173.6	64.0	0.36	6.4	23.0	6.3	111.0
316	233.2	72.0	0.12	0	0	0	0

*NH₄⁺ concentration in stratal water increased by 198 mg·l⁻¹.

**PO₄³⁻ concentration in stratal water increased by 92 mg·l⁻¹.

***Concentrations of NH₄⁺ and PO₄³⁻ increased by 34 mg·l⁻¹ and 107 mg·l⁻¹, respectively; acetate and ethanol (alongside other organic compounds) were added in amount of 5.6 mg·l⁻¹.

TABLE 5—Microbial Methanogenesis from Products of Aerobic Oil Destruction

Experiment	Products of Aerobic Oil Destruction, mg·l ⁻¹ Crude Oil						CH ₄ Formation ml·l ⁻¹ Crude Oil
	Acetic Acid	Propionic Acid	Butyric Acid	Methanol	Ethanol		
1	2100	600	24	300	900		400
2	1051	300	12	150	450		280
Control	36.0	0	0	30	30		40

Probably, part of methane in the control appeared due to degassing of crude oil. In the above two variants of the experiment, 280-400 ml CH₄ per l of crude oil were generated on products of the aerobic oil destruction. This amount is 7-10 times the control amount and confirms the possibility of bacterial methanogenesis on products of microbial oil destruction.

Thus, the addition of ammonium, phosphate and products of aerobic oil destruction intensifies the activity of methanogens in freshened stratal waters of oil fields. This confirms the conclusion about the possible activation of methanogenesis in the zones of contact

between injected and stratal waters by products of aerobic oxidation of residual oil.

Possible activation of recent methanogenesis in the flooded oil field

Oil field experiment on the activation of methanogenic bacteria by products of microbial oil oxidation was performed in water injection well 428. Preliminarily, in conditions of ordinary flooding the control experiment was performed: the well was placed into the regime of self-discharge, and control samples were taken characterizing the water coming from the zones of contact in the formation. Microbiological and chemical data obtained during the control discharge (Table 6) are similar to those yielded by the other experiments on water injection wells (Table 3).

To attain previous conditions after the control discharge (115 m³), 800 m³ of fresh water were injected in this well. Experimental activation of microorganisms was made by the introduction of 35 m³ of aerated water (air volume 1800-2000 m³) supplemented with NH₄Cl and phosphates. The well was closed for 2 months. Then the well was opened, the stratal water was discharged and sampled for analyses.

TABLE 6—Microbial Number and Methanogenesis Rate in Water of Well N 428

Volume of Ejected Water, m ³	C _{org} Content, mg·l ⁻¹		Oil-Oxidizing Bacteria, Cells·ml ⁻¹		Methanogens, Cells·l ⁻¹		Methanogenesis Rate, ml CH ₄ ·10 ⁻⁵ ·l ⁻¹	
	Control	Experiment	Control	Experiment	Control	Experiment	Control	Experiment
4.5	12	66	600	60	2500	6000	3.7	8.8
12.3	10	—	600	—	2500	—	12.0	—
25.8	8	—	250	—	1300	—	19.6	—
54.0	9	117	60	25	250	6000	7.8	8.0
99.2	14	109	25	130	250	6000	6.9	6.3
115	20	184	250	250	250	6000	12.3	5.8
150	—	99	—	25	—	6000	—	12.2
204	—	117	—	25	—	6000	—	6.6
282	—	50	—	250	—	6000	—	6.0
355	—	20	—	25	—	6000	—	47.9
397	—	75	—	25	—	6000	—	130.8
472	—	204	—	25	—	25000	—	37.4
547	—	172	—	25	—	25000	—	51.1
619	—	150	—	25	—	25000	—	56.0

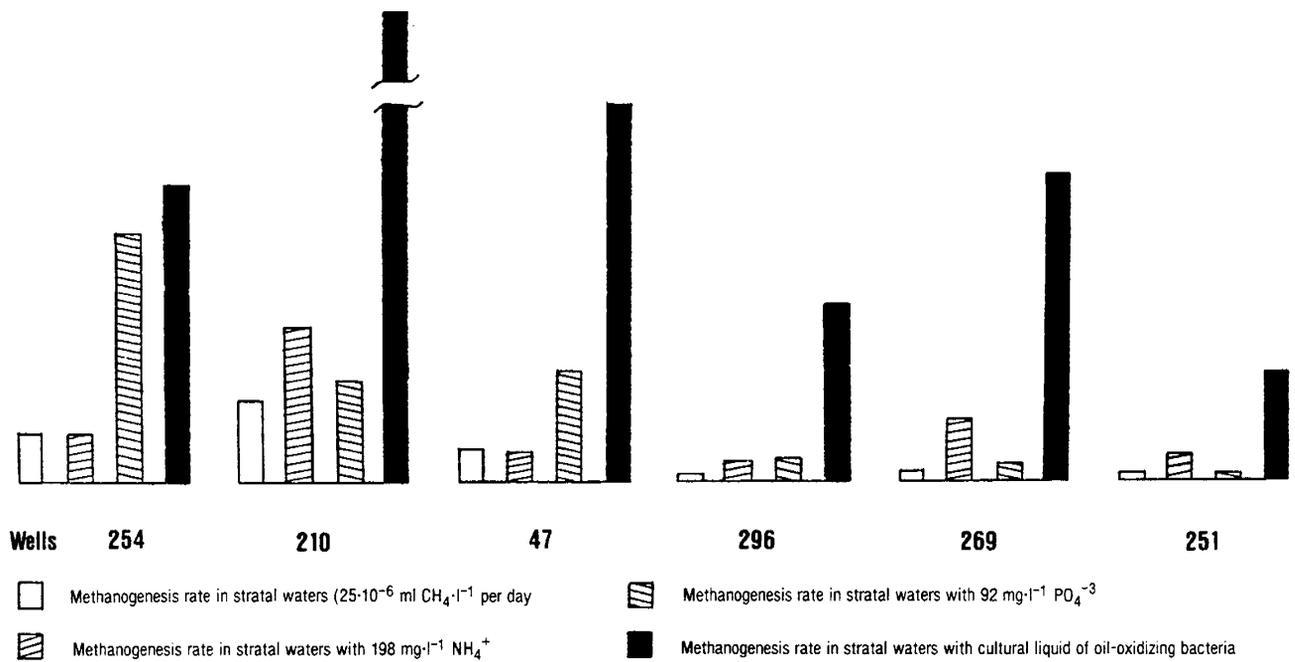


FIG. 5—Effect of additives on methanogenesis rate in stratal waters of Bondyuzhskoe oil field.

Table 6 shows that due to the above treatment of stratal waters, the content of dissolved organic compounds (C_{org}) increased appreciably, the number of aerobic oil-oxidizing bacteria somewhat diminished, the number of methanogenic bacteria increased. In samples taken after the discharge of 300 m^3 the rate of methanogenesis was about one order of magnitude higher compared to the rate of this process in waters of the operating wells (Table 2).

Table 7 presents basic data on the increase in the geochemical activity of methanogens. For the discharge of 115 m^3 of water, the amount of methane generated in experimental conditions was 24 times that produced in control conditions. Altogether, 16.5 m^3 of

methane were generated during the experimental discharge.

An important value characterizing the nature of methane is the ratio between the amount of methane and the sum of heavy hydrocarbons in the gas composition. In the experiment this ratio was 14.6; and 1.7 for the gas of operating wells of the oilfield (Table 7). Higher relative content of methane is a universally accepted indicator of its biogenic origin.

The isotope composition of methane carbon changed significantly. Its $\delta^{13}\text{C}$ values, especially in first samples, were as much as -88.2 percent. The average $\delta^{13}\text{C}$

TABLE 7—Methane and Carbonate Content in Water of Well N 428 and Their Isotopic Composition

Volume of Ejected Water, m^3	Volume of Methane Released, l		CH_4 $\Sigma \text{C}_2 - \text{C}_5$	$\delta^{13}\text{C}$ of Methane, Percent o	$\Sigma \text{CO}_2 + \text{HCO}_3^- + \text{CO}_3^{2-}$, $\text{mg HCO}_3^- \cdot \text{l}^{-1}$		$\delta^{13}\text{C}$ of Carbonates, Percent o	
	Control	Experiment			Control	Experiment	Control	Experiment
4.5	0.5	1.1	17.3	—	138	172	-15.5	-12.9
12.3	2.3	—	—	—	180	—	-26.8	—
25.8	10.4	—	—	—	198	—	-30.5	—
54	37.7	596	16.3	-88.2	300	336	-30.3	-10.2
99	67.0	1531	14.0	-68.9	348	420	-25.8	-11.0
115	81.3	1984	14.0	-55.5	348	420	-20.3	- 9.1
150	—	3314	11.3	-87.3	—	540	—	-14.5
204	—	5016	11.9	-58.7	—	528	—	-11.9
282	—	6831	12.9	-56.7	—	516	—	- 8.6
355	—	8877	13.5	-66.9	—	528	—	- 9.8
397	—	10471	15.0	-62.1	—	540	—	- 6.6
472	—	12921	16.7	-58.5	—	528	—	-11.8
547	—	14742	16.7	-59.0	—	480	—	- 8.1
619	—	16523	18.1	-59.5	—	480	—	- 6.8
Average:			14.6	-64.1			-24.9	-10.1

value of methane released during the experiment made up -64.1 percent. It might be compared with the average $\delta^{13}\text{C}$ value of methane sampled from the nearest operating wells (with mineralization up to $80 \text{ g}\cdot\text{l}^{-1}$) which was -55.7 percent. Such a significant lightening of the carbon isotope composition of methane released during the experimental discharge indicates that at least a major part of methane is of a microbial origin.

This is confirmed by the analysis of the carbon isotope composition of carbonates from stratal liquids. The comparison between $\delta^{13}\text{C}$ values of carbonates obtained in experiments on the methanogenesis activation and $\delta^{13}\text{C}$ of carbonates of stratal waters during the control discharge shows a significant discrepancy between them. Carbon of carbonates from stratal waters of the experimental discharge (average $\delta^{13}\text{C}$ value is -10.1 percent) is substantially heavier than that of the control discharge (average $\delta^{13}\text{C}$ value is -24.9 percent), and of carbon of carbonates from nearest operating wells (average $\delta^{13}\text{C}$ value is -15.4 percent). Such a heavy isotope composition of carbon of carbonates (Table 7) also points to an active bacterial methanogenesis due to CO_2 reduction.

Therefore, the proposed action on the stratal microflora of oil fields enables a significant intensification of its activity. Activation of microbial processes increases the content of organic compounds and the share of newly formed bacterial methane in stratal waters thus, according to some authors,^{5,8,25} enhancing the oil recovery.

SUMMARY

The distribution of microorganisms and rate of bacterial methanogenesis in flooded oil fields was studied. It was shown that the population of microorganisms and methanogenesis rate increased as stratal waters were freshened. It correlated with the decrease of C_{org} content and increase of bicarbonate concentration, as well as with methane isotopic composition getting lighter and bicarbonate carbon isotopic composition getting heavier.

It was found that in the zone of contact between injected fresh waters and stratal waters in the contour of oil fields the process of bacterial oil oxidation took place. The products of oil aerobic destruction stimulated the activity of methane-producing bacteria.

The proposed technology of impact on stratal microflora of oil fields provides a considerable stimulation of its activity. The activation of microbial processes results in the increase of the concentration of soluble organic compounds and content of newly produced methane in stratal water, which according to some authors, enhances the oil recovery.

REFERENCES

1. Andreevsky, I. L. (1959) Application of petroleum microbiology in oil recovery industry. In Collection on Geology, Gostoptekhizdat, 4, v. 131, 403-415 (Russian).
2. Andreevsky, I. L. (1961) Influence of the microflora of the Yaregsky oil field third formation on variations in oil composition and characteristics. In Proceedings of the Institute of Microbiology, Moscow, Izd. Akad. nauk, 9, 75-80 (Russian).
3. Craig, H. (1953) The geochemistry of the stable carbon isotopes. *Geochem. Cosmochem. Acta*, 3, 53-92.
4. Craig, H. (1957) Isotopic standards for carbon and oxygen and correction factors for mass-spectrometric analysis of carbon dioxide. *Geochem. Cosmochem. Acta*, 12, 133-149.
5. Davis, J. B. (1967) *Petroleum Microbiology*. Elsevier Pub. Co. Amsterdam, London, New York.
6. Dostalek, M., Spurny, M. (1957) Release of oil through the action of microorganisms. I. Preliminary experiment on a petroleum deposit. *Cesk. Mikrobiol.*, 2, 300-306.
7. Fritz, W., Walter, L., Walter, S. (1976) Verfahren zur verbesserung der Ausbeute bei der Gewinnung von Erolöl durch Wasserfluten, Patentschrift DT 2410267 C2 (FRG).
8. Gafarov, Sh. A., Markhasin, I. L., Leibert, B. M., Zhdanov, A. G. (1978) Effect of low-molecular fatty acids on the capillary oil ejection by water. In *Physicochemistry and oil field exploitation*. Ufa, 81-86 (Russian).
9. Hitzman, D. O. (1967) Oil recovery process using aqueous microbiological drive fluids, U.S. Patent Office, No. 3,340,930.
10. Ivanov, M. V., Belyaev, S. S., Laurinavichus, K. S. (1976) Methods of quantitative investigation of microbiological production and utilization of methane. In *Microbial Production and Utilization of Gases (H_2 , CH_4 , CO)*. (H. Schlegel, G. Gottschalk, N. Pfennig, eds.) Gottingen, 63-67.
11. Ivanov, M. V., Belyaev, S. S., Laurinavichus, K. S., Obratsova, A. Ya., Gorlatov, S. N. (1982) Microbial distribution and geochemical activity in flooded oil fields. *Mikrobiologia* (in press) (Russian).
12. Jaranyi, I., Kiss, L., Salanczy, G., Szolnoki, J. (1963) Alteration of some characteristics of oil wells through the effects of microbiological treatment. *Intern. Sci. Conf. Geochem. Microbiol. Oil Chem.*, 3-rd, Rept. 1: Geochemistry and Microbiology, 663-650.
13. Johnston, N. (1966) Water flooding process, U.S. Patent Office No. 3,288,211.
14. Jones, L. W. (1967) Aerobic bacteria in oil recovery, U.S. Patent Office No. 3,332,487.
15. Karaskiewicz, J. (1963) Experiments in biological activation of petroleum reservoirs. *Intern. Sci. Conf. Geochem. Microbiol. Oil Chem.*, 3-rd, Rept. 1: Geochemistry and Microbiology, 566-578.
16. Kuznetsov, S. I., Ivanov, M. V., Lyalikova, N. N. (1962) Introduction to Geomicrobiology, Moscow, Izd. Akad. Nauk (Russian).
17. Kuznetsova, V. A., Lee, A. D., Tiforova, N. N. (1963) Determination of the source of contamination of oil-bearing seams D_1 of Romashkinskoye deposit with sulfate-bearing bacteria. *Mikrobiologia*, 32, 683 (Russian).
18. Lindblom, G. P., Patton, J. T. (1963) Substituted heteropolysaccharides, *Canad. Pat. No. 675,416*.
19. Moses, V. (1978) The microbial dimension in enhanced oil recovery. In *Eur. Symp. enhance oil re-*

Microbes and Their Metabolites

covery, Edinburgh, 1978, 271-277.

20. Oil of Tatar ASSR. (1966) Moscow, Khimia (Russian).

21. Reznikov, A. A., Mulikovskaya, E. P., Sokolov, Yu. I. (1970) Methods of Analysis of Natural Waters, Moscow, Nauka (Russian).

22. Rozanova, E. P., Kuznetsov, S. I. (1974) Microflora of oil fields, Moscow, Nauka (Russian).

23. Sulin, V. A. (1946) Waters of oil deposit in natural water system, Moscow, Gostoptekhizdat (Russian).

24. Wolkin, R., Belyaev, S. S., Zeikus, J. G. (1982) The isolation and characterization of methanogenic bacteria from the Bondyuzhskoe oil field, Appl. and Environ. Microbiol. (in press).

25. Zobell, C. E. (1946) Bacteriological process for treatment of fluid-bearing earth formations, U.S. Patent Office No. 2,413,278.

Transport of Bacteria in Porous Geologic Materials

In considering the feasibility of microbial enhancement of oil recovery (MEOR) an exceedingly important parameter is the mobility of microorganisms within a geologic porous system containing water, dissolved salts, and oil. In order to contact trapped oil with bacteria that have favorable oil displacement properties, the microbes must be transported from a wellbore to locations deep within the reservoir. This may be a pivotal factor in the applications of microbes to specific reservoirs.

Two of the papers dealing with microbial interactions with sandstones are reports of work sponsored by the U.S. Department of Energy. The work by Yang et al was initiated specifically to examine the interactions between bacteria and geologic porous media containing oil and brine, and to develop a theory for the transport phenomena of bacteria through porous media under subsurface reservoir conditions.

The work began with synthetic microspheres, one micron in diameter, containing hydrolyzable carboxylic groups which are negatively charged in neutral solutions. When these were injected into a clean, water saturated, Berea core (400 md permeability), strong adsorption was evident indicating reaction of the charged particles with ionic potentials existing in the rock. However, when the microspheres were used with a core containing a residual oil saturation (obtained by waterflood of an oil-brine saturated core) in a Cleveland sandstone core, the microspheres were more readily transported as indicated in Fig. 1.

The concentration of microspheres in the effluent equaled the influent value after 11 pore volumes were injected. Considerable retardation in the transport of the microspheres occurred since the effluent concentration would equal the influent just after 1 pore volume was injected if no adsorption or interaction with the oil phase had taken place.

Using a second Cleveland sandstone core (1-in. diameter, 3-in. long), spores of a salt-tolerant *Clostridium* (isolated at Oklahoma State University), were injected at a concentration of 1×10^5 spores/ml (Fig. 1). The spores exhibited considerably less adsorption behavior since the effluent concentration equaled the influent just after 4.5 pore volumes were injected. Some adsorptive behavior took place even though the spores possess an inert, rigid coat. However, the implication is that the spores of bacteria may be readily transported into oil-saturated sandstones where

they will produce vegetative cells when provided with the proper nutrients for germination.

The third phase of this work involved the injection of live cells into sandstone cores at a concentration of 5×10^7 cells/ml. Cells of *Pseudomonas putida*, *Bacillus subtilis* and a species of *Clostridium* were used in these experiments. In each case, the effluent concentration of cells rose to about 5 percent of the influent and then declined to almost zero within a few pore volumes as a filter cake built up at the face of the core and extensive accumulation of bacteria occurred in pores near the inlet. The buildup of a filter cake in the case of Cleveland sandstone cores is not directly related to the pore size distribution of the sandstone since the mean pore size is about 36 microns.

The formation of extracellular polymeric materials and ionic charges on the surface of bacteria play a larger role than the physical size of the bacteria (1 to 5 microns) in determining the passage through this porous rock. Yen et al also reported that *Bacillus subtilis* and *Pseudomonas putida* were able to penetrate about 4 cm/day without an applied pressure in Berea sandstone cores which were saturated with nutrient broth.

Jenneman et al using species of *Pseudomonas* and *Bacillus* confirmed the work conducted by Yen et al and extended it to an examination of nutrient transport and selective zone plugging based on permeability. In flow experiments with Berea sandstone (mean pore size 15 microns), using 2×10^5 cells/ml of *Pseudomonas*, viable cells were detected in the effluent just after 1 pore volume had been injected. However, the effluent concentration never exceeded 1 percent of the inlet. This indicates a greater effect of the smaller pore-size distribution of the Berea sandstone since the effluent concentration attained with the Cleveland sandstone reached 5 percent of the influent.

The principal objective of the work at the University of Oklahoma is to examine the potential for using microbes *in situ* to selectively plug high permeability zones. Hence experiments were conducted using two cores having different permeabilities in parallel. The cells were selectively transported to the higher permeability core causing diversion of fluid flow to the low permeability core. In this case plugging of the high permeability core was caused by rapid buildup of a filter cake of cells at the face of the core since backflush restored the permeability. Facial plugging can be

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expected when high concentrations of cells are used or large volumes are injected.

Crawford suggested the use of bacteria to plug the high permeability zones of a reservoir which are responsible for bypassing of large quantities of oil due to water channeling. In earlier work, Crawford conducted several experiments using three cores in parallel having low, medium, and high permeability. He found that bacteria would plug the high permeability core to a greater extent than the medium, and the medium permeability core was plugged to a greater extent than the low permeability core.

Crawford discussed the detrimental effects of microbes on oilfield operations. Bacteria are the source of a considerable number of oilfield problems that include corrosion, plugging of water injection wells, scale formation in pipes, fouling of mechanical equipment such as pumps, and plugging of surface flow lines. The detrimental effects of bacteria on oilfields have been presented by numerous authors and a considerable effort is made by the petroleum industry to abate this negative influence of microorganisms.

The papers presented at the MEOR conference show that there are two distinct and contradictory aspects of microbial effects on oil production.

Widespread water injection introduces a large number of microorganisms to petroleum reservoirs which is responsible for considerable detrimental effects in all phases of petroleum production operations. However, the controlled introduction of specific microbial cultures may be instrumental in reversing the negative aspect of microbial systems. Papers presented at the conference suggest the use of *Bacillus* to plug high permeability zones, *Clostridium* to clean out wells and displace oil *in situ* by generation of nascent gases and solvents, production of biopolymers for emulsifica-

tion of crude oil, and the reduction of heavy oil viscosity by bacteria.

The buildup of a filter cake at the face of sandstone has been the experience of the petroleum industry in the field and was discussed in papers presented at the MEOR conference. The papers indicated that the injection of live cells at high concentrations and field fluid injection rates may not be feasible if deep penetration of the microbes is desired.

However, Yen et al showed that microbial spores can be injected with very limited retardation in transport and that they will penetrate deep within the sandstone body. Jenneman et al showed that most of the required nutrients (nitrogen, glucose, phosphate) will also penetrate into a sandstone with limited retardation. Hence, it is possible to inject the spores and then cause them to germinate deep within the rock, or formation, by subsequent injection of essential nutrients.

Updegraff reported that rocks of high permeability show a greater fractional decrease in permeability upon injection of bacteria than those of lower permeability. Therefore, this suggests the possible use of bacteria for selective plugging or stratification rectification to increase sweep efficiency, and hence oil recovery, in waterflooding. He also found that small bacteria penetrate through most reservoir rocks, but not without a serious degree of plugging except in rocks with an appreciable fraction of their pores with a minimum pore entry diameter of 3 microns or more.

Such rocks usually have a permeability greater than 100 md. Updegraff concluded that bacteria may exert a much greater plugging effect when they multiply within the reservoir rock than when cells are injected; therefore, bacterial plugging represents an important limiting factor for microbial enhanced oil recovery.

An Investigation of the Transport of Bacteria Through Porous Media

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ABSTRACT

This paper summarizes the work done at the University of Southern California to study the transport of bacteria through oil bearing geologic formations. It represents the first such systematic attempt to describe qualitatively and quantitatively the effect of various parameters on the retention of bacteria by a reservoir rock.

The bacterial species studied here are *Pseudomonas putida*, *Clostridium sp.* and *Bacillus subtilis*, each chosen to represent a certain class of bacteria that enhance oil recovery in their own way. The transport of these species through sandstone cores and sandpacks was studied. Nutrients were found to be easily transported through the core. In low permeability rocks (<500 md) and at high inflow concentrations, certain species of bacteria tended to agglomerate and plug the face of the core.

However, for nonaggregating species, flowing through high permeability rock at relatively low concentrations (10^8 cells/ml) the dominant mechanism of retention was found to be adsorption rather than pore plugging. *Clostridium sp.* being a nonaggregating species is the most easily transported. Addition of small amounts of nonionic surfactant tends to prevent aggregation and is found to enhance the transport of the cells. The presence of residual oil in the core was found to facilitate transport too.

To attain a better quantitative and ultimately predictive understanding of the adsorption mechanisms, the electrostatic and Van der Waals interactions between the bacteria and rock were studied. The surface charge properties of the detrital grains and the clays in the sandstone and the bacteria were studied in detail. Preliminary model calculations done on estimating interaction energies between bacteria and rock show that such a model does indeed predict most of the observed behavior. The theoretical estimation of the filtration coefficient from the measured zeta potentials of clays and bacteria surfaces, and other system parameters such as rock grain size, porosity, injection rate and Hamaker constant corresponds well with the experimentally observed one. Such a model not only provides us with a deeper insight into the mechanisms of bacterial retention but also may prove to be a predictive tool in the future.

INTRODUCTION

For the past 50 years, different investigators have

applied certain bacteria species to recover additional oil from a number of producing fields. Some examples are listed in Table I, although many are shallow formations tested only in small scale. As far to the mechanism of the enhanced oil recovery is considered, it could be due to one or a combination of the following:

1. Gas products by fermentation (produced from molasses) such as CO_2 , H_2 , CH_4 , will give sufficient reservoir pressure to drive more oil out.

2. Acid products will erode and modify reservoir rocks to increase porosity and permeability.

3. Polysaccharides evolved as metabolic products will become *in situ* surfactants to lower the interfacial tension of trapped oil.

4. Degradation of large molecules in the oil will reduce the viscosity of the produced oil.

5. Internal sulfonation of molecules by bacterial metabolites will result in the production of an *in situ* surfactant.

6. Water-soluble high molecular weight polymers for mobility control will be produced in place.

7. Changes from selective plugging, sequestering and sacrificial agents, etc. will occur.

8. Affinity of bacteria for solid surfaces will force the oil from the solid material through wettability alterations.

9. Generation of more CO_2 as a result of reaction of organic acids produced by bacteria and calcareous material in a reservoir will serve as a swelling agent for oil.

10. Solvents such as alcohols and ketones produced will dissolve more residual oil.

11. Internal emulsification will help to move the oil as in micellar flooding.

The success of an efficient microbial enhanced oil recovery (MEOR) process hinges on the injection of nutrients and microorganisms into a reservoir and the subsequent migration and multiplication of the microorganisms. In a research of the literature, there is no definite data concerning the migration of microorganisms in a typical reservoir. Very little information is available dealing with the transport of microorganisms in porous media. Therefore we initiated the task of studying bacterial transport through porous media.

Transport of Bacteria in Porous Geological Materials

This work was supported by the U.S. Department of Energy with the following objectives:

- a) Examination of the interaction between bacteria and geological porous media containing oil, and brine water;
- b) Study of transport phenomena of bacteria through porous media under applied pressure;
- and c) Facilitation of bacterial transport through geological porous media under naturally-occurring reservoir conditions.

Our primary concern, in the first year, is to study different factors affecting the efficiency of developing this *in situ* bacterial approach. The following factors are of concern:

1. The viability of cells at reservoir conditions and hence the microbial action to release oil;
2. The "injectivity," i.e., the extent to which the bacterial cells and nutrients are able to penetrate the reservoir; and
3. Other secondary effects associated with the injection of bacteria such as the impact on formation properties.

This paper will summarize the effort we made during the first year. The Experimental and Results sections will cover the following: a) Core flooding; b) Chemical additives; c) Special conditioning; and d) Rock characterization.

EXPERIMENTAL

Negatively charged polymeric microspheres—Microspheres carrying hydrolyzable carboxylic groups were synthesized according to the procedures described in Ref. 2. Preparation was by copolymerization of poly-

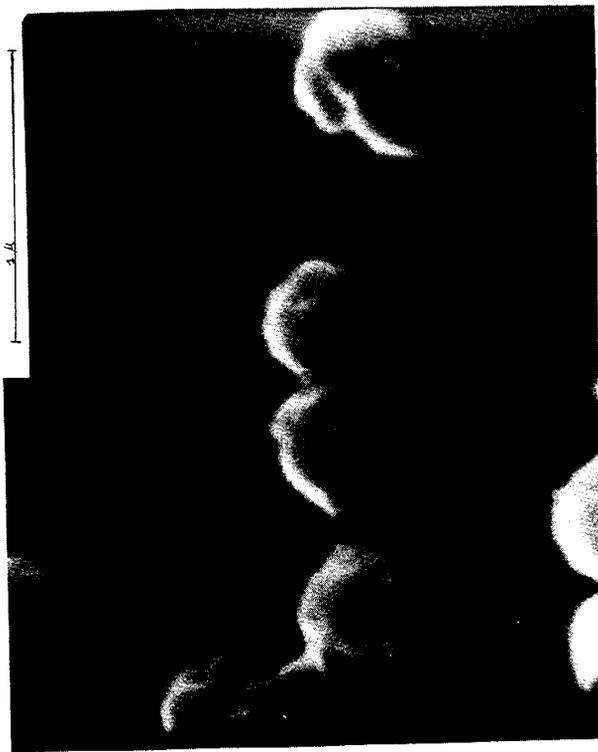


FIG. 1—Scanning electron micrograph of microspheres.

ethylene oxide with 2-hydroxyethyl methacrylate and crosslinked with bisacrylamide. The scanning electron micrograph is shown in Fig. 1.

Sandstone—Berea sandstone cores with permeability 400 md were obtained from Cleveland Quarries Co., Amherst, Ohio. Another sandstone (abbreviated as CS in this paper) with a permeability of 4 darcy was obtained from the road-cut of Lake Keystone, Oklahoma. Both sandstone cores were steam-cleaned according to Donaldson's procedure¹ and dried at 100°C.

Microorganisms—Three bacterial species were chosen. *Pseudomonas putida* (ATCC 12633), an aerobe, is capable of degrading some ring compounds. It was cultivated in liquid thioglycolate medium. *Clostridium sp.*, a spore-forming anaerobe, is capable of producing biogas and solvents. It was cultivated in an anaerobic medium until culture aged and spores developed. *Bacillus subtilis*, spore-forming aerobe, is capable of producing biosurfactant. It was cultivated in nutrient broth. The cultures were then centrifuged and resuspended in carbonless electrolyte solution to maintain a suitable buffer and osmotic balance. Nutrient agar plates of effluent and influent aliquots were scored for *B. subtilis* and *P. putida*. The concentrations of microspores and *Clostridium sp.* spore suspensions were determined by means of counting with a hemacytometer.

Sterilization—System sterilization was achieved by a 70–75 percent alcohol flood for 1 day followed by sterile water. Little contamination was detected by agar plating of the effluent.

CORE FLOODING

Nutrients transport—The apparatus for injecting the suspensions at constant flow rate is shown in Fig. 2. The cores were vacuum saturated with degassed distilled water prior to all experimental runs.

A solution containing 3 percent of fluid thioglyco-

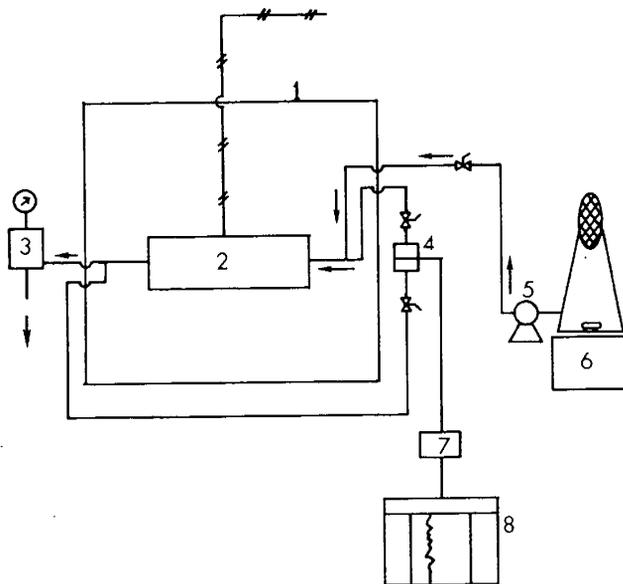


FIG. 2—Apparatus for pumping bacterial suspension into a sandstone core. 1, oven; 2, core holder; 3, back-pressure controller; 4, differential-pressure transducer; 5, pump; 6, magnetic stirrer; 7, demodulator; and 8, recorder.

TABLE 1—Field Tests Using Microorganisms

Investigator	Bacteria	Country	Recovery
Dostalek & Spurney	<i>Desulfovibrio</i> , <i>Pseudomonas</i> in molasses	Czechoslovakia	50 percent, 7 out of 10 wells
Kuznetsov et al.	Aerobic bacteria in 4 percent molasses	USSR	37-40 m ton/d for 4 months
Jaranyi et al.	Anaerobic, thermophiles in molasses	Hungary	126 percent yield for 12 wells
Karaskiewicz	<i>Clostridium</i> in molasses	Poland	20-200 percent
Yarbrough & Coty	<i>Clostridium</i> in 2 percent molasses	USA	200 percent in 4 months
Van Heingen et al.	Bacteria in 50 percent molasses	Holland	30 percent improvement

late medium USP was injected into one CS core. Another solution containing 1 percent glucose was pumped into another CS core at the constant flow rate of 40 ml/hr. The effluent samples were analyzed by Biuret method and Fehling's method, respectively.

Bacteria transport—The experimental conditions of flooding the cores with microspheres and with various bacterial species are summarized in Table 2.

The differential pressure drop across the core was monitored with a differential pressure transducer.

Twenty pore volumes of bacterial suspension were injected into each of the sandstone cores in all the experiments.

CHEMICAL ADDITIVES

Effect on microsphere adsorption—Five grams of silica sand grains ($\sim 250 \mu$ in size) and various chemicals were added to each of the 50 ml suspension containing 10^8 microspheres/ml distilled water, Table 3. The mixture was shaken gently for a period of 1 hr. The equilibrium concentration of microspheres was determined by a visible light spectrophotometer. The percentage of microsphere adsorption was then computed by comparing with the optical density of the original suspension. The relation between optical density and the number density of microspheres in water was precalibrated.

Adsorption and elution of bacteria by ion-exchange resins—The resin was conditioned in the case of the anion exchange resin IRA-410 CP in the chloride form and in the case of the cation exchanger IRC 50 in the hydrogen form. Thirty grams of respective resins were gently stirred for 30 min with a suspension of 8 hr *Ps. putida* cells. The resin suspension was poured into a 1 × 12-in. glass column. Distilled water was flushed through to wash out unabsorbed cells.

The column was then flushed with distilled water (70 ml) followed by 70 ml of 0.01 M phosphate (pH = 6.8) and finally 0.1 M phosphate (pH = 6.8).

Elution of bacteria from sandpack column by phosphates—A washed, centrifuged suspension of *Ps. putida* cells was added to the top of a column packed with acid washed sand. 7.5×10^{10} cells in 50 ml of distilled water were added to the column and this was followed by 200 ml of distilled water. A solution of 250 ml of 0.01 M phosphate buffer was then added to the column. The fractions of the effluents were collected and all densities were determined by agar plating.

A CS core was preflushed with a 6,000 ppm NaCl

TABLE 2—Summary of Core Flooding Experiments

Run No.	Suspension ^a		Suspending Medium ^b	Porous Media ^c	Calculated Value (based on deep filtration model)	
	Species	Conc., Cells/ml (inflow)			Filtration Coef. (cm ⁻¹)	Maximum Retention Capacity (cells/ml rock)
(9/18/81)	<i>Pseudomonas putida</i> (ATCC 12633)	$5 \cdot 10^7$	el	3.5 in. × 1 in.	0.67-0.93	—
(10/20/81)	Microspheres	$6 \cdot 10^6$	di	Oil-containing 3 in. × 1 in.	0.24	2.0×10^7
(11/12/81)	<i>Clostridium</i> sp. spores	$5 \cdot 10^6$	el	3 in. × 1 in.	0.21	3.1×10^6
(11/18/81)	<i>Pseudomonas putida</i> (ATCC 12633)	10^6	el	3 in. × 1 in.	0.61	—
(12/2/81)	<i>Bacillus subtilis</i> cells	10^6	el	3 in. × 1 in.	0.40	—
(1/5/82)	<i>Bacillus subtilis</i> spores	10^6	el	3 in. × 1 in.	0.21-0.30	—

^aThe flow rate is 40 ml/hr for all experiments. ^bel is electrolyte solution; di is distilled water. ^cAll porous media have porosity of 0.22 and a permeability value of 4 darcy.

sterile brine containing 1,000 ppm Tween 80 (polyoxyethylene [20] sorbitan monooleate, a nonionic surfactant). The *Ps. putida* cells were suspended at concentrations ranging $3-7 \times 10^7$ /ml in the medium with the same composition as the preflush medium. The history of the effluent quality was recorded.

SPECIAL CONDITIONING

Transport in residual oil saturated core—One CS core was made to contain residual oil by injecting 5 PV of Long Beach crude, a heavy oil (API gravity, 17°), into the water-saturated sterile core followed by at least 10 PV of sterile water. Subsequent injection of suspension followed in accordance with the procedure described under the section of Bacteria Transport.

Transport in oil-coated sandpack column—This experiment was designed to determine if the filtering ability of sandpack columns depended on the hydrophilic and hydrophobic properties of sand grains.

Seventy-five grams of fine acid sand was packed into a 20 × 300 mm column by gently tapping the column as the sand was poured in. This column was maintained as a control. The second column was treated as follows: 100 mg of Long Beach (Ranger Zone) crude oil (API gravity, 16°) was dissolved in 150 ml of petroleum ether.

The solution was added with constant mixing to the 75 gm of sand. The mixture was stirred in a rotating flask overnight and then put into a 20 × 300 mm column. A third column and a fourth column were prepared in a similar manner except that 500 mg and 1 gm of the crude were used to coat the sand particles, respectively.

An 18-hr culture of *Ps. putida* was prepared and diluted to a final concentration of 1.7×10^6 cells/ml. One hundred ml of the bacterial suspension was added to the top of each of the columns. The column was allowed to run dry and then flushed with 200 ml of distilled water. The eluted bacteria from each column were plated and the number of cells in the eluted water was determined.

Transport in nutrient-saturated Berea sandstone core—An apparatus was designed to link two 250 ml flasks with a 1-in. diameter × 2-in. long Berea sandstone core (Fig. 3). The core was vacuum saturated with nutrient broth. Two such apparatuses each with both flasks containing 150 ml nutrient broth were autoclaved. *B. subtilis* and *Ps. putida* were inoculated into the left side flasks of each apparatus. The time when the flasks turned cloudy was recorded.

ROCK CHARACTERIZATION

Sandstone was ground gently and separated into clay and detrital grain fractions by standard sedimentation methods. The clay fraction ($<2 \mu$) was filtered out after repeated washings with distilled water.

Detrital grains fractions ($>2 \mu$)—The surface charge density at various pH values was computed by means of potentiometric titrations. The pH of the samples in an ionic solution of various KCl concentrations was recorded, as small aliquots of NaOH or HCl were added in known quantities. The difference in the pH of the control (without rock grain sample) and the sample are an indication of the surface charge

$$\sigma = F (\text{pH} - \text{pH}_{\text{control}}).$$

In the double layer model developed by Davis et al,⁷ the surface charge develops due to adsorption of potential determining ions and surface complexation.

The surface charge density and the adsorption-desorption equilibrium constants were determined from the potentiometric data. With these parameters, a set of 11 equations enables us to calculate the unknown quantities in this model.³ Eventually, charge density and electric potential distributions extending from rock surface into the aqueous medium were fully described. The thickness and also the electric capacitance of the inner and outer Helmholtz layer in the double layer were calculated.

The surface zeta potential of clays in solutions of various pH values and electrolytes was measured with a TV screen-equipped Zeta-Reader (Komline-Sanderson Co., Ontario) monitoring the electrophoretic mobility of clays under applied voltage. The apparatus gives the digital readout of zeta potential directly.

RESULTS AND DATA ANALYSIS

Core flooding—The breakthrough curves of glucose and protein from Berea sandstone cores are shown in Figs. 4 and 5. Total breakthrough of these two nutrients occurred after the first few pore volumes of injection.

Bacteria transport—A typical curve for the history of effluent quality such as Fig. 6 can be treated according to deep filtration theory.^{4,5}

The deep filtration coefficient, K_0 , which physically means the retention probability of a bacterial cell traveling with the fluid at unit length in the porous media, of a core having length, L, can be expressed by:

TABLE 3—The Effect of Chemical Additives on Microsphere Adsorption onto Silica Sand*

Run No.	1	2	3	4	5	6	7 (control)
NaCl		10,000		10,000			
CaCl ₂			2,000		2,000		
EDTA (Na) ₄	2,000	2,000	2,000				
Tween 80						20,000	
Percent Adsorption	50 percent	83 percent	75 percent	70 percent	78 percent	19 percent	32 percent

*The values in this table are the concentrations of additive chemicals in ppm; 5 g silica sand was mixed with 50 ml suspension containing 10^8 microspheres/ml.

$$K_o = \frac{\ln\left(\frac{C_i}{C_f}\right)}{L} \text{ (cm}^{-1}\text{)}$$

The maximum retention capacity of bacteria on rock surfaces per unit volume of porous media σ_{\max} can be estimated as

$$\sigma_{\max} \cong t_{\max} C_i U K_o \cong (t_{\text{sat}} - t_{\max}) \frac{UC_i}{L}$$

where U is the superficial linear velocity.

The filtration coefficient and, in some cases, the maximum retention capacity are calculated from the history of effluent quality when the bacteria are injected into the sandstone core. The results are listed in Table 2.

Clostridium sp. spores, which showed the lowest filtration coefficient and the lowest maximum retention capacity among all species tested, seem to be more easily transported through the CS pore. In experimental runs of injecting *Ps. putida*, *B. subtilis* cells, and *B. subtilis* spores at inlet concentration of 10^6 /ml, we always observed a few percent of breakthrough.

If we allowed these experiments to run for an extended period of time, we observed a similar pattern of effluent history as that in Fig. 3. Of course, the retention level on sandstone for these three suspensions is expected to be higher than that of *Clostridium* spores.

We also noticed that when *Ps. putida* cells are injected at 5×10^7 /ml, a filter cake developed at the inlet surface. Meanwhile, the differential pressure drop increased rapidly suggesting a very large retention. Contrary to the curve in Fig. 6, we observed a decrease in effluent concentration (Fig. 7). Probably the already deposited cells and the cells in the filter cake tend to retain more strongly the inflow cells. Furthermore, *Ps. putida* tend to form aggregates consisting of about 20 cells.

Chemical additives

Microsphere adsorption onto sand grains—

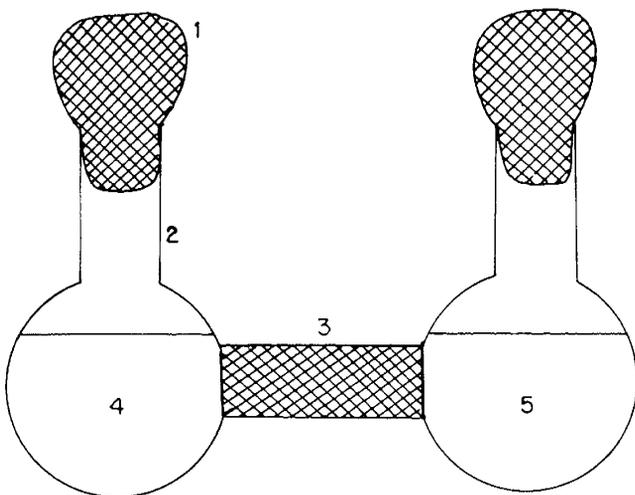


FIG. 3—Apparatus for investigating bacterial transport resulting from presumably combined diffusion, bacterial growth, and migration in the direction of increasing nutrient concentration. 1, cotton plug; 2, 250-ml flask; 3, Berea sandstone core; 4, bacteria culture grown in nutrient broth; and 5, sterile nutrient broth.

The results of adsorption experiments are listed in Table 3. The significance of the results is:

1. The divalent cations boost the adsorption of microspheres to a greater extent than do the monovalent ions even if the former are at a much lower concentration (Runs 2 and 4).

2. The chelating agent, EDTA(Na)₄, may chelate divalent cations. As a result, the extent to which microspheres adsorb is decreased if EDTA(Na)₄ is added to a microsphere suspension containing CaCl₂ (Runs 3 and 5). Adding EDTA(Na)₄ into the suspension containing monovalent cations simply increases the ionic strength. An enhanced adsorption was observed (Runs 2 and 4).

3. The addition of Tween 80 into the suspension seems to decrease the microsphere adsorption (Runs 6 and 7).

Adsorption and elution of bacteria by ion exchange resins—The cell counts of effluent samples is shown in Fig. 8. The initial high values represented cells not adsorbed or loosely attached to the resin grains. They were easily washed off by distilled water. The following peak was eluted with very weak phosphate buffer solution. The combined volume of the effluent was plated out to determine the output/input ratio. The percentage of recovered cells in the effluent was 62.9 percent of the total number of cells applied.

When the column containing bacteria-adsorbed sand grains was flushed with distilled water, the optical density of the first 250 ml effluent was negligible. A noticeable increase in visible turbidity was observed as the phosphate solution was added to the column.

Coinjection of nonionic surfactant with *Pseudomonas putida* culture—The history of effluent quality for coinjection is shown in Fig. 9. The history of effluent quality of injecting *Ps. putida* at inlet concentration of 5×10^7 /ml without adding Tween 80 is shown in Fig. 7. By comparing the two runs in which inflow concentrations are about the same, it seemed that *Ps. putida* cells are more easily transported if Tween 80 is added to the preflush medium and the suspending medium. Contrary to the case in Fig. 7, no significant pore plugging or filter cake forming were observed. The microscopic investigation revealed that cells aggregated to a lesser extent if Tween 80 is added.

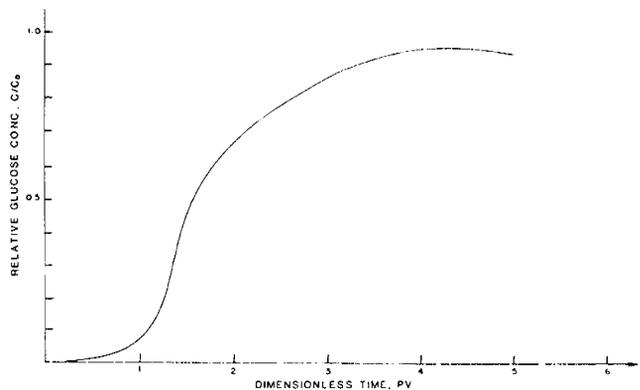


FIG. 4—History of effluent quality as a glucose solution was pumped into a Berea sandstone core.

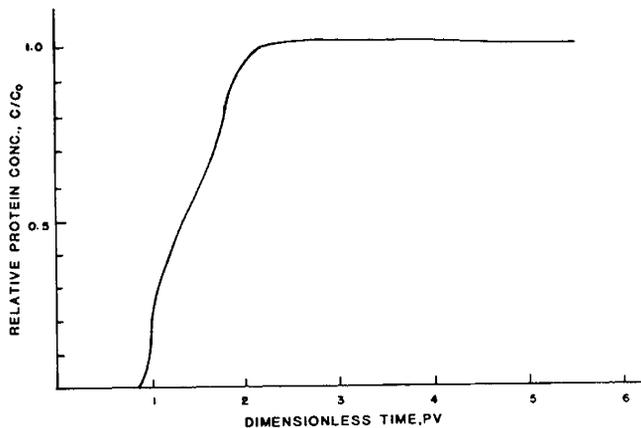


FIG. 5—History of effluent quality as a fluid thioglycolate medium USP medium was pumped into a Berea sandstone core.

Special conditioning

Transport in residual oil-saturated core—The residual oil in the porous rock seemed to aid the transport of microspheres (Table 2, Run 10/20/81). When microspheres are injected into a clean sandstone core (results are not listed in Table 2), the effluent contains very little microspheres. The residual oil might be trapped in narrow pore necks or covers the oil-wet portion of the rock surface. We would expect the filtration and the retention to decrease in residual oil-containing sandstones.

The conditions of each column and the percentage of bacteria recovered from the effluent are summarized in Table 4.

The results of these determinations seemed to show that more bacteria are adhering to sand particles when the sand is not covered with a coating of crude oil than when it is. Also as the amount of oil on the sand increases, the number of bacteria adhering decreases.

Transport in nutrient-saturated Berea sandstone core—The medium in the flask (Fig. 3) which was inoculated with *B. subtilis* turned turbid within 24 hr. The originally sterile medium in the other flask becomes cloudy at 48 hr. In other words, *B. subtilis* is able to penetrate approximately 1.5 in. per day in a nutrient-saturated Berea sandstone. For *Ps. putida*, the respective time when the medium in the flasks turn visibly

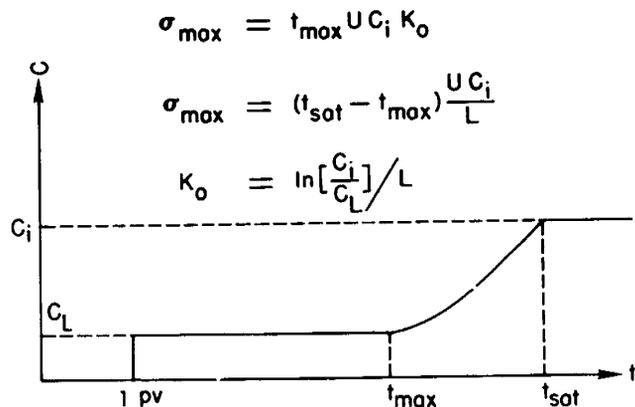


FIG. 6—Typical curve showing history of effluent quality as a bacterial (or colloidal) suspension is injected into a sandstone core.

cloudy was found to be 24 hr and 72 hr. Thus, we might qualitatively conclude that the transport of *B. subtilis* resulting from presumably combined growth/diffusion/migration to the higher-nutrient-concentration area is more favorable than that of *Ps. putida*.

Rock characterization

Detrital grains—The surface charge density of unbaked Berea sandstone and Ottawa sand computed from the results of potentiometric titrations is plotted as a function of equilibrium pH in Figs. 10 and 11.

With the results of potentiometric titrations, the equilibrium constants of the p.d.i. (potential determining ions) absorption and surface complexation reactions are computed and listed in Table 5. The surface charge density and electric potential distributions of Ottawa sand at pH 4.0 is plotted in Table 6.

Clay—The surface of the clay particles acquires a net charge due to a variety of mechanisms as discussed elsewhere.⁶ This surface charge over small pH ranges is pH independent. The velocity of the particles moving under a specified potential difference is measured, and Smoluchowski's equation is used to compute the surface charge. We consider the ionic double layer to be a rigid system in the sense of a Helmholtz double layer, then,

$$\sigma = \frac{4 \pi \mu U'}{E \lambda}, \text{ where}$$

- σ = surface charge ($\mu\text{c}/\text{cm}^2$)
- U' = mobility of particles ($\mu\text{m}/\text{sec}$)
- E = applied potential difference
- μ = viscosity of the medium
- λ = double layer thickness = $\frac{1}{\kappa} \frac{\kappa a_p}{1 + \kappa a_p}$ (cm)
- a_p = radius of particles
- $\kappa = 0.229 \Gamma \times 10^8$ (cm^{-1})
- $\Gamma = \frac{1}{2} \sum c_i z_i^2$ = ionic strength

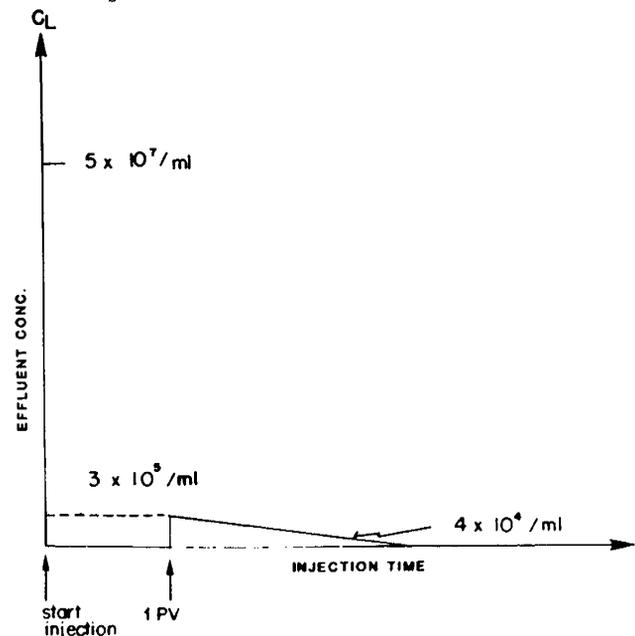


FIG. 7—History of effluent quality as a *Ps. putida* suspension (5×10^7 cells/ml) was pumped into a CS core.

The diffuse double layer influence has been neglected since $kR \gg 1.0$, for our system. The computed surface charge densities are for unbaked Berea sandstone, $\sigma = 19.19 \mu\text{C}/\text{cm}^2$. For baked Berea sandstone, $\sigma = 24.35 \mu\text{C}/\text{cm}^2$.

DISCUSSION

The migration of nutrient is mainly determined by the pumping rate, the inflow concentration, and the adsorption-desorption equilibrium between the molecules in the medium and those on the rock surface. The breakthrough history of nutrients such as protein and sugar from Berea sandstone was investigated. This result reflects the consumption of nutrients by Berea sandstone. Nutrients are more easily transported.

The transport of bacteria is a far more complicated problem. The migration of bacteria under an applied pressure gradient can be described by a deep filtration model. In the past, some qualitative work was done indicating strong retention of bacteria by *clean* Berea sandstone of low or *moderate* permeabilities (<150 md).⁷ This will lead to plugging effect observed and consequently a number of investigators⁸ have suggested selective plugging is the predominant mechanism in MEOR.

Although pore size distribution and pore geometry are constraints for the passage of microorganisms, we feel that for the initial transport investigation we should take a case of porous media which has an average pore size larger than the commonly-used bacteria (>5 μ). Further it was established that in MEOR processes, high permeability and high-porosity of formation rock promise positive results.⁹ However, little research work was done using formation rock of higher permeability (say over 600 md). In our work, a highly permeable sandstone rock (CS) was chosen for most of the experiments. Also, a semiquantitative or quantitative approach was adopted in our studies.

A quantitative picture of bacteria transport in porous rock can be represented as follows: As the suspension of cells flows through the porous passage, some of the cells, under the influence of a number of short-range and long-range forces and some specific cellular attachment mechanisms, may come into contact with the rock grains and be deposited onto the rock grains and on already deposited cells. Viable cells might migrate toward the rock surface where higher nutrient concentration is available, a phenomenon called chemotaxis.

If the porous media contains residual oil, the flow pattern might be much different from the case of clean porous media. Further complications may also arise due to the reentrainment of the deposited cells and their possible redeposition. As a result, the dynamic behavior of the process (namely, the so-called history of the effluent quality and the pressure drop required to maintain a constant rate of flow) varies with time as well as other variables characterizing the interaction among porous rock, hydrodynamics and surface physico-chemical properties of bacteria and rock.

The history of effluent quality of various potential species (suspended in nutrient-free media) from a one-dimensional sandstone rock under constant pumping rate was analyzed. Empirically the filtration coefficient, K_o , can be used as a screening criterion for com-

paring the relative penetrations of various species in rocks. It is possible to evaluate the filtration coefficient from theoretical considerations. In this manner, we could use this empirical coefficient on a semiquantitative or quantitative basis.

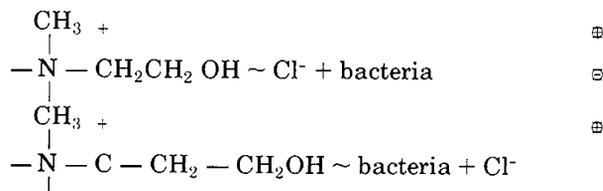
When bacteria are injected into the reservoir along with the nutrient in the real MEOR processes, the nutrient generally migrates ahead of most of the bacteria because the latter is more strongly retained by the rock. In other words, when the injection well is shut-in after some pore volumes of suspension have been pumped in, most bacterial cells are "soaked" in nutrient. We have investigated the transport of bacteria in nutrient-saturated rock, presumably due to combined bacterial growth, diffusion, and migration in the direction of increasing nutrient concentration. Some semiquantitative experiments were carried out.

During the course of our investigation, the experimental results showed that under normal conditions most bacterial breakthroughs were only a few percent of the inflow concentration. In the light of improving or aiding the bacterial penetration or migration we have attempted experiments to either modify the bacterial surface, or to modify the rock surface, or to modify both. The following are methodologies which we are continuing to perform and at this time the research is still in progress.

1. To modify the bacterial surface structure that is responsible for specific attachment.
2. To trap the multivalent cations that might serve as the binding bridge between bacteria and rock.
3. To modify the wettability of both or either of the surfaces.
4. To increase the (negative) surface charge density through specific absorption of certain polyvalent anions by rock.

Based on the above, we have been assessing the effect of phosphate and pyrophosphate on the bacteria transport through the sandpack column, or the sandstone core, or the ion exchange resins.

We reasoned that there may be a similarity between the bacterial transport via sandstone core and the effect of bacteria on an ion exchange resin. The surface of the bacterial cell is known to hold a net negative charge under physiological conditions of growth. And so it would be expected to react with the anion exchange resin in the following manner:



Therefore any chemical which shifts the above reaction to the left-hand side may release the bacteria from ion exchange resin and hence, in our opinion, aid the transport of bacteria in this porous medium by manipulating the surface charge properties.

The filtration phenomenon, as pointed out by many

researchers,⁴ is a strong function of the interaction between the surfaces of bacteria and rock. Theoretically, if this interaction is fully described and if other conditions such as porous structure, flow regime, system geometry, etc. are known, the filtration coefficient can be computed. Two major interactive energies are considered in this paper, namely, the attractive London dispersive energy and the repulsive electrostatic energy. The former is characterized by a Hamaker constant.

We estimated the range of this constant for the interaction between rock and cell across a film of water. The latter is determined by the different nature of surface charges of bacteria and rock and by the interaction of electrical double layers of these two dissimilar surfaces. An expression for this energy term assuming constant-potential flat plate (rock) and constant-charge sphere (idealized cell) is illustrated in this paper.

The surface charge density of clays and bacteria was determined by electrophoretic mobility measurements. The characterization of detrital rock grains was evaluated by potentiometric titration. A sample calculation for estimating the theoretical filtration coefficient from surface interaction characteristics is given.

The following equations are used to estimate the filtration coefficient assuming that micron-sized bacteria are Brownian particles and Happel's model of porous media is valid.¹⁰

$$D_{BM} = K_B T / 6 \mu a_p$$

$$P = (1 - \epsilon)^{1/3}$$

$$W = 2 - 3P + 3P^5 - 2P^6$$

$$A_s = 2(1 - P^5) / W$$

$$N_{Pe} = d_c U / D_{BM}$$

$$\zeta = 0.71 A_s^{-1/3} N_{Pe}^{2/3} (Kr / U)$$

$$f(\zeta) = \frac{1.4 \zeta}{1 + 1.4 \zeta}$$

$$\eta = 4 A_s^{1/3} N_{Pe}^{-2/3} f(\zeta)$$

$$K_o \cong 3(1 - \epsilon) / 2 dc \cdot \eta$$

For the above equations Kr is determined from the integral:

$$Kr = D_{BM} \left\{ \int_0^\infty \left[\frac{\exp(\phi / K_B T)}{R} - 1 \right] dH \right\}^{-1}$$

TABLE 4—Results of Bacterial Transport Through Oil Coated Sandpack Column

	Column No.			
	1	2	3	4
mg Crude/ mg Sand	0.00	0.0013	0.0067	0.133
Total bac- teria applied	1.7×10^8	1.7×10^8	1.7×10^8	1.7×10^8
Bacteria recovered . . .	9.7×10^7	1.09×10^8	1.29×10^8	1.41×10^8
Percent recovery	58.8	64.1	75.9	83.0

The function R now accounts for the hydrodynamic retardation as a sphere approaches a flat surface. At a very close separation distance,

$$R \sim \frac{a_p}{H}$$

The total interactive energy ϕ is the sum of V_A and V_R . For the interaction between a sphere and a flat plate,

$$V_A = - \frac{A a_p}{6 H}$$

$$V_R \cong \pi \epsilon_o \epsilon_r \left[2 \zeta_1 \zeta_2 \left(\frac{\pi}{2} - \tan^{-1} \sinh \kappa H \right) - (\zeta_2^2 - \zeta_1^2) \ln [1 + \exp(-2 \kappa H)] \right]$$

W_R was derived assuming the diffuse double-layer interaction between a constant-charge sphere (bacteria) and a constant-potential plate (rock grain).

In a typical example, the interaction between rock, 1 and bacteria, 2 in water, 3 can be evaluated, e.g., the Hamaker constant A_{132} for the London dispersive attraction between 1 (rock) and 2 (bacteria) across a film of 3 (water) is estimated according to the following:¹¹

$$A_{132} \sim 1.6 A_{131} A_{232}$$

where

$$A_{131} \sim 1.2 - 5.6 \times 10^{-20} \text{ Joule and}$$

$$A_{232} \sim 0.02 - 0.25 \times 10^{-20} \text{ Joule}$$

Thus, A_{131} values approximately range in $0.1-1.9 \times 10^{-20}$ Joule.

With the above consideration, a sample calculation of estimating filtration coefficient can be made. Assume that $\xi_1 = -10$ mv, $\xi_2 = -37$ mv, sodium chloride concentration = 1000 ppm, porosity = 0.22, bacteria radius = 0.5μ , rock grain diameter = 250μ , and Hamaker constant = 1.0×10^{-20} Joule. The final calculated filtration coefficient is estimated as 0.808 cm^{-1} . This value is reasonable when compared to those in Table 2. The sensitivity of filtration coefficient, K_o , or the bacterial retention, to the zeta potentials is quite interesting. The detailed analysis will be reported elsewhere.¹²

For example an increase of the negativity of the charges on bacteria or rock surfaces, by the addition of phosphate will reduce the filtration coefficient, and thus will facilitate the bacterial transport. In this manner we will be able to predict and to extrapolate in what manner and to what extent we can increase the transport of bacteria in actual reservoirs.

CONCLUSIONS

1. Nutrients can move easily through porous media.
2. Viable bacteria, spores, and microspheres can transport through sandstone rock to some extent depending on the filtering strength when the suspension is injected into the core.
3. The adsorption of bacteria on rock surface becomes an important factor in bacterial transport provided the following are true: a) high permeability of rock; b) low inflow concentration; and c) nonaggregating species.
4. *Clostridium* sp. spores are more easily transported through sandstone core than other species tested.

TABLE 5—Equilibrium Constants of Adsorption for H⁺, OH⁻, Na⁺ and Cl⁻

	K_{a1}^{int}	K_{a2}^{int}	K_{cation}^{int}	K_{anion}^{int}	K_{a1}^*	K_{a2}^*	K_{cation}^*	K_{anion}^*
Sand	7.94×10^{-4}	2.51×10^{-12}	1.58×10^{-2}	251.3	1.26×10^{-3}	2.51×10^{-2}	3.98×10^{-10}	3.16×10^{-6}
Unbaked	7.94×10^{-5}	6.31×10^{-13}	25.04	12.603	1.26×10^{-4}	63.1	1.58×10^{-11}	6.31×10^{-6}
Baked	4.1×10^{-7}	1.26×10^{-14}	25.08	12.97	2.44×10^{-6}	1.26	3.16×10^{-13}	3.16×10^{-8}

$$K_{a1}^* = \frac{1}{K_{a1}^{int}}; K_{a2}^* = K_{a2} \times 10^4$$

$$K_{cation}^* = K_{a2}^{int} * K_{cation}^{int} \text{ or } K_{cation}^{int} = K_{cation}^* / K_{a2}^{int}$$

$$K_{anion}^* = K_{a1}^{int} K_{anion}^{int} \text{ or } K_{anion}^{int} = K_{a1}^* / K_{anion}^*$$



5. *B. subtilis* and *Ps. putida* can migrate in nutrient-saturated Berea sandstone cores without applying any pressure gradient.

6. Residual oil in porous rock and oil coating on sand grains can aid transport, so is the coinjection of surface-modifying agents or other chemicals.

7. Surface charge characteristics of rock detrital grains and clays are being investigated.

8. Models for the interactive energy between the constant-potential plate (rock) and the constant-charge density sphere (bacteria) are tentatively adopted.

9. The theoretical estimation of filtration coefficient from the measured zeta potentials on the rock, the clay, and the bacteria surfaces and the system parameters such as rock grain size, porosity, liquid injection rate, and the Hamaker constant were developed.

ACKNOWLEDGEMENT

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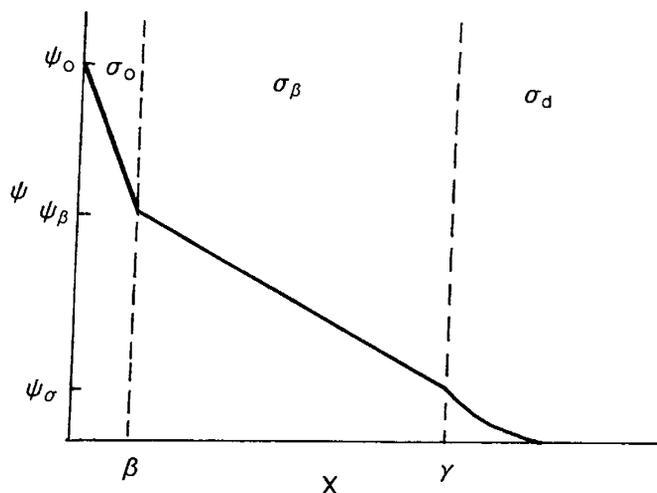
NOMENCLATURE

- A_s : correction factor for the stream function of liquid flowing around a collector in a porous media
- A: Hamaker's constant
- a_p : radius of particles
- C_i : inflow concentration of bacteria
- C_L : effluent concentration of bacteria from a sandstone core
- C_1, C_2 : electrical capacitance in the inner and outer Helmholtz layer, respectively
- d_c : diameter of rock grains
- D_{BM} : Brownian diffusion coefficient
- E: applied voltage across an electrophoretic cell
- F: Faraday's constant
- H: separation distance between a bacterial cell

TABLE 6—Parameters for Surface Potential of Ottawa Sand*

$C_1 = 1.4 \text{ F/m}^2, C_2 = 0.2 \text{ F/m}^2$
pH = 4.0
$\sigma_o = 0.04 \text{ C/m}^2$
$\sigma_\beta = -0.03278 \text{ C/m}^2 \quad \beta = 4.96 \text{ }^\circ\text{A}$
$\sigma_d = -0.007218 \text{ C}^2 \quad \gamma = 34.91 \text{ }^\circ\text{A}$
$\psi_o = 74.658 \text{ mV}$
$\psi_\beta = 46.086 \text{ mV}$
$\psi_d = 10.00 \text{ mV}$

*Diagram below shows various of potential from a rock surface.



and rock surface

- K_B : Boltzman constant
- K_o : deep filtration coefficient
- K_r : apparent 1st-order rate constant of bacterial adsorption reaction
- $K_{a1}^{int}, K_{a2}^{int}, K_{cation}^{int}, K_{anion}^{int}$: equilibrium constants of ion adsorption and complexation

Transport of Bacteria in Porous Geological Materials

on rock surface (Table 5)

L: length of the sandstone core

N_{Pe} : Peclet number

P: parameter used for calculation

U: superficial linear velocity (= flow rate/cross-sectional area)

V_A : London dispersion energy between two many-molecule bodies

V_R : electrostatic repulsive energy between two charged surfaces in an aqueous solution

W: parameter used for calculation

Greek letter

β : thickness of the inner Helmholtz layer

r: distance from the rock surface to the surface of diffuse layer

ϵ : porosity of porous media

ϵ_0 : electric permittivity of vacuuo (= $8.854 \times 10^{-12} \text{ C}^2 \text{ J}^{-1} \text{ m}^{-1}$)

ϵ_r : dielectric constant of water (= 78.5)

η : collection efficiency of particles by an infinitesimal length of porous media

κ : reciprocal length from Debye-Huckel theory

λ : double layer thickness = $\frac{1}{\kappa} \frac{\kappa a_p}{1 + \kappa a_p}$

μ : water viscosity

ξ : parameter used for calculation

ξ_1, ξ_2 : zeta potential of rock and bacterial surfaces, respectively

σ : surface charge density

σ_0 : electric charge in the inner Helmholtz layer

σ_β : electric charge in the outer Helmholtz layer

σ_d : electric charge in the diffuse layer

σ_{max} : maximum retention capacity of bacteria by unit volume of porous media

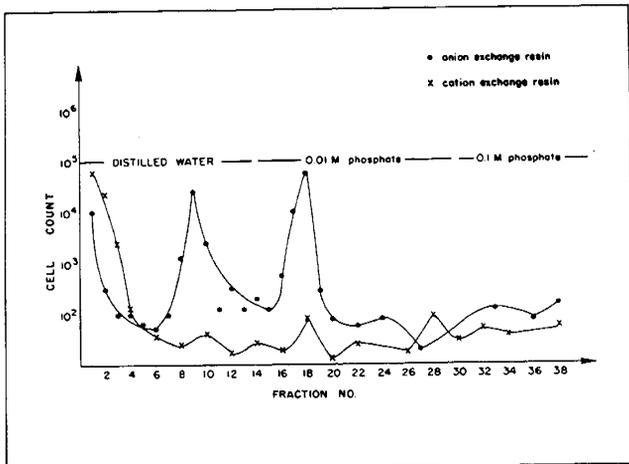


FIG. 8—Cell counts of bacteria collected in the effluent from ion-exchange resin columns.

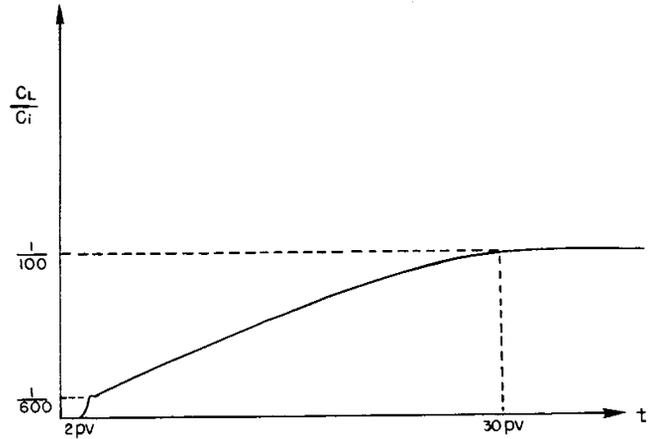


FIG. 9—History of effluent quality as a *Ps. putida* suspension ($3-7 \times 10^7$ cells/ml) containing 6,000 ppm of NaCl and 1,000 ppm of Tween 80 was injected into a CS core.

ϕ : total interactive energy (= $V_A + V_R$)

ψ_0 : surface potential

ψ_β : potential at the surface of inner Helmholtz layer

ψ_d : potential at the surface of diffuse layer (\approx zeta-potential)

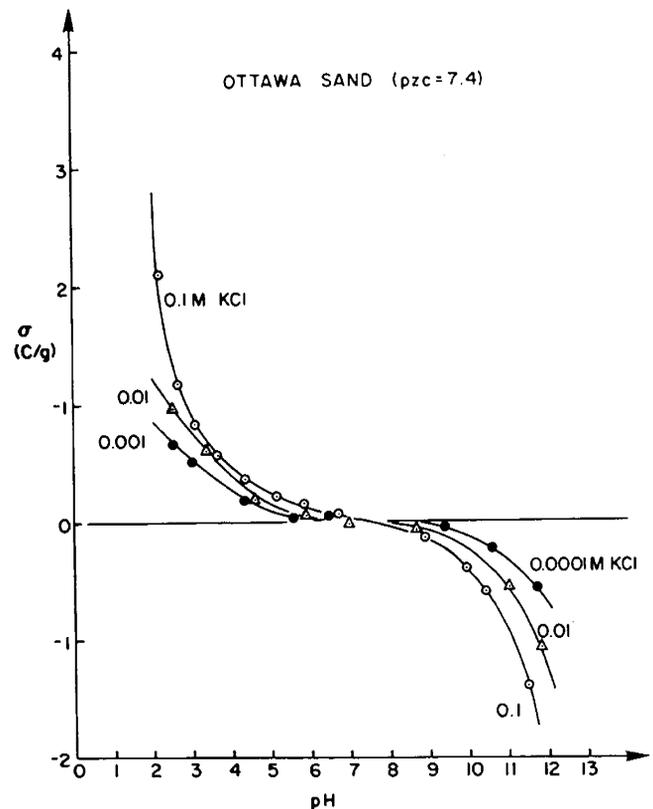


FIG. 10—The surface charge density of Ottawa sand as determined by potentiometric titration.

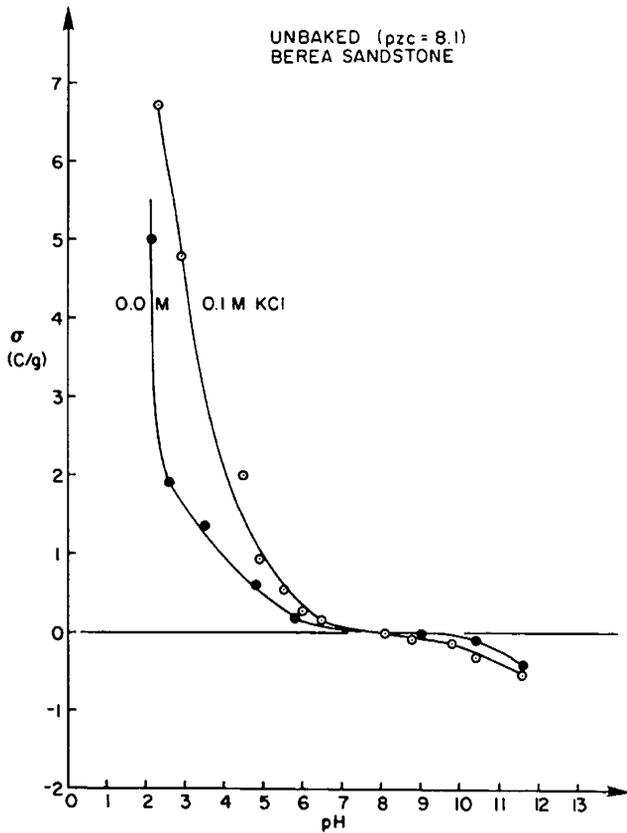


FIG. 11—The surface charge density of detrital grains from unbacked Berea sandstone as determined by potentiometric titration.

REFERENCES

1. E. C. Donaldson, R. F. Kendall, E. A. Pavelka, M. E. Crocker, "Equipment and Procedures for Fluid Flow and Wettability Tests of Geological Materials," Report of the Bartlesville Energy Technology Center, U.S. Department of Energy, 1980.

2. S. P. S. Yen, A. Rembaum, R. W. Molday, W. Dreyer, "Functional Colloidal Particles for Immunoresearch," ACS Sym. No. 24 *Emulsion Polymerization*, Amer. Chem. Soc. 1976.

3. J. A. Davis, R. O. James, J. O. Leckie, "Surface Ionization and Complexation at the Oxide/Water Interface," *J. Colloid and Interface Sci.*, 63(3), pp. 480-499 (1978).

4. C. Tien and A. C. Payatakes, "Advances in Deep Bed Filtration," *AIChE J.*, 25(5), pp. 737-759 (1979).

5. J. P. Herzig, D. M. Leclerc, and P. LeGoff in *Flow through Porous Media*, 6th State-of-the-Art Sym. of I&EC Div. I&EC, Amer. Chem. Soc., Washington, D.C., 6/9-6/11/69, pp. 129-158.

6. M. M. Sharma, unpublished work, 1982.

7. R. T. Hart, T. Fekete, and D. L. Flock, "The Plugging Effect of Bacteria in Sandstone Systems," *Can. Mining and Metallurgical Bull.*, 53, 495-501 (1960).

8. G. E. Jenneman, R. M. Knapp, M. J. McNemey, D. E. Menie, and D. E. Revus, "Experimental Studies of In-Situ Microbial Enhanced Oil Recovery," SPE/DOE 10789, in *The SPE/DOE 3rd Joint Symp. on Enhanced Oil Recovery* of Soc. Petro. Engr., Tulsa, OK, 4/4-4/7/82, pp. 921-931.

9. J. Karaskiewicz, "Recovery of Oil From Reservoirs by the Use of Bacteria," translated from *Nafta (Polish)*, 24(7), 198 (1968).

10. R. Rajagopalan, C. Tien, "The Theory of Deep Bed Filtration," in R. J. Wakeman, ed., *Progress in Filtration and Separation (1)*, Elsevier Scientific Publishing Co., N.Y., 1979, pp. 228-235.

11. J. Visser, "On Hamaker Constant—A Comparison Between Hamaker Constants and Lifshitz-van der Waals Constants," *Advan. Colloid Interface Sci.* 3, pp. 331-363 (1972).

12. L. K. Jang, unpublished work, 1982.

Transport Phenomena and Plugging in Berea Sandstone Using Microorganisms

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INTRODUCTION

The purpose of this study is to examine microbial methods for improving reservoir heterogeneity, particularly permeability variations, in order to improve oil recovery. Reservoir heterogeneity has a significant effect on the oil recovery efficiency of a waterflood or enhanced oil recovery process. Permeability variation greatly influences the volumetric sweep efficiency and its two-dimensional components of areal and vertical sweep efficiency. Meehan et al¹ demonstrated that additional oil recovery occurred if the channeling water in a waterflood was immobilized. The by-passed oil remaining after waterflooding is the target for the application of a reservoir selective plugging process utilizing the *in situ* growth of bacteria.

Numerous investigators have experimentally studied the plugging and transport of bacteria in Berea sandstone.²⁻⁶ However, these studies were concerned with injectivity problems caused by well-bore plugging by bacteria and most of the work was done with non-viable cells in nutrient-poor solutions. Little information exists concerning the transport, growth and metabolism of viable cells in Berea sandstone under nutrient-rich conditions.

The success of an *in situ* microbial plugging process depends on the ability: (1) to selectively transport the microorganisms into the water-sweep portion of the reservoir, (2) to transport the nutrients required for growth by these organisms, and (3) to reduce the apparent permeability of the reservoir rock stratum as a result of microbial growth and metabolism. This would divert the displacing fluid from plugged high permeability zones to the unswept low permeability zones that have higher residual oil saturations. These three aspects will be addressed in this paper to demonstrate feasibility of the process.

MATERIALS AND METHODS

Berea sandstone cores obtained from Cleveland Quarries (Amherst, Ohio) were cut into 2 × 8 in. cylinders with a coring device. Cores were either steam cleaned⁷ for 2 weeks and then dried or used as received. Each core was coated with epoxy and cast in a resin mold (Evercoat Fiberglass Resin). After cutting the core to a specified length, it was placed in the core holder of a flow apparatus (Fig. 1) and vacuum saturated with brine in a CO₂ environment. An oil-brine saturated core was prepared by saturating with brine under vacuum.

The core was then flooded with crude oil to an irreducible water saturation. Brine was then pumped through the core until no more oil was observed in the effluent. The initial brine permeability was then determined. The core and flow tubing (nylon) were sterilized with 2,000 ppm Oxine (a chlorine dioxide solution, Biocide Chemical Co.) for 2 hours. The Oxine was flushed from the system with sterile brine before the start of the experiment. Liquid flow was obtained using a Gilson HP4 Minipuls peristaltic pump. The differential pressure was recorded using Validyne DP-15 transducers with appropriate diaphragms.

Cores used in nutrient flow experiment ranged from 17.0-21.0 cm in length and had permeabilities ranging from 300-400 md. Each had a porosity of 17 percent. These cores were not steam cleaned prior to use. Core No. 5 and Core No. 7 used in the transport and plugging experiments were 4.0 cm in length with permeability of 196 md and 171 md respectively, and each had

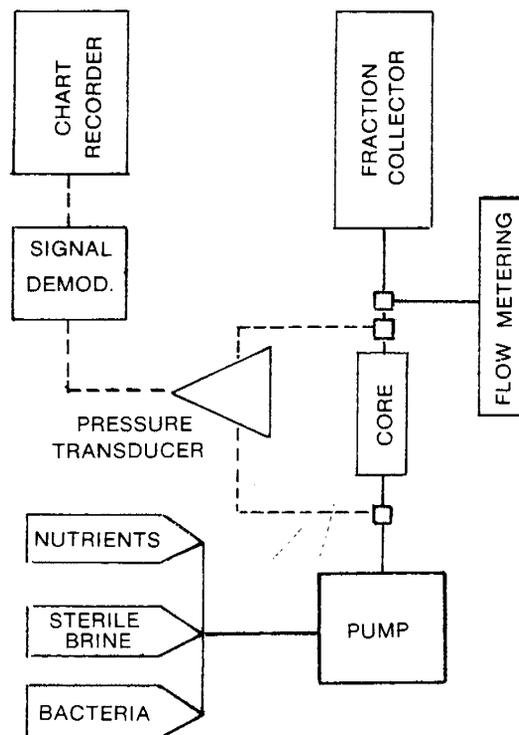


FIG. 1—Flow Apparatus.¹³

a porosity of 17 percent. Cores A (710 md) and B (120 md) were 12.7 cm in length. These cores were arranged in parallel to one another in the core apparatus with no cross flow. Cores A and B were steam cleaned prior to use.

The effluent for nutrient analysis was collected in 15 ml aliquots with a fraction collector. Fractions were combined into volumes about equal to that of the pore volume (PV) of the core. Each nutrient was dissolved in brine containing 2 percent (w/v) NaCl and 0.01 percent (w/v) CaCl₂. Analysis was performed within 12 hours of obtaining the last sample. Glucose,⁸ total phosphate,⁹ ammonia nitrogen,¹⁰ and protein,¹¹ were determined calorimetrically.

The thermotolerant bacterial culture No. 47 was obtained from the culture collection of the Department of Botany and Microbiology, University of Oklahoma. *Pseudomonas* strain I-2 was isolated from tap water using Tryptic Soy Agar (Difco, Co.). Medium E contained the following: 5.0 percent (w/v) NaCl, 0.1 percent (w/v) (NH₄)₂SO₄, 0.025 percent (w/v) MgSO₄, 1.0 percent (w/v) sucrose, 100 mM phosphate buffer (pH 7.0), and 1.0 percent trace metal solution.¹²

Agar (Bacto) was added at 1.5 percent (w/v) final concentration. Strain 47 was grown in Medium E with 1.0 percent yeast extract (Difco) at 50°C. *Pseudomonas* strain I-2 was grown in Tryptic Soy Broth (Difco) at 30°C then subcultured in Tryptic Soy Broth with 2.0 percent (w/v) of NaCl. Nutrients used in parallel core experiment consisted of two types (phase 1 and phase 2). Phase 2 contained Medium E + 0.5 percent yeast extract + 0.1 percent NaNO₃, whereas Phase 1 contained the same nutrients at one-tenth the concentration, except for the NaCl and phosphate buffer which remained the same.

Cells of strains 47 and I-2 used for transport studies were harvested during the log phase of growth by centrifugation (17,000 × g, 10 minutes) and washed once in sterile brine containing 2 percent (w/v) NaCl and 0.1 percent (w/v) CaCl₂. Enumeration of viable cells in the influent and effluent of the cores was determined by plating serial ten-fold dilutions on agar medium having the same composition as that used for liquid cultivation.

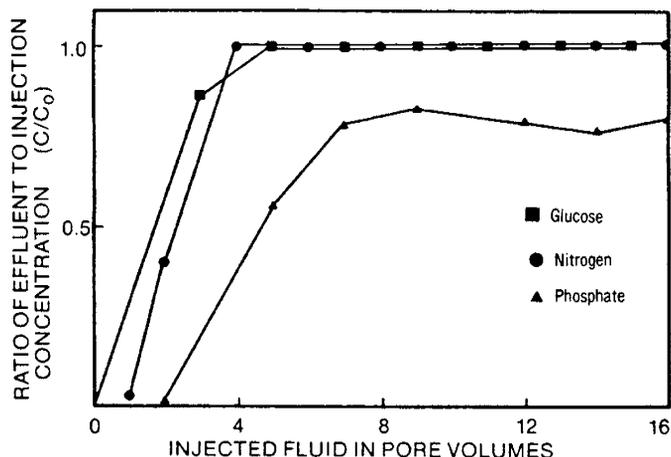


FIG. 2—Transport of nutrients through Berea sandstone, flow rate = 2.03 mls/min. Initial nutrient concentrations are as follows: 100 ppm (NH₄)₂SO₄; 150 ppm Na₂HPO₄.¹³

RESULTS

Nutrients essential for microbial growth were transported through Berea sandstone cores (Fig. 2). Essentially all the glucose was transported through the core after the first several PV. The recovery of ammonia nitrogen was completely stabilized at initial concentration by the third or fourth PV and over 80 percent of the phosphate was detected in the effluent by the tenth PV. The pH of the effluent samples remained constant during the experiment but was about two units higher than the influent pH.

Figure 3 shows the results of protein transport in both brine saturated and oil-brine saturated cores. After 16 PV were injected, less than 40 percent of the influent concentration of protein was recovered in the effluent. There was little difference in protein retention by either brine saturated or oil-brine saturated cores. The effluent pH of the brine saturated system dropped sharply from a pH of 8.5 to 7.0, while that of the system containing oil increased slightly.

Transport of viable cells through Berea sandstone was studied using a *Pseudomonas* isolate (I-2). This bacterium was suspended in a medium containing 2 percent NaCl and 0.01 percent CaCl₂ at a concentration of 1.8×10^5 viable cells/ml. Viable cells were detected in the effluent between the first and second PV (Fig. 4). However, at no time did the concentration of cells in the effluent exceed 1 percent of the inlet concentration. Chlorine dioxide sterilization prior to the experiment effectively reduced the indigenous population enabling the identification of low concentrations of the *Pseudomonas* isolate.

The results of bacterial plugging in the sandstone after the addition of nutrients is shown in Fig. 5. The *Pseudomonas* isolate (I-2) was used in this experiment and the details of the experiment are as follows:

Pseudomonas plugging experiment

Core No. 5 (Fig. 5)

1. Inject 126 PV sterile brine (2 percent NaCl + 0.01 percent CaCl₂) containing 9.3×10^4 cells/ml. (Strain I-2).
2. Inject one PV of Tryptic Soy Broth with 2 percent NaCl and 0.1 percent NaNO₃ and incubate at 25°C for 4 days. (a) pump sterile brine; (b) backflush with sterile brine; (c) pump sterile brine.

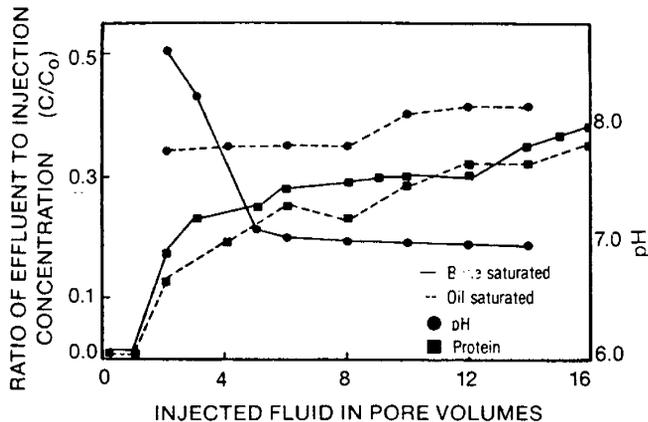


FIG. 3—Transport of protein (Bacto-Peptone) through brine and oil-brine saturated cores, flow rate = 1.96 mls/min. Bacto-Peptone concentration = 100 ppm.¹³

3. Inject Tryptic Soy Broth with 2 percent NaCl and 1 percent sucrose and 0.1 percent NaNO_3 , and incubate at 25°C for 2 days. Repeat (a), (b), and (c) in Step 2.

About 120 PV of *Pseudomonas* strain I-2 was pumped into core 5 (Step 1). Then a PV of nutrient medium was injected and the core was aseptically disassembled and incubated at 25°C for 4 days (Step 2). The core was reattached to the flow system and sterile brine was pumped through in the same direction as the cells were transported (a). After backflushing (b) a 60 percent reduction in the initial permeability was observed and remained stable for about 40 PV. The injection of additional nutrients (Step 3) resulted in an 80 percent reduction of the original permeability. However, backflushing (b) and continued flushing (108 PV) in the direction of cell injection (c) increased the permeability by about 16 percent.

In another experiment, only nutrients were injected into a core in order to determine the amount of plugging attributable to the indigenous microflora. The details of this experiment are as follows:

Indigenous microflora plugging experiment

Core No. 7 (Fig. 6)

1. Inject 1 PV of Tryptic Soy Broth (Difco) containing 2 percent NaCl, 0.01 percent CaCl_2 , and 0.1 percent NaNO_3 . Incubate for 3 days at 25°C . (a) pump sterile brine; (b) backflush; (c) pump sterile brine.

2. Incubation at 25°C for 3 days. (a) pump sterile brine.

After incubation at 25°C for 3 days, the permeability of Core No. 7 decreased by 40 percent (a). When the core was backflushed with 2 PV of sterile brine, the permeability increased by 10 percent (b). Additional pumping of brine in the original direction resulted in an increase in permeability over the first 24 PV and then a gradual reduction in permeability over the next 100 PV. The final effect was a 50 percent reduction in the initial permeability (c). After allowing for further incubation of the core for 3 days, 3 PV of sterile brine was again pumped through the core and permeability increased to 10 percent.

Two cores of differing permeabilities were connected

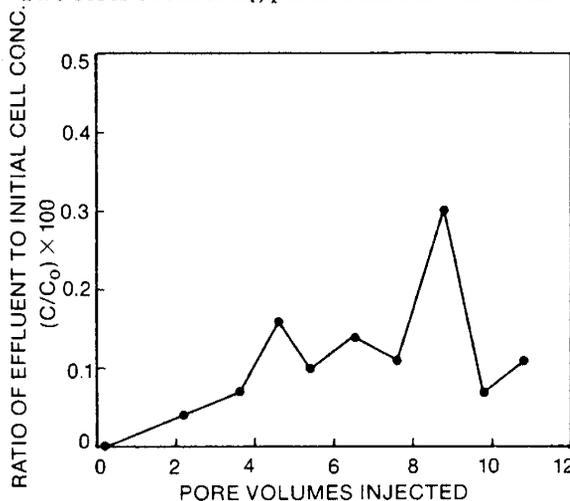


FIG. 4—Transport of *Pseudomonas* isolate (I-2) through Berea sandstone (Core No. 5). 1 PV = 1.3 mls, flow rate = 1.52 mls/min.

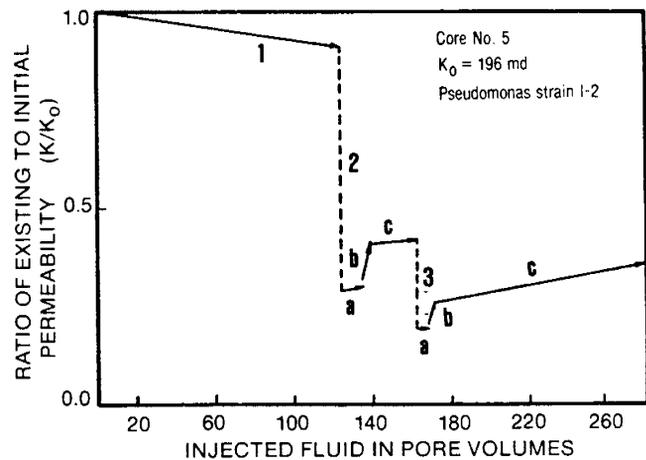


FIG. 5—*Pseudomonas* plugging experiment (I-2), flow rate = 1.52 mls/min.¹³

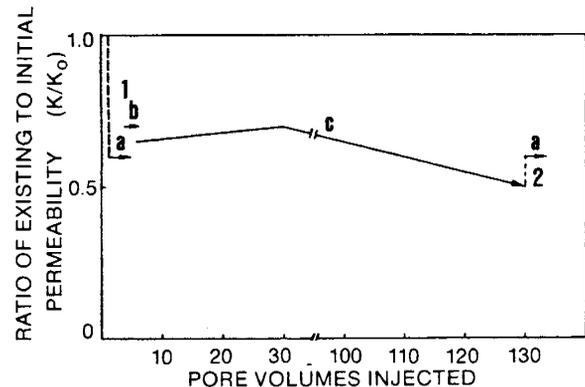


FIG. 6—Plugging in Berea sandstone by indigenous microorganisms (Core No. 7). 1 PV = 13.8 mls, flow rate = 1.52 mls/min.

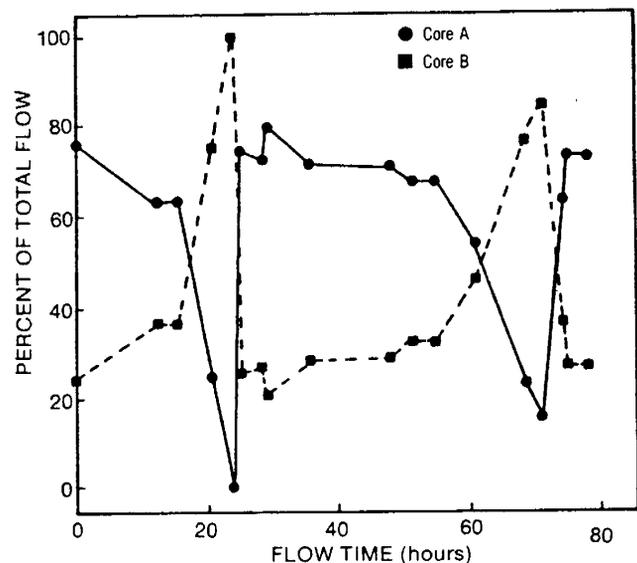


FIG. 7—Multiple core experiment. Core A (710 md), Core B (120 md), 1 PV = 59.0 mls, flow rate = 1.50 mls/min.

in parallel without cross flow and incubated at 50°C to investigate the selectivity of the process (Fig. 7).

Initially 76 percent of the total flow passed through Core A and 24 percent through Core B. Sterile brine containing 5 percent NaCl and a cell suspension of 10^4 cells/ml of a thermotolerant *Bacillus* strain 47 were added. After 24 hours of cell injection, the pattern of flow changed as all the flow was diverted into the low permeability core. Backflushing for 5 hours restored the initial pattern.

A low concentration of nutrients (phase 1) was injected for the next 19 hours of flow time and little change in the flow pattern was observed. After 48 hours of flow, a high concentration of nutrients (phase 2) was injected. This resulted in a change in the flow pattern with 90 percent of the total flow being diverted into Core B. Biomass in the effluent was observed after the 65th hour. The cores were then allowed time to incubate for several days without further nutrient additions. This restored the flow pattern to that initially observed at the start of the experiment.

DISCUSSION

Carbon (glucose), nitrogen (ammonium sulfate), and phosphate (potassium phosphate) in amounts sufficient to support cell growth are transported through sandstone cores with the first several PV. Phosphate is not transported as readily as the other nutrients possibly due to adsorption onto the rock or precipitation by calcium ions. However, enough phosphate was recovered in the first several PV to theoretically support at least 10^9 - 10^{10} cells/ml.

Protein (Bacto-Peptone) does not appear to be as good a carbon and nitrogen source as glucose and ammonia because of the retention of protein inside the core, even in the presence of crude oil. The difference in effluent pH when protein was added to oil-brine saturated versus brine-saturated cores indicates that different proteins (basic vs. acidic) may be retained depending on the type of saturation (oil vs. brine). The increase in pH (2 units) of the effluent samples from that of the influent samples can be explained as an increase in carbonate alkalinity. Therefore, a CO_2 bicarbonate buffering system may be needed to control pH for optimal cellular metabolism.

Viable cells when suspended in brine are transported through Berea sandstone cores. However, under the conditions used (2 percent NaCl and 0.01 percent CaCl_2), only a small percentage of those cells injected are recovered in the effluent. Slight increases in pressure observed during cell injection which can be decreased by backflushing implies that some of the cells are being retained at the inlet face. As to whether this retention is due to adsorption or mechanical entrapment has not been studied. It is possible under other conditions of ionic strength, pH, flow rate, permeability, or cell type that a higher percentage of cells can be transported through the rock.

We are presently most concerned with what happens to the permeability of these cores after growth of the bacteria inside the core. It is hoped that production of biomass (cellular and exocellular) would selectively plug the water-swept high permeability areas and effectively reduce the heterogeneity of the reservoir. The addition of nutrients to cores previously injected

with bacteria (I-2) resulted in cellular growth inside the core resulting in significant reductions in permeability.

These permeability reductions were stable after backflushing and reinjecting numerous PV of brine at relatively high flow rates (greater than 1.0 ml/min). Thus, bacteria are capable of producing enough biomass inside the core to produce significant permeability reductions (60-80 percent). Also, this suggests an alternative means of transporting cells through a rock formation by growing the bacteria through the porous medium rather than transporting the cells in brine.

One problem in these studies is determining how much of the plugging is due to injected cells and how much is attributable to the indigenous populations. Indigenous microbial populations including Actinomycetes and genera such as *Pseudomonas* and *Bacillus* were observed in every core used. Even after cores are steamed for 2 weeks, autoclaved (15 psig, 121°C) for 12 hours and dried at 121°C, viable indigenous populations still remain (approx. 10^3 - 10^4 cells/ml). The use of a chlorine dioxide solution prior to cell injection reduces the counts of indigenous microflora for as long as 24-48 hours.

However, after this time or upon nutrient injection, reestablishment of these populations occurs. If a more selective environment is used (50°C and 5 percent NaCl) then the indigenous populations are suppressed but upon the addition of certain nutrients other types of indigenous microbes are observed. The indigenous populations can account for as much as a 30-50 percent of the reduction in permeability (Fig. 6). These problems complicate the determination of the causative agent of plugging. Presently we are studying the use of antibiotics to inhibit these indigenous microorganisms.

In order to demonstrate the selective transport of cells and nutrients into the higher permeability zones, a multiple core experiment was performed. Two cores of differing permeability connected in parallel and with no cross flow was constructed. Cells were selectively transported to the higher permeability core and caused all the flow to be diverted to the low permeability core. After the original flow pattern was restored by backflushing and phase 2 nutrients injected, a diversion of the flow to the low permeability core again occurred.

This indicates that the nutrients were selectively transported to the higher permeability core and that subsequent plugging due to bacterial growth caused the flow to be directed into the lower permeability core. Bacterial growth was substantiated by the presence of visible biomass in the effluent. After the core was allowed to incubate for 3 days, the pattern of flow was similar to that at the start of the experiment. This can be attributed to cell lysis and death. The occurrence of substantial plugging of the high permeability core when cells alone were injected is due to facial plugging since backflushing restored the original flow pattern.

Facial plugging can be expected when a large number of pore volumes are injected, especially if a relatively large rod similar to *Bacillus* is injected. The purpose of the injection of phase 1 nutrients was to determine if a lower concentration of nutrients would cause distribution of the injected cells throughout the core without causing major permeability reductions. It is evident from this experiment that the latter was achieved but evidence of dispersion of the bacteria in

the core is still forthcoming.

Experiments are presently being conducted to study the extent or depth of plugging that occurs inside these cores. This will be important in a cross flow model where access to different permeabilities can extend the length of the core instead of only at the face.

CONCLUSIONS

1. Nutrients essential for microbial growth can be transported in concentrations sufficient to support good biomass production.

2. Nutrients and cells are selectively transported to high permeability zones.

3. Indigenous microorganisms exist under all conditions tested and are capable of contributing to the plugging activity.

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REFERENCES

1. Meehan, D. N., Crichlow, H. B. and Menzie, D. E.: "A Laboratory Study of Water Immobilization for Oil Recovery," *J. Pet. Tech.* (Feb. 1978) 30:317-322.

2. Merkt, E. E.: *The Effect of Bacteria on the Permeability of Oil Reservoir Rocks*, Thesis, University of Texas, Austin, Texas (1943).

3. Fekete, T.: *The Plugging of Bacteria in Sandstone Systems*, Thesis, University of Alberta, Edmonton, Alberta (1959).

4. Raleigh, J. T., Flock, D. L.: "A Study of Forma-

tion Plugging with Bacteria," *J. Pet. Tech.* (Feb. 1965) 201-206.

5. Hart, R. T., Fekete, T., and Flock, D. L.: "The Plugging Effect of Bacteria in Sandstone Systems," *Can Mining Met. Bull.* (1960) 53:495-501.

6. Kalish, P. J., Stewart, J. E., Rogerts, W. F., and Bennett, E. O.: "The Effect of Bacteria on Sandstone Permeability," *J. Pet. Tech.* (1964) 16:805-814.

7. Sutterfield, F. D.: *Extraction and Characterization of Humic Acids*. Thesis, University of Tulsa, Tulsa, Oklahoma (1973).

8. Sigma Technical Bulletin: *The Colorimetric Determination of Glucose* (January 1971) No. 635.

9. Hach Chemical Company: "Oxidation and Hydrolysis to Orthophosphate for Water and Wastewater," *Hach Water and Wastewater Analysis Procedure Manual*, 3rd Edition, Ames, Iowa (1975) 2-104.

10. Hach Chemical Company: "Nessler Method for Water and Wastewater," *Hach Water and Wastewater Analysis Procedure Manual*, 3rd Edition, Ames, Iowa (1975) 2075.

11. Lowry, Oliver H., Rosebrough, Nira J., Farr, Lewis A., and Randall, Rose J.: "Protein Measurement With the Folin Phenol Reagent," *J. Biol. Chem.* (1951) 193:265-275.

12. Clark, J. B., Munnecke, Douglas M., and Jenneman, Gary E.: "In Situ Microbial Enhancement of Oil Production," *Developments in Industrial Microbiology* (1981) 22:695-701.

13. Jenneman, Gary E., Knapp, Roy M., McInerney, Michael J., Menzie, Donald E., and Revus, David E.: "Experimental Studies of In Situ Microbial Enhanced Oil Recovery," *Proceedings, 3rd Joint SPE/DOE Symposium on Enhanced Oil Recovery*, April 4-7, 1982, Tulsa, Oklahoma. pp. 921-932.

Possible Reservoir Damage from Microbial Enhanced Oil Recovery

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ABSTRACT

Texas has more than 2,500 secondary recovery or water injection projects. The water processing facilities, surface injection equipment, wells and producing facilities are all in place to be used if a profitable microbial enhanced oil recovery method can be developed.

It will be necessary that the potential reservoir damage from microbial EOR be resolved. Great expense is presently entailed to keep the injection water free of all substances which may invite trouble. The water may be aerated or deaerated. Various chemical compounds in the water may be precipitated or solubilized. The water is filtered, polished, corrosion proofed, and the pH adjusted to ensure a suitable injection water. In some cases the well may be acidized to increase water injectivity. Periodically large doses of chlorine may be used to reduce or eliminate live organisms of most all types which may have found their way into the system.

The connecting pores at the face of the well and back into the pay may be only a fraction to a few microns in diameter, and the pores are easily plugged. If the microorganisms should plug the pores at the sand face or in the interior, the water injectivity may be reduced by a factor of two or more. Such will postpone the oil production until the project may become unprofitable and the enhanced oil recovery project would be suspended.

Some oil sands contain bentonite which will swell when one injects fresh water. In many cases the reservoir engineers are required to inject salty, noxious water to keep the water injectivity high. In summary it will be necessary that any proposed microbial EOR method be sufficiently tested to ensure compatibility with presently practiced injection techniques if microbial EOR is to receive wide application in the oil field.

INTRODUCTION

The treatment of water for enhanced oil recovery represents a very high level of technology. The petroleum industry goes to great expense to insure that the water is sufficiently free of most all bacteria and microorganisms that profitable oil recovery processes may be carried out. To install a microbial enhanced oil recovery method, it would appear necessary that the strains of bacteria be developed which would be satisfactory utilizing existing water treatment technology or else the water technology program should be changed or altered to permit the use of the proposed microorganisms.

Microorganisms result in plugging and fouling of the equipment, corrosion and scale formation. Since the injected water may be either aerated or deaerated the microorganisms may be present in the treating facilities. Most all of the oil field waters are filtered and the filters become subject to plugging. The water pumps, surface lines, injection wells and the sand face and the formation itself may all be subject to bacterial plugging. Additionally, masses of the microorganisms may come off and move down the flow line resulting in plugging of the equipment.

To eliminate microbial plugging problems the injected waters are treated with bactericides and germicides. When plugging of the well appears eminent the wells may be treated with high doses of chlorine, or with acid of 5 to 15 percent.

BACTERIAL CORROSION

Bacterial corrosion occurs both from aerobic and anaerobic bacteria. The anaerobic bacteria may produce H_2S forming an acidic water. The resulting iron sulfide forms concentration cells which increase the rate of corrosion. The aerobic bacteria need oxygen. Small quantities of dissolved oxygen will surround bacteria adhering to the sides of the pipe. The oxygen in turn will form an oxide and pitting will result.

Iron sulfide is a corrosion product. The corrosion rate may be increased as the iron sulfide will form concentration cells. The deposits may then result in an increased rate of pitting.

Bacterial plugging results from bacterial growth which may plug the system directly. In addition, there may be a sloughing of the mass of bacteria together with the products themselves, iron sulfide and iron oxide which also plug the system.

MICROBIAL PLUGGED WELLS

Figure 1 shows a top view of a microbial plugged well. The well radius is indicated as R_w in the figure. Well radii for most oil wells usually range from approximately 5 in. to 10 in. in diameter, but a few wells may be smaller and a few may be larger in diameter. The microbial plugged zone may extend from the well radius R_w out to a radius R_o . The partially plugged zone would have some permeability K_1 where the permeability K_1 is less than the permeability of the rock prior to microbial plugging. Beyond the plugged zone is the undisturbed reservoir permeability K_2 . This region extends from R_o to R_e . The region from R_o to R_e has not

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been affected by microbial plugging. When such conditions exist Muskat¹ has shown that the equation showing the relative injection rates before and after plugging is given by Equation (1) below:

$$\frac{Q}{Q_0} = \frac{(K_1/K_2) \text{Log } R_e/R_w}{\text{Log } R_0/R_w + (K_1/K_2) \text{Log } R_e/R_0} \quad (1)$$

where:

- Q = flow rate after microbial plugging
- Q₀ = unrestricted flow rate
- K₁ = permeability in plugged area
- K₂ = permeability in unplugged area
- R_w = well radius, ft.
- R₀ = plugged radius, ft.
- R_e = drainage radius, ft.

Since equation (1) has been set up as ratios of fluid injection rates Q/Q₀, the equation is completely dimensionless, i.e., Q/Q₀ is independent of the unit of measurement; the radii terms are all shown as the ratios of radii so they are independent of the unit of measurement, and K₁/K₂ becomes a dimensionless number. Thus the equation becomes quite useful for relating the fluid injection rate after microbial plugging to the fluid rate before microbial plugging.

The relative fluid injection rates for a microbial plugged well has been calculated using equation (1). It was assumed for these conditions that the well diameter was 8 in. and that the radius of plugging was 50 ft. The drainage radius R_e was set at 330 ft. Normally for many calculational purposes the drainage radius is approximately half the distance between the input and the output well.

Table 1 shows the calculated flow rate after microbial plugging. From the table it will be seen that for the unplugged zone the relative flow rate is 1.0. If the plugged zone is reduced to only 0.5 of the original permeability the relative flow rate or injection rate after plugging is only 0.58. For a plugged permeability of 0.25 the relative flow rate after plugging will be 0.31. For a relative plugging of 0.1, 0.05 and 0.01 the relative flow rates will be 0.13, 0.067 and 0.014, respectively.

The flooding rate has a very great effect on the relative flooding times. As the fluid injection rate begins to decrease this tends to extend the normal flooding time.

TABLE 1—Calculated Flow Rate After Microbial Plugging

Radius of plugging = 50 ft; well diameter = 8 in. drainage radius = 330 ft	
Plugged Permeability (Fraction)	Relative Flow Rate After Plugging (Fraction)
1.0	1.0
0.5	.58
0.25	.31
0.1	.13
0.05	.067
0.01	.014

TABLE 2—Relative Flooding Times for Microbial Plugging

Basis: Normal flooding time = 6 yr; plugged radius = 50 ft; well radius = 8 in.	
Microbial Plugged Permeability (Fraction)	Relative Flooding Time (Years)
1.0	6
5.	10.3
.25	19.3
.1	46
.05	90
.01	425

TABLE 3—Calculated Flow Rate After Microbial Plugging

Radius of plugging = 5 ft; well diameter = 8 in. drainage radius = 330 ft	
Plugged Permeability (Fraction)	Relative Flow Rate After Plugging (Fraction)
1.0	1.0
0.5	0.72
0.25	0.46
0.1	0.22
0.05	0.12
0.01	0.025

TABLE 4—Relative Flooding Times for Microbial Plugging

Basis: Normal flooding time = 6 yr; plugged radius = 5 ft; well radius = 8 in.; R _e = 330 ft	
Microbial Plugged Permeability (Fraction)	Relative Flooding Time (Years)
1.0	6
5.	8.3
.25	13
.1	27
.05	50
.01	240

Table 2 shows the relative flooding times from microbial plugging on the basis that the normal flooding time for an oil field pattern is six yr. The table was calculated on the basis that the plugged radius is 50 ft, and the well radius was 8 in. From the table it is seen that for the unplugged well the relative flooding is 6 yr; however, if the microbial plugged permeability is 0.5 the relative flooding time will be 10.3 yr. If the microbial permeability is 0.25 then the relative flooding time is 19 yr. Clearly flooding times extending beyond this period would be uneconomical.

Table 3 shows the calculated flow rate after microbial flooding for the case in which the radius of plugging by the microbes is only 5 ft; the well diameter is 8 in. and the drainage radius 330 ft. It is seen from this table that if the plugged permeability is of the order of 0.5 to 0.25 the relative flow rates will be of the order

0.72 to 0.46 of the unplugged rate. If the plugged permeability is of the order of 0.1 the relative flow rate after plugging will be of the order of 0.22.

Table 4 shows the relative flooding times for microbial plugging on the basis that the plugged radius extends out 5 ft. The normal flooding time is 6 yr, the well radius is 8 in., the drainage radius R_e is 330 ft. It is seen from this table that if the microbial plugged zone is of the order of 0.50, 0.25 to 0.1 the relative flooding times become 8.3 yr, 13 and 27 yr, respectively. These flooding times compare with a relative flooding time of 6 yr without flooding. It is readily seen that significant microbial plugging can extend the flooding times to such a great extent that the oil recovery program becomes completely unprofitable.

PRESSURE DISTRIBUTION AFTER MICROBIAL PLUGGING

Microbes may easily reduce the permeability of the reservoir rock to only 10 to 85 percent of the permeability without microbial plugging. The reduction may be more or less, but the pressure in the oil reservoir may be calculated. It will be found that the pressure distribution between the injection well and the radius of plugging will be altered. The pressure distribution in the plugged zone will then be given by the equation below:

$$P_r = P_w - \frac{(P_w - P_e) \text{Log } (R/R_w)}{\text{Log } R_o/R_w + K_1/K_2 \text{Log } R_e/R_o} \quad (2)$$

†Note

P_r = pressure at any radius from the well to the outer radius of the microbial plugged zone

P_w = pressure at the well sandface

P_e = pressure at the radius R_e

R = radius of interest at which pressure is desired

R_w = well radius

R_o = outer radius of plugged zone

R_e = drainage radius

R is the radius of interest, for example, if the plugged radius, R_o is of the radius 50 ft then the R would be any radius between the well radius and R_o . The ratio K_1/K_2 represents the permeability in the microbial plugged zone to the unplugged permeability, respectively. In referring to the equation it will be seen that only one term in the equation is related to the permeability in the plugged zone, and this ratio occurs in the denominator. In applying the above equation it will be found that a greater reservoir pressure difference is required to induce flow in the plugged system. To maintain the desired flow rate could require a pressure sufficiently great to fracture the rock.

Scale treatment

The usual scale forming compounds include calcium carbonate, calcium sulfate and barium sulfate. Phosphates are added to the water to prevent scale deposition. Organic phosphate esters and inorganic polyphosphates may be used. The sodium salts of the phosphates or phosphoric acid may be added to the water to prevent scale deposition.

Sodium citrate and sodium gluconate may be added

to the water to sequester cations and prevent scale deposition. The organic materials are subject to decomposition by microorganisms. This could result in scale deposits in the flow system or in the rock itself.

BACTERIA FOR STRATIFICATION RECTIFICATION

Approximately 20 years ago the author prepared two articles showing the possible use of bacteria for the correction of stratification problems when waterflooding a reservoir.^{2,3} Since the pore size distribution of some oil sands ranges from less than 1 micron to the order of 10 to 30 microns in diameter, it is clear that this falls well within the range of many microorganisms. For this reason the microorganism can permeate the rock, and it is entirely possible that the microorganism may plug a portion of it.

Writing in 1961 the author reported on the relation between the time and the percent of the original permeability which was observed for three types of cores subjected to water containing bacteria. The cores were low, medium and high permeability. Basically, it was found that the bacteria would plug the high permeability oil sand to a greater extent than the medium permeability and the medium permeability oil sand was plugged to a greater extent than the cores of low permeability.

From Fig. 2 it is seen that at the time when the high permeability sand was plugged 50 percent, the medium permeability oil sand might have a 65 percent remaining permeability and the low permeability rock could have 90 percent of its original permeability. This is important because in waterflooding it is frequently found that there are zones of high permeability. These zones take the water; these zones result in premature breakthrough of the water. The water bypasses much of the reservoir rock, and the oil recovery is less than it should be. As time continued it was found that the more permeable zones are plugged to a much greater extent than the tight zones.

The fact that the permeable zones are plugged to a greater extent than the tight zones could result in partial rectification of a stratification problem. The actual value of the permeability reduction depends upon the bacteria, the nature of the rock, reservoir conditions, time and many other factors.

Figure 3 shows the effect of bacteria on the preferential plugging of very permeable sands where the permeability ranged up to 1,000 millidarcies. It is seen that the water containing the bacteria preferentially plugged the rock of high permeability to a far greater extent than it did the rock of lower permeability. By utilizing this technique it is entirely possible that the bacteria may rectify a stratification problem or a problem in which the water breaks through prematurely. For example, if one were able to partially resolve the stratification problem with bacteria it is obvious that one would be able to obtain more oil for the same amount of water or conversely obtain the same oil for less water.

SUMMARY

The pore size of oil field rock is sufficiently large to permit the entry of various microorganisms. These

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microorganisms may plug the rock so that the water injection rates become too low for the oil recovery project to be economical. The petroleum industry may inject various bactericides and germicides, chlorine, and acid to restore the injectivity.

It is possible that thief zones taking excess water could be partially plugged by bacteria and result in more oil produced for the same amount of water or the same amount of oil for less water. This possibility should be explored in depth.

An economical MEOR process must either permit the application of existing water technology or plan to modify the water treatment process to permit the MEOR process to proceed.

REFERENCES

1. Muscat, Morris: *Physical Principles of Oil Production*, McGraw-Hill Book Co. Inc., (1949) p. 244.
2. Crawford, Paul B.: "Possible Bacterial Correction of Stratification Problems," *Producers Monthly*, Dec. 1961, p. 10-11.
3. Crawford, Paul B.: "Continual Changes Observed in Bacterial Stratification Rectification," *Producers Monthly*, Feb. 1962, p. 12.

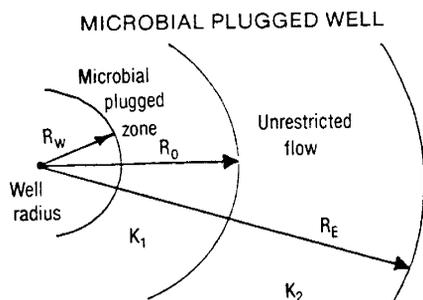


FIG. 1—Top view of pertinent parameters associated with a microbial plugged well.

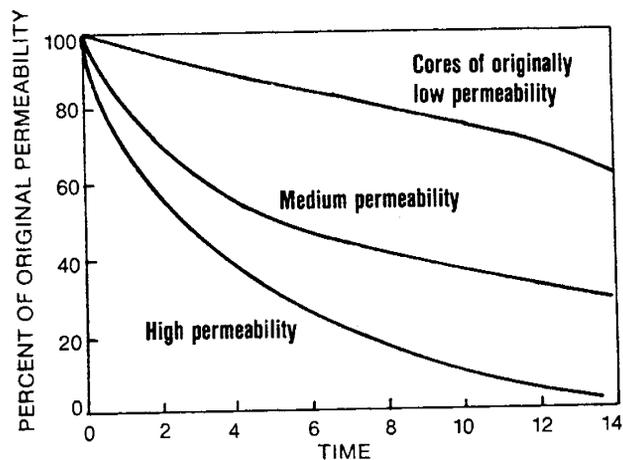


FIG. 2—Relation between time and observed reduction in permeability of three types of cores subjected to waters containing bacteria.

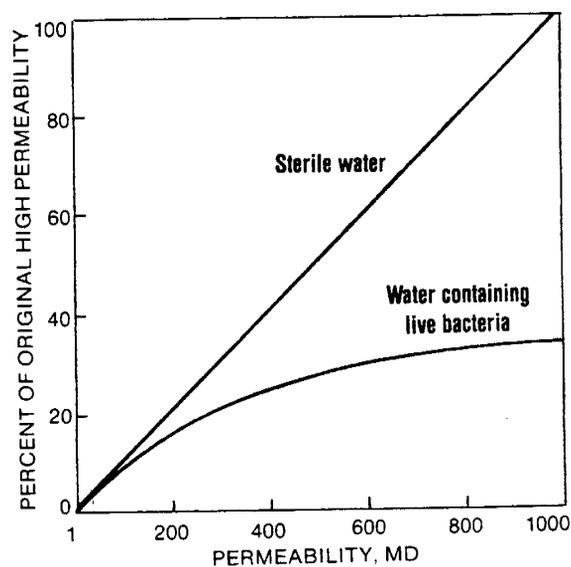


FIG. 3—Legend effect of live bacteria on the preferential plugging of very permeable sands.

Plugging and Penetration of Petroleum Reservoir Rock by Microorganisms

David M. Updegraff

Very early in the history of the petroleum industry it was recognized that primary production alone, meaning production from flow caused by natural reservoir pressure or by downhole pumps, recovered only a fraction of the oil present in the reservoir. A good average value is one third of the oil originally in place. The use of secondary oil recovery methods soon followed. The injection of gas and air was initiated about 1888, and water injection followed soon after. Both processes are still widely used.³⁰

In terms of the amount of extra oil recovered, water injection, or water flooding as it is often called, is by far the most successful secondary oil recovery method. Tertiary recovery methods, also called enhanced recovery methods, such as steam injection, surfactant flooding, polymer flooding and micellar flooding, may recover much more oil, but are much more expensive. Indeed they are relatively rarely used today except in special cases such as recovery of extremely heavy viscous oil because water and gas injection methods are so much more economical.

A water flooding project is best carried out by "unitizing" the entire oil field so that a plan can be worked out for water flooding the entire field. The plan is a careful balance between costs and recovery. For maximum recovery, the number of injection wells should equal or exceed the number of production wells. Also the recovery increases as spacing between injection wells is decreased. Therefore it is desirable to drill a very large number of new wells in the field before flooding. But drilling is expensive, so a compromise must be reached based on maximizing profits over a specified period of time.

PLUGGING

Another factor of great importance in the success of a water flooding project is the quality of the water used. Early water flooding projects often used lake or river water containing large numbers of microorganisms, including bacteria, fungi, protozoa and algae. This factor was probably recognized by early workers, but was not quantitatively investigated until a group of petroleum engineers at the University of Texas, Austin, began work on the effect of microorganisms in injection water used in the Luling and East Texas fields. The M.S. thesis of Merkt (1943),¹⁸ published with additional observations by Plummer, Merkt, Power, Savin and Tapp (1944)²⁴ described these findings in detail. The water was a fairly strong brine in both fields, and was shown by both cultural and microscopic examination to contain many microorganisms.

The waters, containing different kinds and different numbers of microorganisms were injected into cylin-

dric cores of oolitic limestone from the Walnut formation with initial permeabilities varying from 0.4 to 2.1 md and sandstone cores of 6,960 to 50 md. The Luling water contained algae, calcium carbonate precipitates, diatoms, sulfur bacteria (including *Thiobacillus thio-parus*, *Thiobacillus thiooxidans*, and *Thiospirillum*) sulfate-reducing bacteria, hydrogen sulfide and sulfur precipitates. Flowing from 6 to 18 liters of this water through three of the walnut cores 2½ in. in diameter by 3 in. long reduced the permeability of the cores from 6 to 33 percent. A sandstone core with a permeability of 6,960 md was reduced to 76.8 md by flowing 5 gal of distilled water through the core (probably caused by swelling of clays in the rock) and then injection of 5 gal of Luling water reduced the permeability further to 3.4 md. The East Texas water was of much poorer quality, and was black with precipitated ferrous sulfide produced by sulfate-reducing bacteria, and also contained the iron bacterium, *Leptothrix*. A Walnut core permeability was reduced from 1.1 md to 0.31 md. A sandstone core was reduced from 50 to 1.9 md. Unfortunately the plugging effect caused by the bacteria could not be distinguished from that caused by inorganic precipitates in this study (FeS, S and CaCO₃).

Plugging problems encountered during water flooding in the Bradford-Allegany field, as well as in East Texas and Kansas oil fields, were studied by Beck.² He showed the presence of *Desulfovibrio*, iron bacteria and *Pseudomonas* in the injection water, as well as precipitates of ferrous sulfide and ferric hydroxide. Water treatment by means of germicides and sand filtration was recommended to eliminate both bacteria and their iron-containing products.

Another laboratory study on bacterial plugging was carried out at the University of Texas by O'Bryan and Ling,²² but here the emphasis was entirely on ferrous sulfide as a product of the action of sulfate-reducing bacteria, and the decreases in permeability of the cores were attributed to this metal salt alone rather than to bacterial cells.

A more carefully controlled study on the effect of bacteria on the permeability of cores of Berea sandstone was carried out by Hart, Fekete and Flock¹⁴ at the University of Alberta. The sandstone cores, 2.5 cm in diameter and 7-9 cm long, were mounted in plastic, and three taps were drilled through to the core in order to monitor pressure. Cells of *Bacillus subtilis* (1 µm) were suspended in distilled water, killed by autoclaving, and injected into the cores under a constant pressure of 40 psig. Permeability, monitored by flow rate measurements, was measured as a function of the volume of cell suspension (of known bacterial count) injected. The permeability was reduced to 80 percent of

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the initial value by 45 pore volumes of a suspension containing 10^5 cells/ml, while the same volume of suspension containing 10^6 or 10^7 cells/ml reduced the permeability to less than 10 percent of the initial value. Bacteria filtered out on the injection face, and most of the plugging was close to the injection end.

A more comprehensive study was carried out by Kalish, Stewart and Rogers of the Gulf Oil Co. and Bennett of the University of Houston in 1964.¹⁶ Again Berea sandstone cores were employed, carefully chosen to include three permeability ranges: high, 278 to 400 md, medium, 130 to 162 md, and low, 17.7 to 48.3 md. The cores were 1 in. in diameter and 4 in. long for the high and medium permeability cores and 2 in. long for the low permeability cores. Again, killed bacteria were injected but this time suspended in calcium chloride brine instead of distilled water. This avoids the drastic reductions in permeability brought about in some cores by the swelling of clays when contacted with distilled water. Bacteria of different sizes, shapes and states of aggregation were employed to evaluate the effects of these factors on permeability reduction. Permeability was calculated by measuring pressures at both ends of the cores, as well as at intermediate taps, under a constant flow rate, and applying Darcy's law:

$$Q = \frac{kA(P_1 - P_2)}{v \cdot L}$$

where:

Q = flow rate; ml/sec

A = area of core cross section, cm^2

P_1 = pressure at injection end of core, atm

P_2 = pressure at opposite end of core, atm

L = length of core, cm

v = viscosity of fluid, cps

k = permeability, darcies

In every case, bacteria decreased the permeability of the cores, and as expected larger bacteria and those forming aggregates of cells decreased the permeability more. Many of the bacteria filtered out on the inlet face of the core, but in all cases some bacteria penetrated all the way through the cores. Bacteria penetrated high permeability cores more than low permeability cores. Cores were sectioned and stained with crystal violet to reveal penetration patterns, and the pattern was often irregular, showing the effect of non-uniform pore-size distribution. The data were expressed by plotting the permeability ratio, k/k_i where k is measured permeability and k_i is the initial permeability, against the number of bacteria injected. The smooth curves obtained showed many interesting effects, which may be summarized as follows:

1. The adverse effect of plugging is present as a skin, with the most severely plugged zone at the injection face.

2. Large cells plug more severely than smaller cells, and aggregated bacteria such as *Micrococcus roseus* more than single cells.

3. A pronounced concentration effect was observed, with lower concentrations of bacteria showing much

more severe plugging than higher concentrations per unit number of bacteria injected into a core.

4. Low permeability formations develop a higher stabilized k/k_i value than high permeability formations.

5. Increase in pressure results in an increase in permeability, presumably due to moving bacteria out of blocked pores.

6. Plugging can be alleviated by flowing hydrochloric acid through the cores.

A study of this excellent paper is a must for anyone planning work in microbial enhanced oil recovery. Pore-size distribution curves were made on the cores as well, and correlate well with results. The measurement and significance of pore-size distribution will be discussed in the next section of this paper.

A later study by Raleigh and Flock^{26,27} of the University of Alberta employed very similar methods. Killed cells of *Bacillus subtilis* ($1 \times 4 \mu\text{m}$) suspended in sodium chloride brine were employed. Cores of four distinct texture types were employed: Berea sandstone, Cardium sandstone, Devonian limestone and Indiana limestone. All cores were evaluated for oil and water wettability by capillary imbibition tests and for pore size distribution and pore geometry factor (G). Plots of permeability ratio against pore volumes of bacterial suspension injected showed a steady decline in permeability with volume injected in all cases. The rate and degree of plugging did not correlate with any measurable rock property, porosity, permeability, pore size distribution, wettability or pore geometry factor. Evidently there is such wide variation in the geometry of the interconnected pores in petroleum reservoir rocks that such correlations are not observable.

Allred¹ published a review of his own work with Conoco Oil Co., as well as the work of others on the control of microbial corrosion and plugging in water flooding projects. A water flooding project in California employing sea water which contained 20 to 150 million bacteria per ml reduced the permeability of cores by about 80 percent. The same water, treated with mercuric chloride showed no reduction in permeability. The mercuric chloride did not remove the bacteria but prevented them from multiplying within the reservoir rock. Thus the plugging observed in this case was not due to the injected bacteria, but to the multiplication of bacteria within the porous reservoir after injection. This highly permeable reservoir had a pore size distribution between 1 and $12 \mu\text{m}$ of minimum pore entry diameter. Allred also emphasized the severe plugging which could be caused by iron bacteria, slime-forming bacteria, algae and sulfate-reducing bacteria, which corrode iron pipes and precipitate the iron as ferrous sulfide.

Sharpley²⁹ reviewed the subject of bacterial plugging in water flooding projects and its alleviation by germicides. Sulfate-reducing, iron, and slime-forming bacteria were described as the worst offenders. Counts greater than 10,000 per ml of any kind of bacteria may give rise to plugging according to the American Petroleum Institute.

PENETRATION OF BACTERIA THROUGH POROUS MEDIA

In contrast to the above studies, many workers have been interested in determining the penetration or lack of penetration of bacteria through porous mineral materials. More than 50 years ago, Russian workers established that bacteria are found in porous rock thousands of feet below the surface of the earth.¹⁷ Accordingly it is now accepted that they are capable of extensive migration through such media. Porous filters made of baked clay, unglazed porcelain, fused kieselguhr and fine sintered glass have long been used by microbiologists to remove bacteria from a variety of liquids, including blood, urine, culture media and plant and animal extracts. The history of these developments is fascinating. A review by Morton¹⁹ attributes the development of such filters to Tiegel (1871) who used clay. Later Chamberland (1884), working with Louis Pasteur, developed more refined porcelain filters.

The Russian botanist, Iwanowski, discovered viruses by the use of such a filter, and published his findings in 1903.¹⁵ He ground up tobacco leaves with water, filtered the suspension, and demonstrated by microscopic means that the filtrate contained no bacteria or other visible particles. He then applied the filtrate to healthy tobacco leaves and produced the tobacco mosaic disease. He correctly reasoned that there must be some infectious agent, too small to be seen with the microscopes of the time, which will pass through filters which retain all known bacteria. We now know this entity as tobacco mosaic virus, a slender, rod shaped RNA virus 0.018 μm in diameter and 0.3 μm long. At that time there was no known way of determining the pore sizes of such filters. The first successful attempt to do so was made by Einstein and Muhsam.¹² (Yes, this was *the* Albert Einstein.) They applied the theory of capillary pressure, and correctly pointed out that the important parameter to determine is the *minimum* pore entry diameter rather than the maximum or average pore diameters. The filter was immersed in ether, and the walls of the filter pores were wet by the solvent, and quickly imbibed it. They then measured the air pressure required to drive the solvent from the pores and give rise to air bubbles. The capillary pressure thus measured was then employed to calculate the minimum pore entry radius r_0 , using the formula:

$$P = \frac{2\sigma}{r_0}$$

where σ is the capillary constant for ether and P is the measured capillary pressure. The pore radius thus calculated was 0.36 μm , equivalent to a minimum pore entry diameter of 0.72 μm . The authors pointed out that this theory assumes that the pores are all of circular cross section, and hence the values given can be considered only an approximation. They also calculated the minimum pore entry diameter of the filter using Poiseuille's law which relates the flow rate of a liquid of known viscosity to the pore entry diameter:

$$\frac{\Delta P}{\Delta S} = \frac{K\mu v}{d^2}$$

where:

ΔP = change in pressure

ΔS = distance of flow (core length)

K = a constant

μ = viscosity

v = velocity of flow

d = pore diameter

The calculated value was approximately 10 times too large, because the theory assumed the porous medium to be a bundle of capillary tubes of constant diameter and circular cross section.

Beckhold³ also calculated the pore size of bacteriological filters from capillary pressure data, but he employed a value 10 times too large for the capillary constant, and thus obtained a value 10 times too large for the pore diameters. This happened to agree with results calculated by Poiseuille's law, and also with optical measurements made on thin sections of filter media with a microscope by Peragallo,²³ and thus the erroneous values persisted in textbooks until at least 1946 (see Porter, 1946, p. 210)²⁵ where the mean pore diameters of bacteriological filters are given as 1.5 μm for the finest Chamberland filters to 12 μm for Berkefeld filters. This led many workers to attribute removal of bacteria by such filters to adsorption forces and electrostatic attraction. Actually the pore entry diameters are of the same order of magnitude as bacterial cells, and simple sieving action suffices to explain their effectiveness.

If the pore diameters of these ceramic filters are indeed about the same as the diameter of small bacteria, it should be possible to force such bacteria through them. Craw⁷ found precisely these results. By employing high pressures, greater than 50 psi, he was able to force a few bacterial cells through many of the filters tested. And when he placed bacteria in rich nutrient medium inside the filter tubes, and then immersed the filters in sterile nutrient media, the bacteria grew through the filters and multiplied in the outer compartment containing the nutrient medium within 2 to 8 days with every kind of filter tested. Also the time required for the motile bacteria used (*Serratia marcescens*) to penetrate the filters correlated well with the "grain" (pore size) of the filter determined by microscopic examination. The larger the "grain" the shorter the time.

The earlier workers also noted the phenomenon of plugging of these filters with bacterial cells, resulting in a flow rate which decreased with the volume of fluid injected. Much later Ritter and Drake²⁸ of the Mobil Oil Co. developed a more elegant method of determining the pore size distribution of porous media such as catalysts and fine porous glass and ceramic filters. The method was based on capillary pressure, and employed a dilatometer to measure the volume of mercury injected into the pores as a function of pressure. A plot was prepared of absolute pressure in psi against the volume of mercury injected into the sample ($V_0 - V$), where V_0 is the total pore volume and V is volume of mercury injected at pressure p). An equation was then developed relating these quantities:

$$d(r) = \frac{p}{r} \frac{d(V_0 - V)}{dp}$$

where:

$d(r)$ = differential pore radius

p = pressure

$$d(V_0 - V) = \text{differential of volume injected}$$

$$dp = \text{differential of pressure}$$

The derivative in the above equation was determined by graphical differentiation, and employed to construct a pore-size distribution function curve as indicated in Figs. 1 and 2 below of Ritter and Drake.²⁸ The curves show that the ultrafine sintered glass and the fine ceramic plate material (both used as bacteriological filters) have a negligible amount of their pore volume with a radius greater than 5,000 Å (0.5 μm). Thus the minimum pore entry diameter is less than 1 μm, approximately the same size as small bacteria.

This powerful new method was soon applied to petroleum reservoir rocks by scientists with the same company, Burdine, Gournay and Reichertz.⁵ It was now possible to read from the curve the fractional part of the pore volume occupied by pores of any pore entry diameter. This was an enormously important development in petroleum reservoir engineering, and is now used routinely by all of the major oil companies. Data were taken on 100 samples from many different kinds of petroleum reservoir rocks, and distribution function curves were shown for four different samples with very different properties. Two equations were developed for relating the pore-size distribution curves to permeability, and were shown to give results close enough to measured permeability values to suffice for engineering purposes in most cases. Some anomalous rocks, usually of low permeability, gave results far from calculated values. The table below summarizes the kind of data which can be obtained.

Sample No.	Permeability, md	Porosity, Percent	Median Pore Entry Diameter, μm
826A	440	23.7	2
682	20	17.0	6
173C	20.1	14.3	5
192A	1,440	20	14

The writer had the rare privilege of working in the same laboratory with Bourdine, Gournay and Reichertz during his studies on microbial enhanced oil recovery,

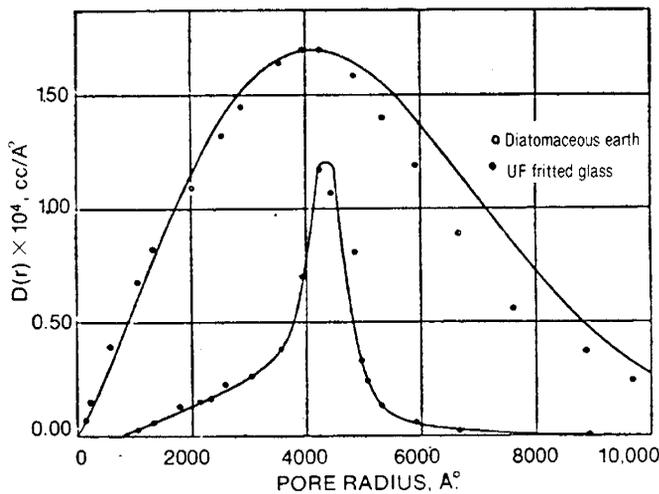


FIG. 1—Distribution functions for diatomaceous earth and fitted glass. Reprinted with permission, Reference 28. Copyright 1982, American Chemical Society.

and hence had access to this data file. As summarized in Updegraff and Davis (1954),^{31,32} it was found that pores must be at least twice the diameter of cocci or short bacilli in order for the cells to pass through without serious plugging. Empirically it was observed that reservoir rocks containing a large fraction of pores greater than 3 μm in diameter will pass large numbers of sulfate-reducing bacteria up to 0.6 μm in diameter and 3 μm long without serious plugging. Such reservoir rocks generally have a high permeability, greater than 100 md, and a porosity greater than 15 percent. Severe plugging may be expected in cores where most of the pore volume is contributed by pores less than 2 μm in diameter. This is the case in many low permeability formations.

Crawford⁹ applied computer technology to develop an advanced mathematical procedure for calculating porosity and permeability of a porous matrix. Both pore diameter and connecting channel diameter are considered in this advanced model.

Several workers have studied the rate of penetration of bacteria of various sizes and shapes through petroleum reservoir rocks, both in the presence and absence of liquid flow through the rock sample. Myers and McCready²⁰ at the University of Alberta studied the penetration of *Serratia marcescens* into cores of two types of sandstone and two types of limestone. Core samples 2 in. in diameter and 7 to 14 in. long were cut, mounted in epoxy resin, cleaned and dried, and the lower end was immersed in a suspension containing 3.7×10^9 cells/ml in distilled water. No pressure was applied to the liquid, but in every case the cores rapidly imbibed the liquid by capillary action. Bacteria passed completely through all cores, even Mesozoic sandstone and Mississippian limestone with permeabilities of less than 0.1 md. The authors noted that the imbibition rate declined sharply with time, suggesting severe bacterial plugging of all cores. The cores were split longitudinally and examined at the end of the experiment. Numbers of bacteria declined markedly with depth of penetration into the core.

Myers and Samiroden²¹ employed the same method to study the penetration of *Serratia marcescens* into 35 cores, some partially saturated with crude oil. In all except two very low permeability cores (Cardium sandstone, 2.5 to 6.7 md, and Pekisko limestone, 0.5 to 13.0

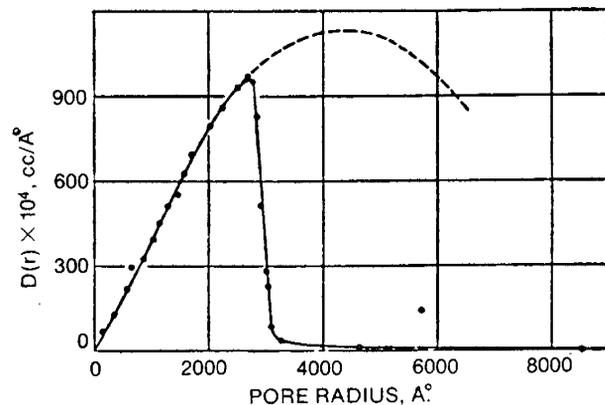


FIG. 2—Distribution function for porous plate. Reprinted with permission, Reference 28. Copyright 1982, American Chemical Society.

md) the bacteria penetrated the cores in 48 hr or less. Crude oil did not prevent penetration of the bacteria. The rate of penetration did not correlate with the permeability or porosity of the cores.

Recently Clark⁶ reinvestigated the question of transport of living bacterial cells through cores of Berea sandstone. Cells were suspended in 2 percent sodium chloride plus 0.01 percent calcium chloride, and injected into cylindrical core samples 5 cm in diameter and 19 cm long, with permeabilities of 200 to 400 md. Surprisingly, some species of bacteria penetrated through the cores while others did not. Size of the bacteria did not appear to be a factor.

SELECTIVE PLUGGING

In the studies described by Fekete and Flock,¹³ Kalish, Stewart and Rogers,¹⁶ and Raleigh and Flock²⁷ it was generally found that the permeability ratio was reduced the most by bacterial plugging in the case of high permeability cores, to a lesser extent with medium permeability cores and an even lesser extent with low permeability cores. On the basis of this finding Crawford⁸ suggested that bacteria could be used for stratification rectification in a water flood. In many water floods, permeability stratification permits rapid passage of water from the injection well through a high permeability stratum, thus bypassing large amounts of the oil in lower permeability strata, resulting in poor sweep efficiency of the flood, and decreased oil production. Crawford's suggestion was that injection of bacteria could lead to rapid penetration of the more permeable zones with concomitant rapid plugging, but less plugging in the low permeability zones. This should reduce water flow through the original high permeability zone and improve the sweep efficiency, thus resulting in greater oil production. Such a process is, unfortunately, very difficult to evaluate in laboratory models.

SUMMARY

The most important physical properties of petroleum reservoir rock, from the point of view of enhanced oil recovery processes, are permeability, porosity and pore size distribution. Since measurements of pore size distribution curves indicate that a large fraction of the pores in most petroleum reservoir rocks are of the same order of size as many common bacteria, from 0.5 to 1.5 μm in diameter, it is hardly surprising that bacteria plug most petroleum reservoir rocks to some degree. In general rocks of high permeability show a greater fractional decrease in permeability upon injection of bacteria than those of lower permeability. This suggests the possible use of bacteria for selective plugging or stratification rectification to increase sweep efficiency and hence oil recovery, in water flooding.

Small bacteria penetrate through most reservoir rocks, but not without a serious degree of plugging except in rocks with an appreciable fraction of their pores with a minimum pore entry diameter of 3 μm or more. Such rocks are usually of high permeability, greater than 100 md.

Davis^{10,11} concluded that bacterial enhanced oil recovery should only be considered for reservoirs with an average permeability greater than 100 md, while Bubela,⁴ after reviewing the literature, put this figure

at 150 md. Actually it would be necessary to conduct extensive tests with cores from the reservoir and bacteria of choice to be sure that the organisms penetrated the formation without serious plugging. Updegraff and Wren³² showed that sulfate-reducing bacteria readily penetrated highly permeable sand packs (7 to 80 Darcies) without serious plugging.

Bacteria may exert a much greater plugging effect when they multiply within the reservoir rock than when cells are simply injected. Since microbial EOR requires bacteria to multiply within the reservoir rock, this process requires a great deal more study. Bacterial plugging represents an important limiting factor for microbial enhanced oil recovery.

REFERENCES

1. Allred, R. C., 1976, Microbial Problems in Secondary Oil Recovery. In the Role of Microorganisms in the Recovery of Oil. 1976 Engineering Foundation Conference. NSF/RA-770201.
2. Beck, J. V., 1947, Prevention of Microbiological Growths in Water Flood Operations. Producers Monthly Oct. 1947, pp. 21-25.
3. Beckhold, H., 1931, Porengrösse von Bakterienfiltern und Siebwirkung. Zeitschr. f. Hyg. 112: 413-420.
4. Bubela, B., 1978, Role of Geomicrobiology in Enhanced Recovery of Oil: Status Quo. APEA Journal 1978: 161-165.
5. Burdine, N. T., L. S. Gournay and P. P. Reichertz, 1950, Pore size distribution of petroleum reservoir rocks. Pet. Trans., AIME, 189: 195-204.
6. Clark, J. B., 1981, The use of microorganisms in enhanced oil recovery processes. DOE Contract No. DE-AC-19-80BC10300. Quarterly Report.
7. Craw, J. A., 1908. On the grain of filters and the growth of bacteria through them. Jour. Hyg. VIII: 70-73.
8. Crawford, P. B., 1962, Continual changes observed in bacterial stratification rectification. Prod. Monthly (Feb. 1962) 12.
9. Crawford, P. B., 1965, Rock properties computed from random pore size distributions. Jour. Sed. Petrol. 35: 917-921.
10. Davis, J. B., 1967, Petroleum Microbiology, Elsevier, New York.
11. Davis, J. B. and D. M. Updegraff, 1954, Microbiology in the petroleum industry. Bacteriol. Rev. 18: 215-238.
12. Einstein, A. and H. Muhsam, 1923, Experimentelle Bestimmung der Kanalweite von Filtern. Deut. Med. Woch. 49: 1012-1013.
13. Fekete, T., 1959, The plugging of bacteria in sandstone systems. M.S. Thesis, University of Alberta, Edmonton, Alberta, Canada.
14. Hart, R. T., Fekete, T., and Flock, D. L., 1960, The plugging effects of bacteria in sandstone systems. Can. Inst. Min. Trans. 63: 318-324.
15. Iwanowski, D., 1903-04, Ueber Mosaikkrankheit der Tabakspflanze. Z. Pflanzenkrankh. XIII: 1.

Transport of Bacteria in Porous Geological Materials

16. Kalish, P. J., Stewart, J. A., Rogers, W. F., and Bennett, E. O., 1964, The effect of bacteria on sandstone permeability. *J. Pet. Technol.* 16(7): 805-814.
17. Kuznetsov, S. I., M. V. Ivanov and N. K. Lyalikova, 1963, Introduction to Geological Microbiology. English Translation, McGraw-Hill, New York.
18. Merkt, E. E., Jr., 1943, The Effect of Bacteria on the Permeability of Oil Reservoir Rocks. M.S. Thesis, University of Texas, Austin, Texas.
19. Morton, H. E., 1938, Bacterial filters and filtration techniques, *Am. J. Clin. Path., Tech. Suppl.* 2: 185-205.
20. Myers, G. E. and McCready, R. G. L., 1965, Bacteria can penetrate rock. *Can. Jour. Microbiol.* 12: 477-484.
21. Myers, G. E. and Samiroden, W. D., 1967, Bacterial penetration in petroliferous rocks. *Producers Monthly* 31(4): 22-25.
22. O'Bryan, O. D. and Ling, T. D., 1949, The effect of the bacteria *Vibrio desulfuricans* on the permeability of limestone cores. *Texas Journal of Science* 1(3): 117-128.
23. Peragallo, I., 1937, Recherches experimentales sur les bougies filtrantes. *Ann. Inst. Pasteur*, 58, 48-57.
24. Plummer, F. B., Merkt, Jr., E. E., Power, H. H., Savin, H. J. and Tapp, P., 1944, Effect of certain microorganisms on the injection of water into sand. *Petroleum Technol. Pubs., AIME*, No. 1678, 1-13.
25. Porter, J. R., 1946, *Bacterial Chemistry and Physiology*. Wiley; New York.
26. Raleigh, J. T., 1962, The effect of rock properties on bacteria plugging in reservoir rocks. M.S. Thesis, University of Alberta, Edmonton, Alberta, Canada.
27. Raleigh, J. T. and Flock, P. L., 1965, A study of formation plugging with bacteria. *J. Petrol. Technol.* 17(2): 201-206.
28. Ritter, H. L. and Drake, L. C., 1945, Pore size distribution in porous material. *Ind. Eng. Chem., Anal. Ed.* 17: 782-786.
29. Sharpley, J. M., 1961, Bacteria in flood water: What are they—what they mean. *Petroleum Engr.* 33(2): B-55-B-67.
30. Torrey, P. D., 1950, A review of secondary recovery in the United States. In *American Petroleum Institute, Secondary Recovery of Oil in the United States* (Second Edition). American Petroleum Institute, N.Y., pp. 3-29.
31. Updegraff, D. M., 1955, Microbial Corrosion of Iron and Steel. *Corrosion* 11(10): 44-48.
32. Updegraff, D. M. and Wren, G. B., 1954, The release of oil from petroleum-bearing materials by sulfate-reducing bacteria. *Appl. Microbiol.* 2: 309-322.

Application to Heavy Oils

The ultimate recovery by primary and secondary methods from the Lloydminster oil field in Canada (Alberta/Saskatchewan border) is estimated to be no higher than 8 percent. The oil in place is about 2.5 billion cubic meters of oil at 600 meters depth with an API gravity range of 13-17°. Hence, this is a significant target for enhanced oil recovery.

The principal impediment to production of the oil is water channeling due to the high viscosity of the oil, 400-9,000 centipoise at 25°C, and the high permeability (4 darcies) poorly consolidated sand. Jack suggested that there are two approaches to microbial enhancement of oil recovery (MEOR) that can have a significant impact on the oil recovery efficiency: (1) repressurization with gases produced by microbes *in situ* and (2) release of oil in portions of the reservoir by anaerobic fermentation of molasses which can cause *in situ* production of gases, acids, surfactants and solvents in relative quantities that depend on the nutrients, bacteria, and immediate environmental conditions.

Preliminary laboratory experiments conducted by Jack et al indicated that the major mechanism for oil release from the sand is the nascent formation of gas *in situ* which brings about a marked decrease of the oil viscosity accompanied by swelling as the nascent gas dissolves in the oil. The effect is very pronounced in the laboratory, but may not be as efficient under field conditions because of low gas transfer rates and the absence of convective mixing.

However, an intensive search for gas/acid forming bacteria capable of establishing viable colonies in 6 percent salt concentrations (NaCl and CaCl₂) was undertaken by Jack starting with field samples of *Clostridium* and other mixed cultures. A large number of the cultures, including *Clostridium*, grew poorly and were discarded. However, isolates of Gram-negative, facultative, rods were obtained which release 1.6 moles of gas per mole of sucrose used. They were identified as *Enterobacter cloacae* and are currently under study for possible field applications.

During the preliminary screening of bacteria, Jack et al obtained 11 cultures that produced copious amounts of slime (biopolymer). The laboratory experiments with 6-darcy permeability glass beads showed that the bacteria could be used to effectively plug a water channel; however, the injection of bacteria that are actively producing

slime invariably resulted in plugging at the face of the porous system; hence, a nutrient solution that supports growth but suppresses slime formation was developed for injection of the bacteria.

Once in place, the bacteria were induced to produce slime *in situ* by introduction of a second growth medium. Thus by controlling the nutrient solutions the anaerobic bacteria may be injected deep within the water channel and then activated by a second growth medium to produce the water blocking biopolymers *in situ*. This would cause diversion of the injected water to zones of by-passed oil and thus enhance recovery of the heavy oil.

According to Jack et al, emulsification of a viscous crude oil *in situ* is not feasible at this stage of development because transport of bacteria within the formation and mixing of the biopolymer with the crude oil in place are not technically feasible. However, emulsification of a produced heavy oil as it is pumped from the wells to storage tanks, or in the tanks by active microbes, would facilitate later transfer from the tanks to pipelines and pumping through the pipelines because of its non-wetting properties.

Emulsan, discussed by Gutnick et al, when separated from its production medium as a product might also be used extensively to clean tanks and lines of residual oil and sludge. Singer et al report the preparation of a crude oil emulsifying biopolymer which exhibits the additional property of viscosity reduction by as much as 95 percent. The work began with a challenge to answer the question: "Can bacteria be isolated which reduce the viscosity of heavy crude oils?"

Singer et al initiated a specific screening procedure to determine the ability of isolated colonies of bacteria to emulsify heavy crude oils (API gravity <15°). Crude oil was added to a basal nutrient medium, mixed with agar and gelled on petri plates which were observed daily for dispersion of the oil on the plates. Thus about 200 isolates were quickly selected. Subsequent studies of interfacial tension lowering and ability to form stable emulsions with hexadecane narrowed the isolates to 63. Interfacial tension (IFT) measurements of the spent culture broths were used to evaluate the production of surface active products. All of the isolates grown with hexadecane yielded broths with lowered interfacial tensions; however, one isolate (labeled H-13) stood out from the rest by registering an IFT decrease from 49

(hexadecane and broth) to 12 and was selected for more intensive study.

The culture was added to a basal salt medium and Venezuelan Monagas crude oil which was incubated aerobically for 7 days. The oil phase was separated and found to be 2.5 times greater in volume than the original oil indicating considerable swelling from emulsified water and extracellular products. This oil emulsion exhibited physical properties quite different from the original heavy oil. It did not adhere to the surface of glass and it behaved as a thixotropic liquid showing a decrease in apparent viscosity with time of application of shear force; but upon standing the original highly viscous condition returned. Viscosity measurements with a rotating Brookfield viscometer showed a difference between the crude oil and the thixotropic oil phase separated from the incubated culture of 90 to 98 percent, from 6,500 to less than 650 centipoise.

Analysis of the spent growth medium showed the presence of polysaccharides and glycolipids which are considered to be the surface active components responsible for the emulsification of heavy crude oil. Further analytic characterization of the extracellular products and taxonomic identification of H-13 is currently in progress.

Westlake confirmed the analytic results of the interactions of microbes and crude oils. He reviewed several articles that demonstrate the changes under laboratory and field conditions. Aerobic bacterial attack of crude oils generally results in loss of n-alkanes, isoprenoids, and low-molecular-weight aromatic and sulfur heterocyclic compounds. Westlake reported that the loss of these compounds is associated with the changes noted in Table 1 and Fig. 2, such as lowering of the pour point and increase of density.

Cooper discussed a different type of surface active compound which is produced by *Corynebacterium lepus*. This strain produces two types of biosurfactants which are effective for the release of bitumen from tar sands. When the biosurfactants are isolated at the end of the fermentation, one obtains a mixture of lipids. However, if the active agent is isolated during exponential growth, the product is a mixture of corynomycolic acids (α -alkyl- β -hydroxycarboxylic acids). The compounds were also found to be very effective reagents for crude oil-water emulsion coalescence and could prove to be commercially significant for de-emulsification of produced fluids, especially in steam recovery operations.

The Potential for Use of Microbes in the Production of Heavy Oil

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INTRODUCTION

Our laboratory project began in mid-1981 at the University of Calgary under the auspices of the Arctic Institute of North America with the kind cooperation of Professor J. W. Costerton, Department of Biology. Funding is by the NOVA/Husky Research Corporation, a company incorporated in December, 1981 to serve the research needs of NOVA, an Alberta corporation and Husky Oil Ltd. The aim of our program is to develop microbial systems for the production of oil.

Our research efforts are focused on a major system of reservoirs as the target site for application of new technology.

TARGET RESERVOIRS

The target reservoirs are located in a 10,000 square mile area about the town of Lloydminster on the Alberta/Saskatchewan border. These are the most southerly deposits in the Cretaceous Manville Group heavy oil sand belt and have been estimated at 9.9 to 16 billion barrels of oil-in-place. The oil is an asphaltic crude with API gravity of 13° to 17°, 400 to 9000 Cp viscosity at 72°F. The high viscosity of the oil and severe channeling problems in waterflood production result in a total production yield of about 8 percent by primary and secondary production.^{1,2,3} These reservoirs represent a significant opportunity for EOR.

Husky Oil holds a dominant position in the Lloydminster area (1.7 million acres);⁴ a fact which accentuates the importance of this target in our microbial biotechnology research program.

Reservoir properties—In terms of using bacteria *in situ*, the Lloydminster reservoirs present two major challenges; high salinity of the connate brine (6.5 percent) and the high viscosity of the heavy oil itself. Other parameters are amenable to MEOR application; high porosity (30 percent) high permeability (up to 4 Darcies) and low pressures (2,000 feet, depth).⁵ The inorganic matrix is poorly consolidated sand.

The nature of heavy oil—In chemical terms, Lloydminster oil is deficient in simple low molecular weight alkanes and aromatics, has a H/C ratio of 1.6 and a molecular weight consistent with the average molecular formula, C₂₉ H₄₈ N_{0.1} S_{0.5} O_{0.2}. Nuclear magnetic resonance studies on the whole oil indicate that 28 percent of the carbon in the whole oil is aromatic in

nature and 72 percent is alkyl with 72 percent of the alkyl carbons not in simple linear alkyl groups. The "average" aromatic nucleus (from the ratio of peripheral to bridgehead carbons) is a condensed four ring system.

Westlake et al.^{6,7} have shown that Lloydminster heavy oil has the composition of a highly biodegraded crude oil and that further biodegradation results in only modest changes in its composition and properties. This observation coupled with the general literature evidence against rapid anaerobic attack on pristine hydrocarbons^{8,9} suggests that heavy oil is not a readily available nutrient source *in situ*. Therefore, our research is based on the *in situ* fermentation of an injected substrate such as sugar beet molasses.

The sulfur content in Lloydminster heavy crude is 3.5 percent with the highest sulfur concentration (7.0 percent) being found in the asphaltene fraction which makes up 15 percent of the oil weight. It is important to note however that most of the sulfur is actually in the more abundant maltene fraction.

Potential applications of bacteria in heavy oil production

The production problems in Lloydminster heavy oil operations present a number of opportunities for applied microbiology (Table 1). Two *in situ* applications; flow diversion in waterflooding and MEOR by repressurization through gas fermentation are of particular interest here.

The poor production yield obtained by waterflooding in Lloydminster reservoirs results from a very poor sweep efficiency. In effect the injected water creates

TABLE 1—Potential Applications of Microorganisms in Heavy Oil Production

- 1) Flow diversion in waterflooding
- 2) De-emulsification of EOR production emulsions
- 3) Viscosity reduction for pipeline transport without condensates
- 4) De-sulfurization of heavy oil (asphaltene and/or maltenes)
- 5) Control of sulfate-reducing bacteria
- 6) MEOR through *in situ* fermentation in some cases

channels through the reservoir bypassing oil-rich zones of lower permeability. Selective plugging of these channels by bacteria could be used to divert water flow through the oil rich zones and improve recovery.¹⁰

The more conventional MEOR approach has been to ferment molasses anerobically in the reservoir to generate agents of oil release *in situ*.¹¹ These could include gases, acids, surfactants and solvents, Fig. 1. In the sand reservoirs at Lloydminster, acids will play a minimal role since formation carbonates are not an important factor in controlling oil flow. The major agent for stimulated oil release will probably be gas formation which could repressurize the reservoir and cause the oil to swell and drop in viscosity¹² as CO₂ dissolves into it. This latter effect is very pronounced in laboratory experiments with heavy oil¹³ but the extremely high initial viscosity of these heavy oils and the questionable rates of gas transfer into the oil phase in the absence of mechanical mixing underground could limit the efficacy of this MEOR approach in Lloydminster fields.

In our research program, we have sought out by classical selection and adaptation procedures bacterial cultures capable of growing anaerobically on 3 percent sugar beet molasses in full strength oil field brine (6.5 percent salinity) at the temperature found in the Golden Lake oil reservoir, Lloydminster. Successful cultures were screened for their ability to serve *in situ* as selective plugging or MEOR agents.

RESEARCH PROGRAM

Preliminary screening

Our first task was to undertake a preliminary screening to determine if bacteria could produce useful MEOR agents (gases, acids and surfactants) or plugging agents (polymers and biomass) under the simplest possible conditions—by anaerobic fermentation of 3 percent sugar beet molasses in coproduced brine at reservoir temperatures.

Classical adaptation, selection procedures were carried out beginning with 38 field samples and selected catalogue cultures of *Clostridium*. An initial attempt to jump cultures quickly onto the simple molasses/brine medium failed. It proved necessary to enrich the medium with trace metals, yeast extract, nitrogen and phosphorus sources before challenging the cultures with full strength brine. Even in this enriched medium, only 11 of the field samples and none of the *Clostridia* survived at 6.5 percent salinity (Table 2). This initial work was carried out in Vancouver by E. Lee at B.C. Research under contract.

At the University of Calgary, the surviving mixed cultures were separated into more than 70 pure bacterial strains on the enriched medium and then transferred gradually onto the bare medium of 3 percent

TABLE 2—Sources of Microorganisms

Culture	5	Richmond oil sump
	9	Marine sediments
	11	Marine sediments
	13	Marine mud from a sewage outfall
	18	Garden compost
	000016	Anaerobic sludge

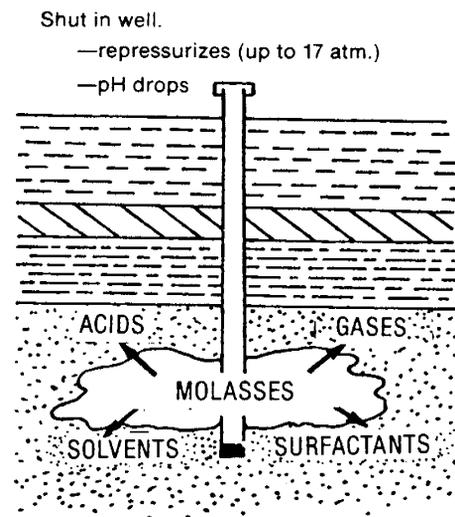
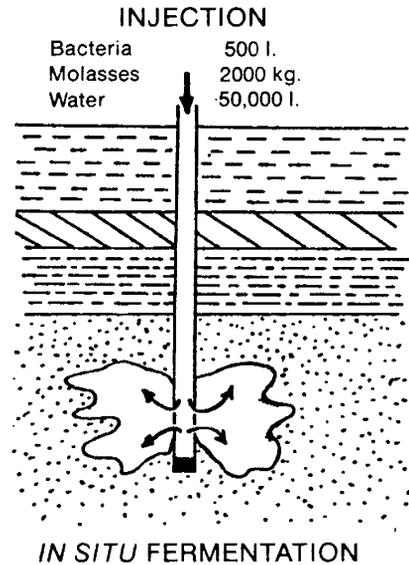


FIG. 1—Microbially enhanced oil recovery involves the injection of bacteria and nutrients into the reservoir followed by a fermentation period in which gases to repressurize the reservoir, acids to dissolve carbonates and solvents and surfactants which facilitate oil release can be produced.

sugar beet molasses in full strength brine. All handling was carried out in a Forma Scientific Anaerobic Work Station Model 1054. Acid and gas production were monitored by observing the gas collection in an inverted vial within each screw cap culture tube. (The presence of a CaCO₃ buffer allowed the accumulation of CO₂ through the dissolution of carbonate by produced acid even in the absence of fermentation gas production.) Surfactant production was monitored in the culture broth by Fisher Autotensiomat (based on the Du Nuoy ring method). Slime (polymer) production was observed visually on agar plates. Gas analysis was carried out on a Fisher Gas Partitioner.

For the cultures capable of growing anaerobically on 3 percent molasses full strength oil field brine (6.5 percent salinity) the following observations were made.

—28 of the cultures including the only *Clostridium* isolated from field samples grew poorly and were discarded.

—no culture was found to produce significant surfactant.

—all cultures produced acid. Indeed most of the moderate gas formation was actually due to acid dissolution of the carbonate buffer—less than half these cultures produced gas directly by fermentation.

—only moderate gas formation was found unless trace metals, yeast extract, N and P sources were added to the medium.

—11 cultures produced copious biopolymer independent of additives to the medium.

Gas producers—A second screening was carried out in an enriched medium (Table 3) for the isolation of gas producing bacteria. The most potent isolates were Gram-negative facultative rods which released 1.6 moles of gas/mole of sucrose utilized (CO₂:H₂:N₂::70:10:20). API20A and 20E identification kits characterized these isolates as *Enterobacter cloacae*. Studies are currently in progress on the ability of these isolates to grow under pressure and to develop pressure through gas production.

Slime formers—The 11 slime (biopolymer) producing isolates from the preliminary screening were considered promising as *in situ* plugging agents for flow diversion in waterflooding in Lloydminster but it remained to be demonstrated that such slime could plug high permeability zones (~4D) and that given that slime forming bacteria could plug such zones that they could be successfully injected without plugging the well bore of the injection well.

Well-bore plugging

Jerry Shaw, a graduate student in Geology at the University of Calgary working under Professors N. C. Wardlaw and J. W. Costerton with NOVA/Husky Research Corporation support has demonstrated the efficacy of slime forming bacteria in a model glass bead core system. (Figs. 2 and 3). The model cores, Fig. 3, are Blast-O-Lite glass beads, sieved to a uniform 90μ size and fused into 5 cm × 1 cm cores of 5 to 7 Darcies permeability. Bacterial suspension is passed through the core at a constant 3.5 psi pressure differential from the reservoir (Fig. 2) and the effluent flow rate recorded against elapsed time and cumulative volume.

Using this system, Shaw has demonstrated that extracellular polysaccharide produced by an actively sliming *Pseudomonas* sp. can plug a glass bead core run under modest injection pressures (up to 7 psi) far more effectively and efficiently than dead cells of the same culture.¹⁴ This observation is relevant to waterflooding operations where injectivity is lost due to microbial growth in the well bore region.

We have adapted the original apparatus of Shaw for anaerobic work by the addition of an extra valve and a packet of palladium catalyst inside the culture reservoir, Fig. 2. The bacteria are grown in a Forma Scientific Anaerobic Work Station Model 1024, placed in the reservoir and are pressurized in the reservoir under an atmosphere of mixed gases (5 percent H₂, 5 percent CO₂, 90 percent N₂—Medigas, Calgary). In the presence of the palladium catalyst reaction of oxygen with the hydrogen atmosphere sustains an anaerobic condition.

Since many MEOR schemes are based on the underground fermentation of injected molasses, Fig. 1, the

first anaerobic runs employed a sugar beet molasses medium made up in artificial brine. This artificial brine was based on the analytical composition of a known coproduced brine but was made up at half-strength (~3 percent salinity) to avoid precipitation problems. This unfiltered molasses/brine medium did cause considerable plugging without any bacterial inoculum, presumably due to particulates (beet pulp) in the molasses itself (Fig. 4). Prefiltering the molasses medium through Sartorius filters (0.2μ) considerably alleviated plugging (Fig. 4).

The ability of a slime forming Gram-negative anaerobic rod to plug the core is demonstrated in Fig. 5. In this case, the scanning electron microscope revealed a surface plug of biopolymer in which bacteria could be seen (Fig. 6).

The conclusions are that:

—molasses will require prefiltration before injection as part of a MEOR system.

—that slime forming anaerobes capable of growing on 3 percent molasses in full strength oil field brine can effectively and efficiently plug high permeability (6 Darcies) zones.

—that injection of actively sliming bacteria will result in well bore region plugging and a serious loss of injectivity.

Flow diversion in waterflooding

The above study indicates that slime forming anaerobic bacteria could be used to plug the high permeability channels which ruin the sweep efficiency of water-

TABLE 3—Autoclaved Medium

All the following ingredients were mixed and the pH was adjusted to 7.1-7.3 using NaOH. The solution was distributed into screw cap tubes containing a shell vial and autoclaved 20 min at 15 psi.

Ingredients	Specified As
Molasses	30 g
K ₂ HPO ₄	0.7 g
KH ₂ PO ₄	0.3 g
(NH ₄) ₂ SO ₄	1.0 g
FeSO ₄	0.005 g
ZnSO ₄	0.002 g
MnSO ₄	0.005 g
CoCl ₂	0.001 g
NH ₄ Mo ₇ O ₄	0.001 g
Na ₂ B ₄ O ₇	0.0011 g
CdSO ₄	0.001 g
CuSO ₄	0.0007 g
Yeast extract	0.02 g
Cysteine HCl	0.25 g
*Mops or Hepes (buffers)	0.04 m
Lloydminster brine to 1 liter	
plus NaOH (1N) to pH 7.1 to 7.3	

*Mops (3-(N-Morpholino) propane sulfonic acid) pH 6.5-7.9, MW 209.3
 Hepes (N-2-Hydroxyethylpiperazine-n'-2-ethanesulfonic acid) pH 6.8-8.2, MW 238.3

floods in the heavy oil reservoirs at Lloydminster. However, the above study also demonstrates that injection of actively sliming bacteria is not practical due to well bore plugging problems. It is necessary therefore to be able to control the production of slime in the anaerobic bacteria employed so that they can be injected in a growth phase or medium which supports growth but which does not support slime formation and once in place can be made to produce slime *in situ*.

For the slime forming anaerobes isolated on 3 percent sugar beet molasses in full strength brine in the preliminary survey, at least two modes of turning slime production "on" and "off" have been found.

For one set of five isolates, the key involves controlling the composition of the medium.

Manipulation of the medium results in more than a ten-fold control of extracellular polysaccharide formation for at least two of the isolates in liquid culture. 18-1, a Gram-positive facultatively anaerobic coccus from garden compost has been studied further in the model core system.

The effect of slime control for isolate 18-1 is apparent in Fig. 7. When the culture grown in a medium which supports slime formation is passed through the model core, rapid and effective plugging occurs. In contrast, an equivalent cell suspension (3×10^8 cells/ml) grown in a medium which does not support polysaccharide formation does not cause severe plugging. Indeed if these two curves Fig. 7 are compared to the curves generated by Jerry Shaw for aerobic *Pseudomonas* sp. actively sliming and dead, the results are found to be almost identical (for equal numbers of cells passed through the core). In the non-sliming mode isolate 18-1 gives the same extent and rate of plugging as dead *Pseudomonas* cells.

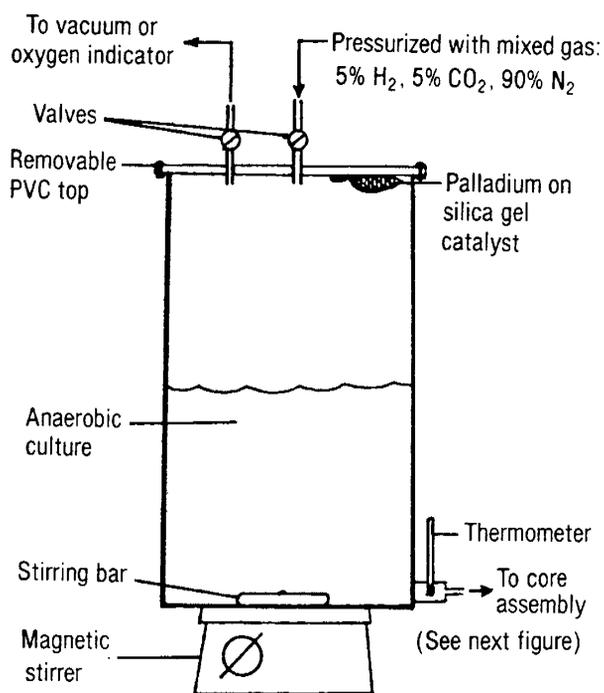


FIG. 2—Anaerobic reservoir for bacterial suspensions being fed to the model core system.

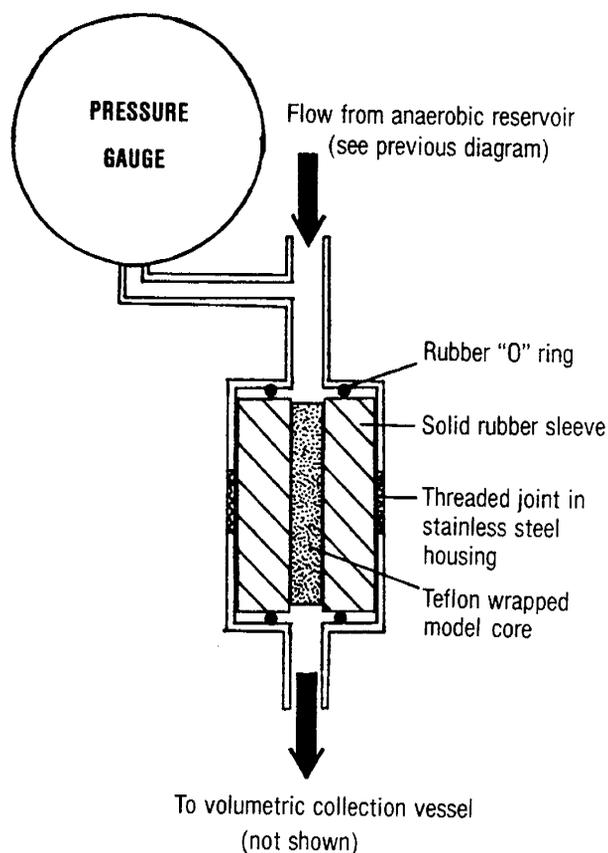


FIG. 3—The model core system used in these injectivity studies.

Since the actual field use of such a system would require injection of the non-sliming culture followed by slime formation *in situ*, two further experiments were performed.

In Fig. 8, a bacterial suspension of 18-1 was introduced into the core in a polymer forming medium over a two hour period. At "A," the input was switched from

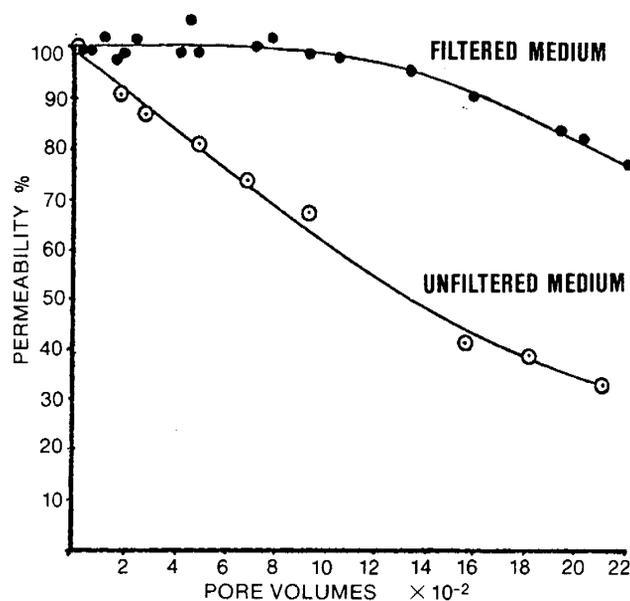


FIG. 4—Comparison of plugging for molasses/brine medium before and after filtration through 0.2μ filters.

bacterial suspension to a sterile medium.

This resulted in a washout of most of the bacterial suspension from the core and a return to the initial permeability. At "B," the core was sealed and left standing overnight to allow growth and slime formation by the bacterial cells remaining in the core. On restoring input pressure to the core, the permeability was found to have declined by about 50 percent. Thus a small population of residual cells had grown up overnight to substantially plug the core.

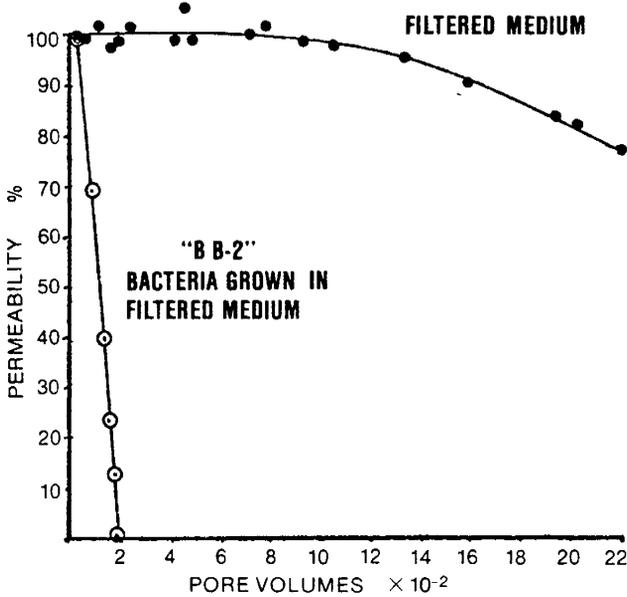


FIG. 5—Plugging caused by a stationary phase culture of anaerobic bacteria BB2 compared to the pristine filtered medium.

ing medium for 72 hr. In this time, visible slime appeared on the core due to polysaccharide formation as medium permeated the core through the dialysis tubing. On returning the core to the apparatus, the permeability was found to be 11 percent of the initial value.

From these preliminary results it appears that anaerobic bacteria capable of growing under the conditions of the heavy oil reservoirs in the Lloydminster region can produce extracellular slimes (polysaccharides) in a controllable fashion and that these bacteria can be injected into such a reservoir successfully in a non-slime inducing medium. Further, slime formation can be induced *in situ* by the subsequent introduction of an appropriate slime inducing medium.

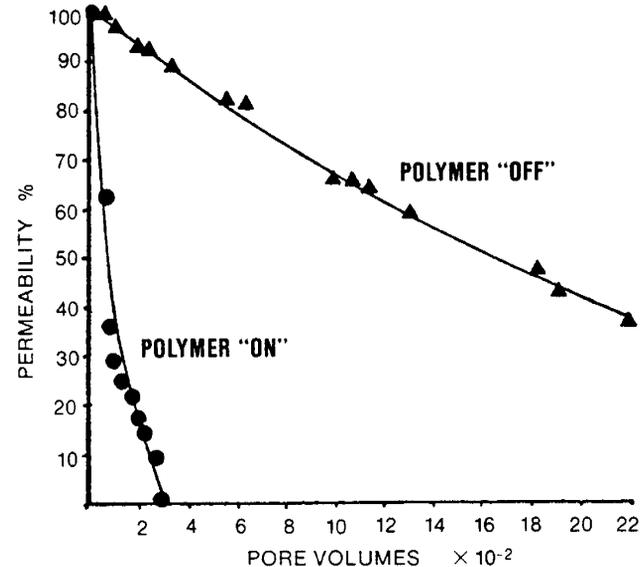


FIG. 7—Comparison of plugging by cell suspensions ($3 = 10^8$ cells/ml) of isolate 18-1 grown in media which induce and do not induce polysaccharide production.

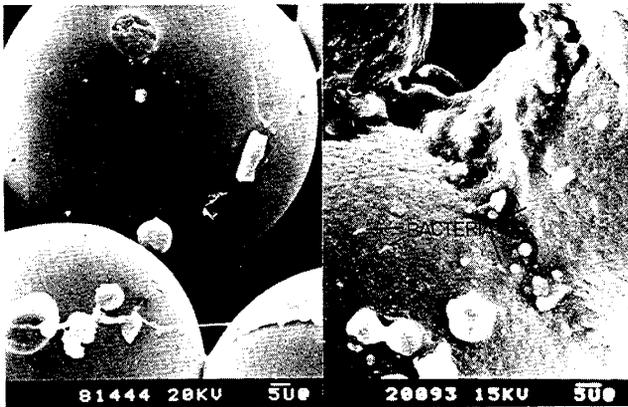


FIG. 6—Scanning electron microscope photomicrograph of a pristine glass bead core (left half of figure) compared to the slime layer formed on the top of the BB-2 run in Fig. 5 (right half of figure). Isolated bacteria are evident in the slime.

A similar experiment was conducted in which a core was inoculated by passing about five pore volumes of a non-sliming bacterial culture through the core. (The initial permeability was also determined at this time.) The core was then removed aseptically, placed in a sterile dialysis tubing and suspended in a slime induc-

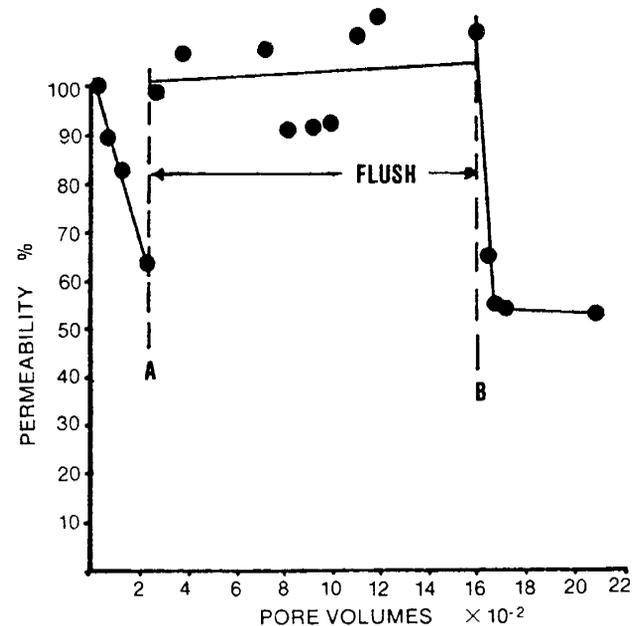


FIG. 8—Growth and slime production by isolate 18-1 in a core initially inoculated with an actively sliming bacterial suspension ($3 = 10^8$ cells/ml) (to point "A") then flushed with sterile medium ("A" to "B") and incubated overnight without flow ("B"). (Permeability reduction on restoring flow is evident after "B".)

These bacteria could provide a feasible system for plugging the high permeability channels which normally thwart heavy oil production by waterflooding in the Lloydminster fields. Further work is in progress.

ACKNOWLEDGEMENTS

We wish to thank Jerry Shaw who developed the model core system and his supervisors Professor N. C. Wardlaw and J. W. Costerton. We also gratefully acknowledge the assistance of Brenda Bramhill who provided cores and advice and Ian Lee who provided assistance on the SEM.

REFERENCES

1. Orr, R. D., Johnston, J. R. and E. M. Manko, 1977. Lower Cretaceous geology and heavy oil potential of the Lloydminster area. A preprint of a paper presented at the 28th Annual Meeting of the Petroleum Society of the Canadian Institute of Mining, May 30 to June 3, 1977.
2. White, W. I. and W. P. von Osinski, 1977. Geology and heavy oil reserves of the Manville Group, Lloydminster-North Battleford area, Saskatchewan. As above.
3. Vigrass, L. W., 1977. Trapping of heavy oil at Lloydminster. As above.
4. Husky Oil Ltd., 1980. Annual Report.
5. Private communication; Bob Meldau and Mary Sperling. Husky Oil Enhanced Oil Recovery Unit, Santa Maria, California.
6. Rubinstein, I., Strausz, O. P., Spyckerelle, C., Crawford, R. J. and D. W. S. Westlake, 1977. The origin of the oil sand bitumens in Alberta: a chemical and a microbiological simulation study. *Geochimica et Cosmochimica Acta*. 41:1341-1353.
7. Crawford, R. J., Spyckerelle, C. and D. W. S. Westlake, 1978. Biodegradation of oil reservoirs. Pages 163-176 in *Oil Sand and Oil Shale Chemistry*, O. P. Strausz and E. M. Lown, editors. Verlag Chemie, N.Y.
8. Jobson, A. M., Cook, F. D. and D. W. S. Westlake, 1979. Interaction of aerobic and anaerobic bacteria in petroleum degradation. *Chemical Geology*, 24:355-365.
9. Ward, D. M. and T. D. Brock, 1979. Anaerobic metabolism of hexadecane in sediments. *GEomicrobiology Journal*, 1:1-10.
10. Van Heiningen, J., Jan De Haan, H. and J. D. Jansen. Netherlands Patent 89580 issued November 15, 1958.
11. Updegraff, D. M. Recovery of petroleum oil. U.S. Patent 2807570, issued September 24, 1957.
12. Jack, T. R., 1980. Enhanced oil recovery by microbial action. A paper presented at the Second International Chemistry Conference of North America, Las Vegas, August 28, 1980.
13. Miller, J. S. and R. A. Jones, 1981. Physical characteristics of heavy oil after CO₂ saturation are studied in DOE tests. *Oil and Gas Journal* (July 6):135-145.
14. Shaw (Chin), J., 1982. M.Sc. Thesis. University of Calgary, Calgary, Alberta.

Microbial Processes in the Recovery of Heavy Petroleum

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INTRODUCTION

Enhanced oil recovery has advanced to a stage where recovery processes are technically feasible in limited application to specific, highly favorable reservoir conditions. Enhanced oil recovery technology remains, however, in a research mode with several specific areas in need of continuing extension and development.

A detailed discussion on the application of microbial processes to oil production will necessarily fall short of precise or even general solutions. Even as the petroleum engineer lacks total and comprehensive knowledge supported by definitive data-bases on the chemical and physical structure of the oil reservoir, the microbiologist as well lacks specific information necessary to critically address possible role(s) microorganisms may play in enhanced oil production. The areas of hydrocarbon and petroleum microbiology have compiled an extensive literature over the past few decades.⁵

These advances are reflected in the acquisition of definitive data-bases relating to the physiological, biochemical and genetic properties of a variety of hydrocarbon-utilizing microorganisms. The transfer of this knowledge has yet to be realized in direct application to EOR or oil production although a consensus of opinion exists among many microbiologists that a potential use of microorganisms and their products has application to oil field problems.

The available literature concerning microbial activities within the reservoir environment is scant. Ample reports exist concerning the proliferation of sulfate-reducing bacteria and the souring of sweet crudes, plugging phenomena plus other deleterious problems.⁵ Recent studies have indicated the presence of microbial populations in oil reservoirs with the taxonomic classification and cataloguing of their various physiological and biochemical properties remaining to be accomplished. The realization has surfaced that reservoirs are not, necessarily, sterile, biologically refractory environments. The source of the indigenous microflora is further unknown with possible point source contamination from surface origins or, alternatively, of subterranean origin.

It is further recognized that *in situ* alteration of oil can occur as a result of some level and rate of microbial activity. Questions arise as to whether producing wells decrease in productive capacity over time through mi-

crobial processes that tend to significantly alter the viscosity characteristics of oil. If so, definitive answers to such processes would allow for meaningful treatment regimes that would control microbial activity, enhancing long-term productivity.

An alternative consideration is the possible stimulation of the indigenous microflora towards greater metabolic activity by the addition of appropriate nutrients that allow for hydrocarbon-utilizing microorganisms to attack specific fractions of oil and, in turn, produce chemicals effective in increased oil production. Evidence has appeared that indicates utilization and release of hydrocarbons and oil by microorganisms in subsurface environments by the introduction of air and mineral nutrients.⁹

The application of surfactants to EOR has long represented an active developmental area in oil production. The consideration and application of biosurfactants and/or bioemulsifiers has been generally overlooked, primarily due to the lack of a definitive and cohesive literature on the subject. A review discusses the broad generalities of biosurfactants in context of theory, sources, types and applications.¹⁰

The production of surface active agents by microorganisms has been recognized for years; however, a systematic characterization of these microbial products has been slow to emerge. Recent studies have documented the production of numerous surfactant compounds produced by hydrocarbon-utilizing microorganisms. The development and application of such microbial-produced biosurfactants may represent valuable and potentially useful compounds in the recovery and production of oil.

The principal question addressed at the onset of this research was "can bacteria be isolated which reduce the viscosity of heavy crude oils?" The experimental approach to obtaining answers to this question is based on three simple assumptions:

1. that microorganisms can grow on heavy crude oil as a sole source of carbon and energy;
2. that microorganisms can grow on asphaltenes or resin acids as a sole source of carbon and energy; and
3. that microorganisms produce surface active products of potential usefulness and utility.

Application to Heavy Oils

The rationale serving to justify this approach was that two basic mechanisms exist by which microbial processes or activities would reduce the viscosity of heavy oils:

1. reduction of the average molecular weight of the heavy oil; and
2. the production of specific metabolic products.

The reduction of the average molecular weight of heavy oils would necessitate the microbial depolymerization and/or the degradation of the high molecular weight constituents such as asphaltenes or resins acids to smaller molecular weight components, thus, effectively reducing the average molecular weight of the bacterial-treated oil. The production of surface-active compounds by microorganisms growing at the expense of hydrocarbons or components of oil would serve to generate macro- and/or micro-emulsions of oil-in-water or water-in-oil type having lower viscosities than the parent crude. The following report summarizes our studies to date on the viscosity reduction of heavy oils by microorganisms.

MATERIALS AND METHODS

Culture conditions—Bacterial isolates were grown in either 0.8 percent nutrient broth, 0.5 percent yeast extract (NBYE) or in a mineral salts medium (basal salts E) containing in g/l: 10 g K_2HPO_4 ; 5 g NaH_2PO_4 ; 2 g $(NH_4)_2SO_4$; 200 mg $MgSO_4 \cdot 7H_2O$; 1 mg $CaCl_2 \cdot 2H_2O$; 1 mg $FeSO_4 \cdot 7H_2O$, and 25 ml n-hexadecane or 50 gms crude oil per liter. Bacterial cell growth was monitored by optical density or by dilution-plating techniques. The crude oils used in this study were obtained from the following sources: West Texas crude, East Texas crude; Venezuelan Monagas crude, Venezuelan Cerro Negro crude; Monterey crude; Alaskan North Slope crude.

Tensiometric studies—The spent culture media of bacterial cells grown on either hexadecane/mineral salts medium or NBYE medium were centrifuged and filtered to remove bacterial cells prior to measurements of interfacial or surface tension. Surface tension was measured using Fisher Autotensiomat recording Du-Nuoy Tensionmeter. Oil/water interfacial tension was measured using either the drop weight method, or with a spinning drop tensiometer.

Viscosity—Crude oil was separated from the aqueous culture medium in a separatory funnel. Viscosity of water-saturated oil, or oil dried for 2 days in a vacuum desiccator, was measured using a Brookfield Rheolog/Thermosel viscometer.

Analytical methods—Spent culture medium or cells were extracted with chloroform/methanol (2:1) followed by back extraction with 0.9 percent NaCl to remove water-soluble material. The chloroform phase was designated the crude lipid extract. Alternately, the spent culture medium was extracted twice with an equal volume of diethyl ether following acidification. The ether-soluble material was then designated as the crude lipid extract. The crude extract was fractionated on a silicic acid column. Neutral lipids were eluted with chloroform; glycolipids with acetone; and phospholipids with chloroform/methanol (2:1). Glycolipids were quantified using the anthrone method² using glucose as a standard. Phospholipids were quantified

using the Bartlett phosphate method.³ Dry weights of lipids were obtained using a Cahn Electro-Balance. Extracellular protein and polysaccharide were determined after dialysis of the spent growth medium by the Lowry method for protein⁷ and the anthrone method for sugar.

Chromatography—Polar lipids (phospholipids and glycolipids) were separated by thin-layer chromatography (TLC) on 0.4 mm layers of silica gel G using a solvent system containing chloroform/methanol/5 N NH_4OH (65:30:5). Glycolipids were detected using the orcinol spray reagent. Phospholipids were detected using the phosphate spray reagent (Dittmer and Lester, 1964).⁶ Purification of glycolipids by preparative TLC was performed using 0.5 mm layers of silica gel G.

Neutral lipids were separated on silica gel G using petroleum ether/diethyl ether/acetic acid (80:20:1) as the solvent system. Neutral lipids were visualized by spraying with 50 percent H_2SO_4 and charring 30 min at 120°C. The glycolipid sugar was chromatographed following deacylation by base saponification and desalting, on Whatmann 3 MM paper using butanol/pyridine/ H_2O (3:2:1) as the solvent system.

Gas-Liquid chromatography (GLC)—Fatty acid methyl esters derived from the purified glycolipid were analyzed by GLC using a Tracor 560 gas chromatograph equipped with a flame ionization detector and an 8 ft \times 4 mm glass column with 5 percent DEGS-PS.

Acetylated sugars derived from the purified glycolipid were analyzed using a Varian 1200 gas chromatograph equipped with a flame ionization detector using a 3 percent ECNSS-M column ($1/8$ in. \times 10 ft stainless steel column).

RESULTS AND DISCUSSION

Enrichment and selection of microorganisms—The enrichment of microorganisms was accomplished through conventional enrichment techniques. A variety of soil samples ranging from randomly selected normal soils to soils subject to chronic exposure with oil were supplemented with Venezuelan Monagas crude, Venezuelan Cerro Negro crude, West Texas crude and asphaltenes derived from each of these crudes. The culture flasks were incubated aerobically at room temperature with shaking for varying periods of time. Serial transfers of these primary enrichments were made at selected intervals to fresh oil-containing media and maintained through a minimum of 5 serial passages.

Such procedures are neither new nor novel, having been used for decades as the standard technique for isolation of hydrocarbon-utilizing microorganisms. The final serial transfer was streaked onto nutrient broth-yeast extract (NBYE) agar plates for pure culture isolation. Each of the 300 bacterial isolates obtained in this manner was tested for ability to grow in liquid culture at the expense of oil or fractions derived from oil.

A screening procedure was derived to determine the ability of bacterial isolates to disperse and/or solubilize crude oil. Crude oil was added to the complete basal salts minimal medium containing 2 percent agar and sonicated while the temperature was maintained at 70°C. This oil suspension was poured into petri plates (15 ml/plate) and allowed to solidify at room tempera-

ture. The oil plates were then inoculated with the bacterial isolates, incubated at room temperature and inspected daily for clearing of the oil.

This procedure allowed for the rapid presumptive screening of large numbers of bacterial isolates which produced products that were effective in the dispersion of crude oil. We were able to identify approximately 200 isolates which caused dispersion of oil. Out of 200 isolates, 77 bacterial isolates have been studied with respect to their ability to form emulsions. There were 63 isolates (82 percent) which formed stable emulsions with hexadecane and 14 (18 percent) isolates which formed unstable emulsions.

Tensionmetric characteristics of spent growth media—The surface tension and interfacial tension of spent culture broths derived from selected cultures grown on hydrocarbon and non-hydrocarbon media were determined for the purpose of evaluating the production of surface active products (Table 1).

TABLE 1—Tensionmetric Properties of Spent Culture Broths

Sample	Surface Tension ¹		Interfacial Tension ²	
	NBYE	Hexadecane	NBYE	Hexadecane
	dyne/cm			
E-6	63.0	39.4	22.8	34.5
H-10	46.7	57.6	18.6	24.4
H-11	48.2	59.3	19.4	26.3
H-12	50.6	57.2	22.9	29.0
H-13	49.2	30.4	20.5	11.8
I-2	44.3	43.4	11.3	13.2
J-14	48.1	52.0	16.0	21.4
K-3	47.8	53.7	13.9	25.7
K-4	44.8	52.8	13.9	21.6
L-4	40.9	59.3	15.4	25.7
L-9	36.0	64.6	8.2	36.8
L-10	49.8	62.8	24.6	30.2
M-1	49.5	60.4	21.4	25.7
M-8	41.8	45.0	7.1	18.8
M-9	41.7	49.6	10.2	20.5
M-10	46.8	52.2	16.7	24.2
N-5	72.8	58.3	30.4	37.2
P-1	42.4	48.1	12.2	16.3
P-2	42.0	49.3	11.1	19.2
P-4	42.4	48.2	10.4	13.9
P-5	43.5	47.9	10.0	22.2
P-6	53.6	60.0	17.2	31.0
P-7	43.4	56.7	10.0	24.4
P-10	40.0	48.0	9.7	15.8
P-11	42.4	52.3	10.6	20.7
R-2	53.9	63.7	24.6	31.9
R-5	44.3	54.5	11.8	22.9
R-6	43.2	56.3	9.5	24.4
NBYE (control)	44.6	—	18.0	—
Basal salts (control)	—	72.0	—	49

¹ Surface tensions measured at 25°C.

² Interfacial tensions measured against hexadecane at room temperature.

The isolates group into essentially 3 classes with respect to their ability to alter surface tension: (1) those organisms which decrease the surface tension of spent

media; (2) those organisms which raise the surface tension of spent media; and (3) those organisms which do not change the surface tension of spent media. These classes are most evident in isolates grown at the expense of NBYE where 39 percent of the isolates yielded a spent culture broth with decreased surface tensions. The interfacial tension of spent culture broths derived from isolates grown on NBYE showed 64 percent of those isolates decreased interfacial tension; whereas, 29 percent increased interfacial tension.

Nutrient broth-yeast extract appears to contain surface active component(s) which are metabolized by specific isolates with a corresponding increase in the tensionmetric characteristics of the spent culture broth. It is not known whether spent culture broths derived from those isolates that yielded lower tensions produced additional biosurfactant, or altered constituents present in NBYE to surface active constituents. If these isolates metabolized the surface active component(s) present in NBYE as well as producing additional biosurfactant through metabolism, then the effective net yield of surface active component(s) was significantly greater than indicated by the measured tensions.

In contrast, all the isolates grown at the expense of hexadecane yielded spent culture broths having surface and interfacial tensions that were decreased from control values. One specific isolate (H-13) appeared to decrease the tensionmetric properties of the spent culture broth to a greater degree than the rest and was selected for further study.

For comparison, a number of known hydrocarbon-utilizing bacteria as well as nonhydrocarbon-utilizing bacteria were assessed for tensionmetric properties imparted to the spent culture broth following growth in chemically defined media (Table 2).

TABLE 2—Surface Tension of Spent Media from Hydrocarbon and Nonhydrocarbon Utilizing Bacteria

Microorganism	Hexadecane-grown	Glucose-grown
	dyne/cm	
<i>Mycobacterium vaccae</i>	51.5	—
<i>Candida tropicalis</i>	52.6	—
<i>Pseudomonas putida</i>	52.1	—
<i>Mycobacterium rhodochrous</i>	53.8	—
<i>Nocardia</i> species	52.8	—
<i>Corynebacterium</i> species	52.6	—
<i>Acinetobacter</i> sp. 17987	53.9	—
<i>Acinetobacter</i> sp. HO1-N	50.8	—
Average	52.5	—
<i>Escherichia coli</i> K-12	—	53.4
<i>Escherichia coli</i> B	—	49.5
Average	—	51.4
E-6	39.4	—
H-13	30.4	—
Average	34.9	—
Control (basal salts)	72.0	72.0

These data illustrate the validity and application of the screening and evaluation protocol developed for the purpose of recognizing and selecting microorganisms capable of producing surface active products. These data also demonstrate that bacterial growth on hydrocarbon does not necessarily result in production of surface-active agents in quantities detectable by increased surface activity. In fact, of the alkane-oxidizers tested, none decreased the surface tension of the growth medium to a greater extent than glucose-grown *E. coli*. However, most alkane-grown isolates decreased the interfacial tension to values well below controls (Table 1). Thus, the measurement of interfacial, rather than surface tension may be more reliable for detection of surfactant production.

Viscosity reduction of bacterial-treated heavy crude—Selected isolates were grown in 3-liter Fernbach flasks containing 1-liter of the complete basal salts medium plus 50 gms of Venezuelan Monagas heavy crude. All isolates were pregrown on 0.5 percent hexadecane for a minimum of 2 days and an inoculum volume of 100 ml added to the Fernbach flask. This culture was incubated aerobically on a rotary shaker for 6-7 days at room temperature. The oil was separated from the aqueous medium by allowing phase separation to occur in a 2-liter separatory funnel.

A number of visual characteristics were associated with these cultures following growth on heavy oil. First, uniform dispersion of the crude oil occurred throughout the aqueous medium. This dispersion was routinely stable for a number of hrs with eventual phase separation occurring in 24-48 hrs. Simple swirling of the flask resulted in the immediate and uniform dispersion of the oil. Second, following phase separation the volume of oil recovered was 2-2.5 times greater than the volume of the original oil. Third, the crude oil recovered was significantly less adherent to glass surfaces, tending to separate cleanly from such surfaces. Fourth, the recovered oil exhibited significantly greater fluid properties, with flow characteristics considerably different from the original crude oil.

Visual characteristics are of considerably less value than the more quantifiable properties such as viscosity measurements. Table 3 summarizes our results relating to the viscosity changes occurring with Monagas heavy crude following treatment with selected bacterial isolates.

These results demonstrate a viscosity reduction for bacterial-treated Monagas crude ranging from greater than 50 percent for dry samples to 98 percent for wet samples. Bacterial growth of all isolates occurred with Monagas crude as the sole carbon and energy source. Colony forming units were estimated at 10^{10} - 10^{11} cells/ml, representing a 4-5 log increase in population cell density. The component(s) of Monagas crude supporting growth of these bacterial isolates is presently under analysis.

Surfactant production by bacterial isolate H-13—H-13 is a mixed bacterial culture capable of growth on a variety of heavy crude oils and pure hydrocarbons, both paraffinic and aromatic (Table 4).

H-13 was identified as a surfactant-producing culture by its ability to significantly lower the surface tension of the hexadecane-mineral salts growth medium

TABLE 3—Viscosity Changes in Bacterial-Treated Monagas Crude

Sample	Temperature	Viscosity (Centipoise)	Percent Decrease
Monagas crude control (dry) ¹	40°C	>25,000	
	60°C	4,690	
Monagas crude control (wet) ²	40°C	6,510	
	60°C	1,070	
F-3 treated Monagas crude (dry)	40°C	14,000	44
	60°C	2,040	56
F-3 treated Monagas crude (wet)	40°C	151	98
	60°C	64	94
H-10 treated Monagas crude (dry)	40°C	5,912	76
	60°C	1,003	79
H-10 treated Monagas crude (wet)	40°C	5,500	15
	60°C	978	9
H-13 treated Monagas crude (dry)	40°C	10,160	60
	60°C	1,670	64
H-13 treated Monagas crude (wet)	40°C	452	93
	60°C	163	85
H-13A treated Monagas crude (dry)	40°C	9,462	62
	60°C	2,785	41
H-13A treated Monagas crude (wet)	40°C	145	98
	60°C	76	93
L-9 treated Monagas crude (dry)	40°C	11,162	55
	60°C	1,323	72
P-2 treated Monagas crude (dry)	40°C	8,038	68
	60°C	1,263	73
P-10 treated Monagas crude (dry)	40°C	7,650	69
	60°C	1,253	73

¹ Samples designated dry represent the equilibration of Monagas crude with minimal basal salts medium for 5 days, recovery of the oil and pumped under vacuum with a water aspirator for a minimum of 24 hours.

² Samples designated wet represent the recovered oil as a stable emulsion.

TABLE 4—Growth of H-13 on Hydrocarbons and Crude Oils

Carbon Source	Growth
<i>Alkanes</i>	
Octane	+
Dodecane	+
Tetradecane	+
Hexadecane	+
Octadecane	+
<i>Aromatics</i>	
Naphthalene	+
Anthracene	+
Phenanthrene	+
<i>Crude Oils</i>	
Venezuelan Monagas crude	+
Venezuelan Cerro Negro crude	+
West Texas crude	+
Monterey crude	+
Alaskan crude	+
East Texas crude	+

from 72.0 dynes/cm to 30.4 dynes/cm; and to effectively reduce the interfacial tension of the same growth medium from 49.0 dyne/cm to as low as 6.0 dyne/cm. In addition, we observed that a minimum of 25.0 ml of hexadecane per liter of growth medium was dispersed in the form of a stable emulsion by H-13 and to examine the physiology of surfactant production.

The surfactant produced by H-13 during growth on hexadecane is surface active under a variety of experimental conditions, as measured by the interfacial tension of the spent growth medium versus hexadecane (Table 5). Surface activity of the spent growth medium was stable under conditions of high salinity and high or low pH.

The spent growth medium was analyzed for the presence of extracellular lipids, protein and polysaccharides. The chloroform-soluble material was analyzed for neutral lipids, phospholipids, and glycolipids. Glycolipids were detected in the chloroform-soluble fraction; whereas, trace quantities of glycerides and phospholipids were detected. The spent growth medium contained negligible amounts of protein as contrasted to large amounts of polysaccharide. The polysaccharide may be related to the extremely mucoid character of H-13.

The extracellular glycolipid was further analyzed to assess its physical-chemical properties and role as a potential surfactant; and to examine the physiology of glycolipid synthesis by H-13.

Physiology of surfactant production by H-13—H-13 was grown on 2.5 percent hexadecane-mineral

TABLE 5—Surface Activity of Spent Medium Derived from Hexadecane-Grown H-13

Spent Growth Medium	Interfacial Tension (dyne/cm)	
	Hexadecane	
Nondialyzed, pH 7.0	11.8	
Dialyzed, pH 7.0	16.7	
Dialyzed, pH 7.0 + 0.1 M MgCl ₂ or 0.1 M CaCl ₂	16.0	
Dialyzed, pH 7.0 + 2 pct NaCl. . .	24.2	
Dialyzed, pH 7.0 + 10 pct NaCl. .	22.9	
Dialyzed, pH 3.0	25.3	
Dialyzed, pH 3.0 + 2 pct NaCl. . .	25.7	
Dialyzed, pH 3.0 + 10 pct NaCl. .	22.9	
Dialyzed, pH 10.0	23.1	
Dialyzed, pH 10.0 + 2 pct NaCl. .	23.1	
Dialyzed, pH 10.0 + 10 pct NaCl. .	20.3	
Control, uninoculated medium. . .	49.0	

TABLE 6—Extracellular Glycolipid Production By H-13

Carbon Source	Glycolipid Produced
	(mg/liter)
2.5 percent Hexadecane	295.0
1 percent Glucose	8.2
0.4 percent Succinate	6.6
0.8 percent Broth-Yeast Extract	9.7

salts medium. Samples were removed periodically for determination of cell number by dilution-plate count techniques, and for measurement of the interfacial tension of the medium from which bacterial cells were removed. In addition, cells and spent medium were extracted separately with chloroform/methanol to quantify glycolipid content. Results of this experiment are shown in Fig. 1.

The interfacial tension of the growth medium measured against hexadecane decreased continually over 7 days, at which time the value of 6.0 dynes/cm was attained. This increase in surface activity correlated directly to the increase in glycolipid concentration in the growth medium. Maximal extracellular glycolipid concentration (295 mg/liter) was attained in one week, well into the stationary phase of growth. The cellular glycolipid content also increased over the 7 day growth period to a maximum of 250 mg per 10 gms cell wet weight. Extracellular glycolipid was not produced by H-13 grown on carbon sources other than alkane (Table 6).

Whether the extracellular glycolipid is a secondary metabolite, a byproduct of hexadecane metabolism, or is synthesized by H-13 as a pre-requisite for the transport and cellular uptake of hexadecane is not determined. Production of extracellular surfactants by hydrocarbon-utilizing bacteria is a well-documented phenomenon.^{4,10}

Such surfactants are considered to function in the formation of oil-in-water emulsions, aiding in transport of the water-insoluble hydrocarbon to the bacterial cell. The cellular glycolipid of H-13 may increase the hydrophobicity of the bacterial cell surface, allowing direct cell-hydrocarbon contact, or formation of hydrocarbon-water micro-emulsions at the cell surface.

TABLE 7—Reduction of Crude Oil Viscosity By H-13

Treated Crude Oil (wet)	Viscosity (cps)			Percentage Reduction
	40°	60°	80°	
Monagas crude— control	6,510	1,070	—	—
H-13 treated Monagas crude	452	163	—	85-93
H-13A treated Monagas crude	145	76	—	93-98
H-13 spent medium plus Monagas crude ¹	3,252	678	—	37-50
Cerro Negro crude— control	>25,000	7,662	1,390	—
H-13 treated Cerro Negro crude	—	737	375	73-90
“De-Asphaltened” Monagas crude	161	70	—	93-98

¹ Spent medium derived from hexadecane-grown H-13 was centrifuged and filtered through a 0.45 μ Millipore filter. This cell-free spent medium was supplemented with 30 gm of Monagas crude, sparged with nitrogen and shaken agitated at 300 RPM for 18 hr on a rotary shaker.

Pure culture bacterial isolates derived from the H-13 mixed culture were grown on hexadecane individually and were analyzed for glycolipid content and surface activity of the spent medium. Isolate H-13A produced 93 percent of the glycolipid detected in the medium of

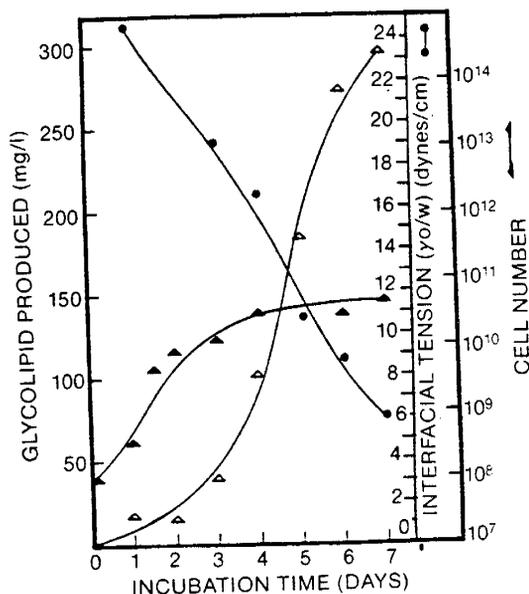


FIG. 1—Physiology of surfactant production by H-13 on hexadecane. Cell number Δ — Δ was measured using dilution-plate count techniques. Glycolipid content Δ — Δ was measured using the anthrone method. Interfacial tension of spent medium 0—0 was measured by the drop weight method.

the mixed culture and, correspondingly, reduced the interfacial tension of the spent medium to 19.0 dynes/cm within 2 days of growth, a value comparable to that obtained with H-13. Thus, H-13A was identified as the active bacterial agent responsible for glycolipid production by the H-13 mixed culture. Taxonomic identification of H-13A is currently in progress.

Glycolipid characterization

The extracellular glycolipid was isolated from the cell-free spent growth medium by extraction with diethyl ether following acidification. This crude lipid extract was fractionated by silicic acid column chromatography, with the glycolipid eluting from the column with acetone. This fraction contained several minor glycolipid components (5) and 1 major glycolipid, as determined by thin-layer chromatography (Fig. 2). The major glycolipid component was further purified by repeated preparative thin-layer chromatography.

Physical-chemical characterization of the glycolipid

Solubility—The crude glycolipid preparation was determined to be water-soluble and hexadecane-insoluble. Crude glycolipid was dissolved in the mineral salts medium, pH 7.0, and was partitioned with an equal volume of hexadecane with 100 percent of the anthrone-positive material remaining in the aqueous phase.

Structure—The purified major glycolipid contained 0.52 mg glucose/mg dry weight. No phosphate or amino groups were detected in either the minor or major glycolipids. The major glycolipid was hydrolyzed, reduced and acetylated by the method of Albersheim et al.¹ for

analysis of the sugar composition by gas chromatography. Only glucose was detected by GLC. A sample of the purified major glycolipid was deacylated by base saponification and the fatty acid analyzed by gas chromatography yielding a complex spectrum of fatty acid. The deacylated glycolipid was shown to contain a disaccharide composed of glucose monomers and a monosaccharide-glucose. Further chemical structural analysis of the glycolipid is in progress.

Critical micelle concentration—The critical micelle concentration (CMC) of the crude glycolipid fraction and of the purified major glycolipid were determined by measuring the interfacial tension of increasing amounts of glycolipid dissolved in the mineral salts medium versus hexadecane (Fig. 3). The CMC of the crude glycolipid fraction dissolved in BSE was determined to be 1.5 mg/ml (0.15 percent by weight) with a minimum interfacial tension of 0.25 dynes/cm as determined by the drop weight method. This value was confirmed by a spinning drop tensiometer as shown in Fig. 4. A value of 0.25 dynes/cm was obtained for a concentration of the glycolipid greater than 1.5 mg/ml.

Upon equilibration for longer time periods (24 hr), interfacial tension as low as 2.1×10^{-2} dynes/cm were measured for the crude glycolipid fraction. The CMC for the crude glycolipid fraction (1.5 mg/ml) is comparable to that obtained with synthetic surfactants.

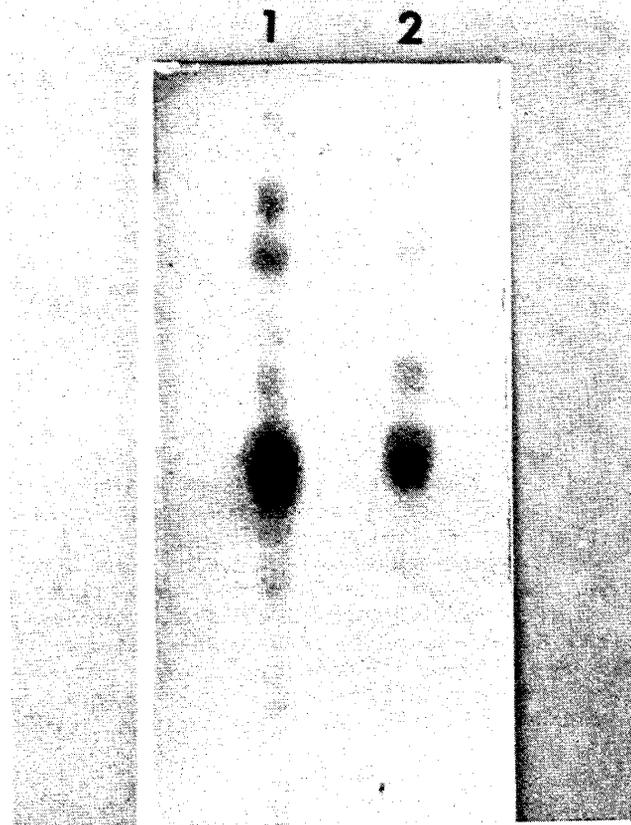


FIG. 2—Thin layer chromatogram of the crude glycolipid fraction (lane 1) and purified major glycolipid (lane 2). TLC of glycolipids was performed using chloroform/methanol/5 N NH_4OH (65:30:5) as solvent system on silica gel G. Glycolipids were detected with the orcinol spray reagent, followed by heating to 120°C 5 minutes.

Sodium dodecyl sulfate (SDS) has a CMC of approximately 0.23 percent by weight.

Upon equilibration for longer time periods (24 hr), interfacial tension as low as 2.1×10^{-2} dynes/cm were measured for the crude glycolipid fraction. The CMC for the crude glycolipid fraction (1.5 mg/ml) is comparable to that obtained with synthetic surfactants. Sodium dodecyl sulfate (SDS) has a CMC of approximately 0.23 percent by weight.

The CMC of the purified major glycolipid was estimated to be 1.0 mg/ml with a minimum interfacial tension of 1.41 dynes/cm. The interfacial tension of the minor glycolipid fraction at a concentration above the CMC for the crude glycolipid fraction (7.1 mg/ml) was 0.44 dynes/cm. A CMC for the minor glycolipid fraction was not determined. However, these data suggest that a mixture of minor and major glycolipids is required for maximal surface activity. The addition of the minor glycolipids to the major glycolipids may alter the hydrophilic/lipophilic balance (HLB) of the surfactant blend increasing its surface activity towards hexadecane.

Formulations containing synthetic surfactants plus long chain alcohols or other lipophilic agents have been shown to increase surfactant effectiveness or efficiency. The effectiveness or efficiency of biologically formed surfactants may be similarly improved by the addition of such components or by *in vivo* alternation of the surfactant molecule by manipulation of bacterial growth conditions. The fatty acid composition of the cellular and extracellular lipids of alkane-grown *Acinetobacter*, as well as other hydrocarbon-grown microorganisms, reflects the chain length of the alkane growth substrate.⁸ Alternation of surfactant fatty acid chain length would alter the HLB of that surfactant. Growth of H-13 on octadecane, eicosane, or longer chain alkanes would presumably result in the production of more hydrophobic, oil-soluble glycolipids with long chain fatty acids increasing the surfactant efficiency. Growth of H-13 on octane or dodecane could result in the formation of more hydrophilic glycolipids with short chain fatty acids. These hypotheses are currently being tested.

Reduction of heavy crude-oil viscosity by H-13

Two heavy crude oils, Venezuelian Monagas crude and Cerro Negro crude, were chosen for treatment by H-13. The oils served as the sole source of carbon and energy for bacterial growth. The growth of H-13 on Monagas crude resulted in a 93 percent reduction of oil viscosity; whereas, the viscosity of Cerro Negro crude was reduced 90 percent (Table 7). The pure culture isolate (H-13A) reduced the viscosity of Monagas crude by 98 percent (Table 7). Viscosity reduction in this range is comparable to that obtained when Monagas crude is chemically de-asphalted by solvent extraction (Table 7).

Surfactant production appears to contribute to oil viscosity reduction by H-13. Several observations suggest the formation of a surfactant-stabilized oil-in-water emulsion in cultures of H-13 grown on heavy crude oil: 1) the volume of H-13-treated oil is two to three-fold greater than the initial oil volume; 2) the oil phase recovered after growth of H-13 had a conductivity of 2.43×10^{-3} mho/cm, a conductivity comparable to

that of 0.02 M KCl, indicating an oil-in-water emulsion; 3) 18 h treatment of crude oil with cell-free spent medium from hexadecane-grown H-13, containing 295 mg/liter of extracellular glycolipid, resulted in a 50 percent reduction of crude oil viscosity (Table 7). Thus, glycolipid formation appears to enhance the reduction of oil viscosity.

CONCLUSIONS

The initial objective to isolate microorganisms that alter the viscosity of heavy oil has been successfully accomplished. The premise that viscosity changes which occur through microbial processes requires either the reduction of the average molecular weight of the heavy oil through extensive degradation of high molecular weight asphaltenes or the production of surface active agents by microorganisms growing at the expense of the oil determined the experimental approach for enrichment and selection of microorganisms.

The preliminary recognition of bacterial isolates which produce surface active products was achieved through screening bacterial isolates that reduced the tensiometric properties of spent media as well as reducing the viscosity of heavy oils. These procedures yielded the isolation of 200 bacterial isolates which grow at the expense of crude oil as well as effect its dispersion. The bacterial isolate H-13 was selected for further detailed study due to its superior comparative characteristics in decreasing the tensiometric properties of spent culture broths derived from growth on hexadecane or heavy oil.

The physiological characteristics of H-13 encompass a number of attributes worthy of note. This isolate has the ability to grow extremely well at the expense of n-alkanes (8-18 carbons), polycyclic aromatic hydrocarbons as well as a number of crude oils. In addition, H-13 produces a surface active extracellular and cellular glycolipid complement when grown at the expense of n-alkanes as the sole source of carbon and energy. The application of H-13 to viscosity reduction of heavy oil resulted in significant decreases in the viscosity of

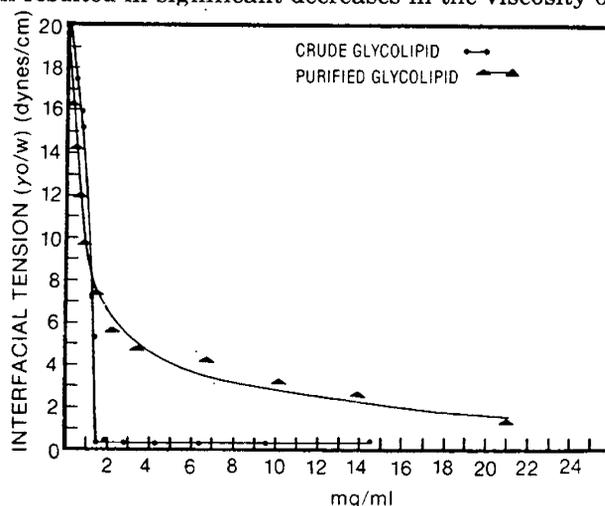


FIG. 3—Critical micelle concentration (CMC) of the crude 0—0 and purified Δ — Δ glycolipid fractions. The CMC of the crude glycolipid fraction was estimated to be 1.5 mg/ml while the CMC of the purified glycolipid was estimated to be 1.0 mg/ml.

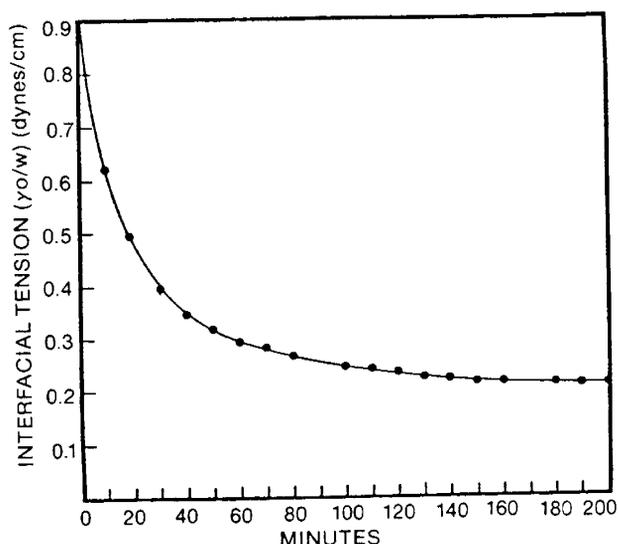


FIG. 4—Spinning drop tensiometry of the crude glycolipid fraction versus hexadecane. At equilibrium a minimum interfacial tension of 0.25 dynes/cm was achieved.

bacterial-treated oil. The mechanisms for these changes appear to be largely due to extracellular surface active glycolipid which forms stable oil-in-water emulsions.

These emulsions are characterized by significant decreases in viscosity as well as improved flow characteristics. The production of surface active products leading to emulsification by H-13 may represent only part of the mechanism(s) responsible for viscosity reduction of heavy oil. Preliminary studies indicate that H-13 grows at the expense of purified asphaltene as a sole source of carbon and energy. Further studies are necessary to establish the structural changes and/or modifications of asphaltene that are occurring in this bacterial culture.

The application of H-13 and/or surface active products derived from H-13 to heavy oil production merits further consideration for its potential contribution to oil recovery technology. Two experimental approaches appear feasible for introducing this emulsion-forming biological system into heavy oil containing formations: 1) direct inoculation of the formation with the microorganisms; and 2) direct injection of biosurfactant.

The *in situ* cultivation of the bacteria within the formation would necessitate providing inorganic nutrients plus adequate levels of oxygen to enable the biological agent to grow at the expense of oil. There exists, however, no guarantee that the bacteria will survive the reservoir environment, let alone produce surfactant. The alternate approach of biosurfactant production outside the formation with subsequent delivery as a surfactant flood merits further development as a potentially useful EOR technology.

The modification of the glycolipid produced by H-13 through physiological and genetic manipulation is experimentally feasible and of scientific merit with respect to strain improvement for increased product yield as well as alteration of the hydrophilic-lipophilic balance (HLB) value of the molecule. Glycolipid synthesis is induced by growth of H-13 on n-alkanes, suggesting the regulation of surfactant biosynthesis. The selection of constitutive mutants for glycolipid biosyn-

thesis would eliminate the requirement for cost-intensive alkanes as growth substrates and allow for yield improvement through physiological control of culture conditions.

SUMMARY

This research project has established:

1. The isolation of microorganisms that utilize heavy crude oils as sole sources of carbon and energy.
2. The isolation of bacterial isolates that produce extracellular surface active compounds.
3. The identification of a bacterial isolate (H-13) that is capable of reducing the viscosity of heavy crudes in excess of 95 percent.
4. One mechanism of viscosity reduction by H-13 is the formation of stable oil-in-water emulsions.
5. The biosurfactant produced by H-13 is a glycolipid.
6. The CMC of crude glycolipid is 1.5 mg/ml with a measured minimum interfacial tension of 2.1×10^{-2} dynes/cm.
7. The CMC of purified glycolipid is 1.0 mg/ml with a minimum interfacial tension of 1.41 dynes/cm.
8. The minor glycolipid fraction exhibited an interfacial tension of 4.4×10^{-1} dynes/cm.

The application of specifically tailored microbial processes through physiological and genetic means can be accomplished to produce useful changes in oil. The application of genetic and bioengineering concepts to EOR and oil production are dependent upon the acquisition and development of basic and definitive data-bases. The future interface between petroleum engineering and the application of microbial processes appears complementary in terms of contributions that the microbiologist can offer to oil recovery and production.

REFERENCES

1. Albersheim, P., D. J. Nevins, P. D. English, A. Karr. 1967. *Carb. Res.* 5:340.
2. Ashwell, G. 1957. p. 73. *In*: S. P. Colowick and N. O. Kaplan (eds.). *Meth. Enzymol.* Vol. 3. Academic Press, Inc., N.Y.
3. Bartlett, B. G. 1959. *J. Biol. Chem.* 234:466.
4. Cooper, D. G. and J. E. Zajic. 1980. *Adv. Appl. Microbiol.* 26:229.
5. Davis, J. B. 1967. *Petroleum Microbiology.* Elsevier Pub. Co., 640 pp.
6. Dittmer, J. C. and R. L. Lester. 1964. *J. Lipid Res.* 5:126.
7. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. *J. Biol. Chem.* 193:265.
8. Makula, R. and W. R. Finnerty. 1968. *J. Bacteriol.* 95:2102.
9. VanLoocke, R. 1979. *Exptl. Sci. and Technol.* 13:346.
10. Zajic, J. E. and C. J. Panchal. 1976. *CRC Crit. Rev. Microbiol.* 5:39.

Microbial Activities and Changes in the Chemical and Physical Properties of Oil

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INTRODUCTION

Recent interest in the interaction of bacteria and fungi with petroleum and petroleum products resulted from the recognition of the role played by microorganisms in the removal of oil spilled in marine and terrestrial environments. The results of these studies have been recently summarized by Atlas,³ Colwell and Walker⁵ and McGill, Rouatt and Westlake.²² However, the role of bacteria in the biogenesis of oil deposits and their potential use in enhanced oil recovery processes were not considered in these reviews.

An anonymous report in *World Oil*¹ suggests that approximately 10 percent of the world's oil supply has been reduced in economic value because of microbial activity. Winters³⁵ discusses changes in the chemical and physical properties of *in situ* oils as related to potential reservoir microbial activities. In their review on petroleum transformation in reservoirs Milner, Rogers and Evans²³ consider biodegradation as one of the natural processes involved in petroleum biogenesis.

Philippi²⁶ suggests that heavy to medium heavy naphthenic crudes are derived from the microbial transformation of primary paraffinic oils. Rubinstein et al.²⁹ studied microbially induced laboratory changes in the chemical and physical properties of conventional oils and concluded that the Alberta oil sand bitumens have arisen from the biodegradation of conventional crude oils. The use of microbes and microbial products in enhanced oil recovery has been the subject of recent meetings (1979)* and review articles (Forbes¹⁶; Clark, et al⁷).

The greatest rate of change in the chemical and physical properties of oil as a result of microbial activity has been observed from laboratory and field studies to take place under aerobic conditions and in an environment containing non-growth limiting concentrations of nitrogen and phosphate. That is, conditions which allow for growth of aerobic microorganisms. There is no evidence in the literature which indicates that anaerobic microorganisms are able to initiate or readily decompose hydrocarbons as found in crude oils.

I will discuss in this paper the changes in the chemical-physical properties of oil resulting from the

action of microorganisms and very briefly discuss their possible role in oil genesis and enhanced oil recovery.

CHANGES IN THE CHEMICAL COMPOSITION OF OIL

Our understanding of the chemical changes in oil brought about by microbial activity has followed developments in our ability to separate and resolve some of the compounds found in oil. Changes in the chemical composition of petroleum as a result of microbial activity have been obtained by gravimetric, column and gas chromatographic techniques (Jobson, Cook and Westlake).¹⁸ The asphaltene components of oil are insoluble in *n*-pentane and may be separated from the other components by celite chromatography.

The deasphalted oil is fractionated on silica-gel columns into a saturate component (eluted with *n*-pentane), an aromatic component (eluted with benzene) and a polar N,S,O-fraction which requires a slightly polar solvent (benzene-methanol) to be eluted from silica-alumina gel columns. Changes in the chemical composition of oil have been based on loss in weight of fractions and on the resolution of some of the components in the saturate (e.g. *n*-alkanes and isoprenoids) and aromatic (e.g. mono-, di- and tri-cyclic ring systems) fractions by gas-liquid chromatography.

Walker, Colwell and Petrakis³² applied these techniques together with low resolution mass spectrometry in a study of the microbial alterations in the chemical composition of a South Louisiana crude oil.

Saturates and aromatics

Changes in the components of the saturate and aromatic fraction of oil resulting from microbial activity have been determined gravimetrically and by means of conventional packed column gas chromatographic procedures. Such studies (Jobson, Cook and Westlake¹⁸; Atlas²) showed that the *n*-alkane components of the saturate fraction and to a lesser extent the isoprenoids phytane and pristane were readily removed by microbial activity.

Some utilization of components present in the aromatic fraction was also indicated. Walker, Colwell and Petrakis³² combined the above techniques with computerized low resolution mass spectrometry and studied the microbially induced chemical changes in a South

*Conference on Microbiological Processes Useful in Enhanced Oil Recovery—San Diego, Calif., U.S. Department of Energy. Contract No. DE-ATI-78MC08333.

Louisiana crude oil. They reported that most of the normal and branched chain alkanes were degraded and that there was an increase in long chain *n*-alkanes (C₂₈-C₃₂).

The susceptibility of cycloalkanes to degradation, which so far can only be detected by mass spectrometry, was reported in the order 6<1<2<3<5<4-ring compounds although 4.75 mg, 3.25 mg, 2.0 mg, 1.5 mg, 1.5 mg and 1.2 mg of 1, 2, 3, 4, 5 and 6 ring compounds respectively were utilized. They also concluded that the susceptibility of aromatic components to degradation decreased with the increase in the number of rings; monoaromatics>diaromatics>triaromatics>tetraaromatics>pentaaromatics and that aromatic nuclei containing sulfur were twice as refractory as non-sulfur analogues.

Rubinstein et al,²⁹ Rubinstein and Strausz²⁸ and Crawford et al¹¹ using gas chromatographic and mass spectrometric techniques examined the chemical composition of oil and bitumens from northern Alberta together with those from microbially altered conventional oils. They showed that bacterial populations readily removed the *n*-alkanes from Prudhoe Bay and Bellshill Lake oils. The acyclic isoprenoids and the mono-, di- and tricyclic hydrocarbons were removed at a slower rate while the tetra- and pentacyclics (i.e. the steranes and hopanes) were not utilized.

Approximately 50 percent of the weight of the aromatic fraction was lost through microbial degradation and the loss occurred at a slower rate than was observed with the saturate fraction. Small ring systems were utilized more quickly than larger ring systems and rings with only a few alkyl groups were utilized more readily than multi-alkylated ring systems. An increase in the C/H ratio and the percent oxygen of recovered oil was observed in these studies.

As a result of the comparison of the chemical and physical characteristics of Alberta bitumen with those produced by microbial action on conventional oils, they concluded that the Alberta bitumen deposits were derived by microbial modification of crude oils. Wyndham and Costerton³⁷ demonstrated the bacterial colonization of hydrocarbon surfaces derived from Alberta bitumens and reported their growth, as determined by increase in epifluorescent count, on all non-asphaltenic compounds.

Fedorak and Westlake¹² using gas chromatography with glass capillary columns, showed that marine samples supplemented with nitrogen and phosphate utilized the simple aromatics (e.g. naphthalene and 2-methylnaphthalene) more quickly than *n*-alkanes. These studies also showed that marine bacterial populations from a pristine environment in a non-nutrient enriched medium removed aromatic compounds more extensively than *n*-alkanes.

The pattern of aromatic utilization progressed from the less complex to more complex molecules in the following order: C₂-naphthalenes; phenanthrene; dibenzothiophene; C₃-naphthalenes and methylphenanthrenes; C₂-phenanthrenes. The patterns of release of ¹⁴C₂ from Prudhoe Bay oil spiked with one of the following: ¹⁴C-hexadecane, ¹⁴C-naphthalene, ¹⁴C-phenanthrene or ¹⁴C-anthracene confirmed the gas chromatographic observation on the removal of simple aro-

omatics like naphthalene before *n*-alkanes (Westlake and Cook³⁴). These studies also showed that phenanthrene was more readily metabolized than anthracene.

The data of Fedorak and Westlake¹³ revealed that sulfur heterocycles, which are eluted in the aromatic fraction, can be utilized as readily as many of the components in the aromatic fraction and that many sulfur heterocycles were used without nutrient supplementation. The approximate order of susceptibility to microbial degradation was C₂-benzo[b]thiophene>C₃-benzo[b]thiophenes=dibenzothiophene>C₁-dibenzothiophenes>C₂-dibenzothiophenes. The selective removal of sulfur heterocycles by microbial activities has been suggested for the improvement of oil feed stocks (Shockley, Attaway and Finnerty,³⁰ 1982). Finnerty (1981¹⁴) also recently reported on the bacterial desulfurization of a high sulfur crude oil.

Examples of changes in glass capillary gas chromatographic profiles of the saturate, aromatic and sulfur heterocycle fractions of the relatively low sulfur Rainbow and Redwater oils brought about by the growth of mixed bacterial cultures are presented in Figs. 1, 2 and 3 respectively. The low molecular weight components of saturate fraction up to tetradecane (Fig. 1), the monoaromatics, the alkylbenzenes (region 1) and part of naphthalene (Fig. 2) would be lost by volatilization under the conditions used in these experiments. The *n*-alkanes and isoprenoids from the saturate fractions (Fig. 1), and the identifiable aromatic compounds (Fig. 2) which were utilized are listed in Table 1.

The lower molecular weight sulfur heterocycles (Fig. 3 and Table 1) C₂-benzo[b]thiophenes (peak A), C₃-benzo[b]thiophenes (peaks B and C), dibenzothiophene (peak D) and C₁-dibenzothiophenes (peaks E, F and G) were readily removed from both crude oils by microbial

TABLE 1—Aromatic and Sulfur Heterocycle Compounds in G.C. Profiles

Compounds		Compounds	
Aromatics ^a		Sulfur Heterocycles ^b	
Symbol	Compound(s)	Symbol	Tentative Identification
1	alkylbenzenes	A	C ₂ -benzo(b)thiophene (3) ^c
2	naphthalene	B	C ₃ -benzo(b)thiophenes (2)
3	2-methylnaphthalene	C	
4	1-methylnaphthalene	D	dibenzothiophene
5	biphenyl	E	C ₁ -dibenzothiophenes (3)
6	C ₂ -naphthalenes	F	
7	4-methylbiphenyl	G	
8	C ₃ -naphthalenes	H	C ₂ -dibenzothiophenes (4)
9	dibenzothiophene	I	
10	phenanthrene	J	
11	methylphenanthrenes	K	
12	C ₃ -phenanthrenes	L	unknown
chr	chrysene (marker)	M	benzo(b)naphthathiophenes (2)

^a see Figs. 2 and 5; ^b see Figs. 3 and 6; ^c number of isomers reported by Wiley et al (1981).

action. The C_2 -dibenzothiophenes (peaks H, I, J and K) were more completely removed from the Redwater oil than from Rainbow oil whereas the unknown sulfur heterocycle (L) and (peak M), possibly a benzo[b]naphthothiophene, were more resistant to microbial attack under these experimental conditions.

utilized α -aromatics but not n -alkanes (unpublished data DWSW). Monoprenoid compounds are more readily utilized under mesophilic than psychrophilic conditions (unpublished data DWSW). That is, some specificity is demonstrated by microorganisms as to the types of hydrocarbons metabolized.

Asphaltenes and polar N,S,O-compounds

Alterations in the content of these fractions as a result of microbial activities are based on weight changes as there are no readily usable analytical techniques for resolving the components in these fractions.

Earlier studies (Jobson, Cook and Westlake;¹⁸ Zajic, Supplison and Volesky,³⁸ Walker, Colwell and Petrakis,³² suggested that there was an increase in these fractions as a result of the microbial attack on petroleum. Rubinstein et al²⁹ however reported that there was no increase in the weight of the polar fraction as a result of the microbial degradation of Prudhoe Bay oil.

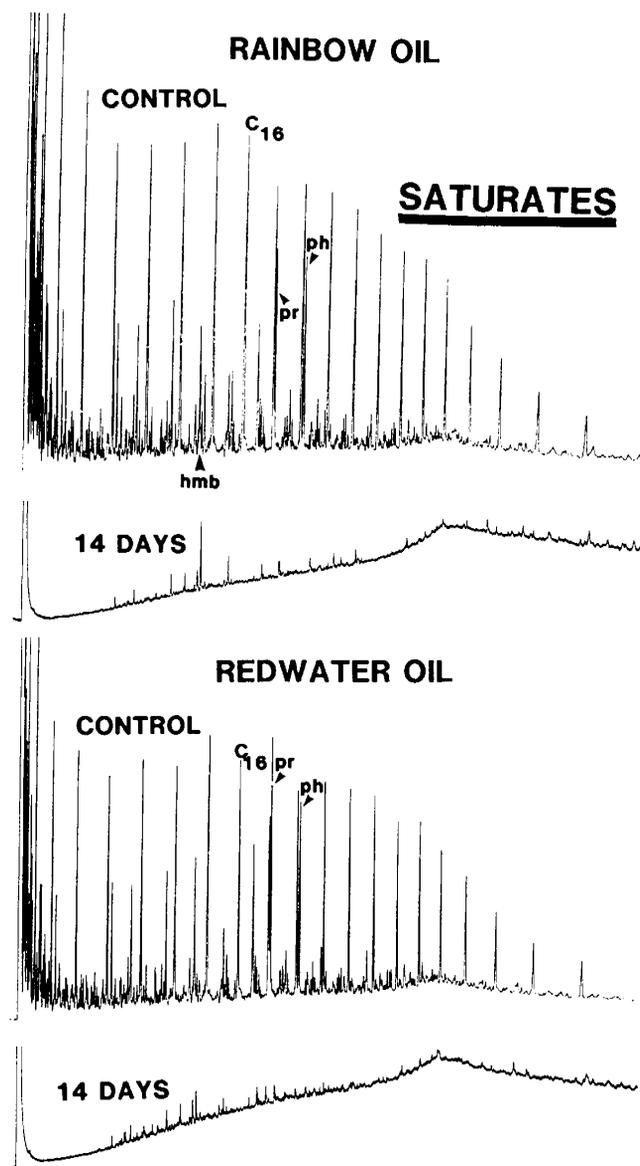


FIG. 1—Gas chromatographic profile of the saturate fractions of Rainbow and Redwater oils using the methods of Fedorak and Westlake (1982).

The nature of the compounds utilized in an oil by a mixed bacterial population depends on the source of that population, the nutrient status of the environment (Fedorak and Westlake¹²) and the chemical composition of the oil (Westlake et al³³). The resolution of mixed cultures of oil-degrading populations into pure cultures has shown that such cultures utilize hydrocarbons in specific fractions of oil.

For example, based on chromatographic studies, culture *Arthrobacter* sp. 20S-5 utilized n -alkanes but not aromatics whereas culture *Flavobacterium* sp. 8W-2

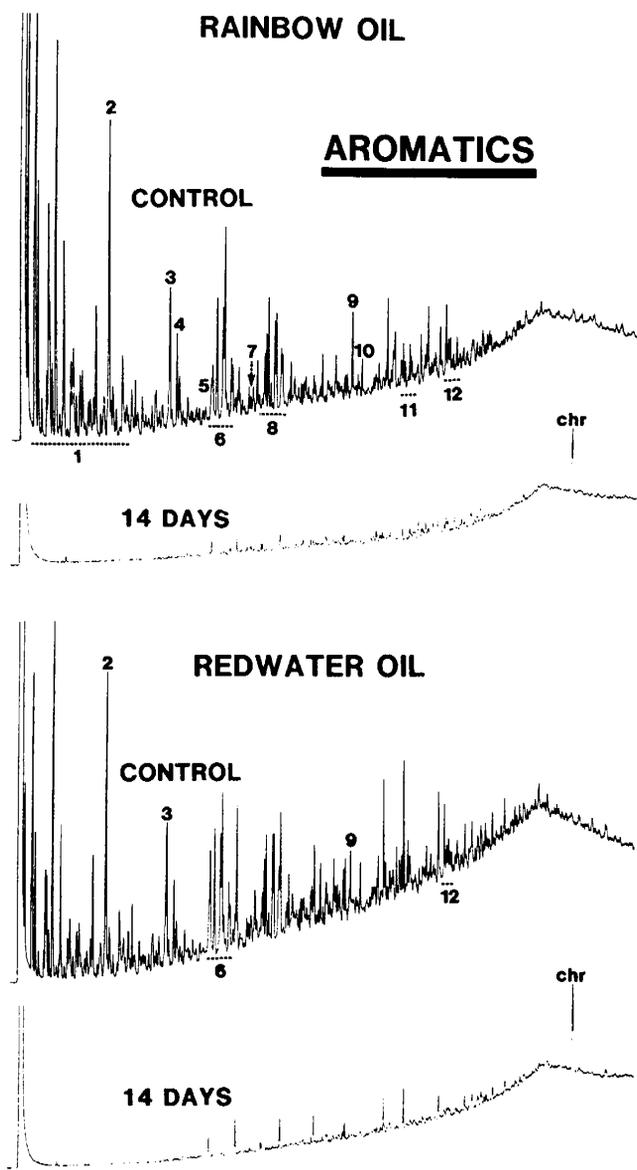


FIG. 2—Gas chromatographic profile of the aromatic fractions (flame ionization detector) of Rainbow and Redwater oils using the methods of Fedorak and Westlake (1982).

They suggested that the differences between their results and previous data could be attributed to the use of different bacterial cultures. It is also possible that such differences are a result of insufficient oxygen levels (i.e. aeration) being provided to bacterial populations growing on oil. Cultures growing under such conditions quickly become acidic reaching pH values in the range of 4.5 to 5.0 which are characteristic of the presence of organic acids (unpublished data DWSW).

Such compounds could be recovered in the polar fraction. Rubinstein et al²⁹ however did observe a reduction in the nitrogen and sulfur content of recovered oil. The loss in sulfur content was attributed to the metabolism of sulfur compounds e.g. thiophenes found in the aromatic fraction of oil.

CHANGES IN THE PHYSICAL PROPERTIES OF OIL

Changes in the physical properties of oil are primarily due to alterations in its chemical composition such as would be brought about by microorganisms. Physical properties such as pour point, viscosity, gravity, optical rotation and the physical state of oil (i.e. dispersed, non-dispersed, or emulsified) can be affected by microbial activity.

In a study on the biogenesis of Alberta tar sands Rubinstein and Strausz²⁸ followed changes in the pour point, viscosity, A.P.I. gravity and optical rotation of

conventional oils as they were subjected to biodegradation. Changes in the pour points of oils were correlated with the chemical composition of the oil. Samples containing relatively large amounts of polar material such as Lloydminster crude and Athabasca bitumen had relatively high pour points, +3° and +8°C respectively.

The presence of a large aromatic component compensated for the polar compounds and resulted in a significant lowering of the pour point. The saturate content also affected the pour point as the incubation of Prudhoe Bay oil with *Saccharomyces lipolytica*, which utilizes only *n*-alkanes (i.e. it would "dewax" the oil), resulted in a drop in the "pour point" from -2° to -12°C. Further biodegradation of this recovered oil sample using a mixed bacterial culture resulted in the pour point being increased to +7°C.

The proportion of polar material also was considered to be the major factor affecting the viscosity and A.P.I. gravity of oil and bitumen samples (Rubinstein and Strausz²⁸). For example, Prudhoe Bay oil containing approximately 10 percent polar material has a viscosity of 208,131 SUS (Saybolt Universal Seconds) units. Partially degraded Prudhoe Bay oil, that is after growth of *S. lipolytica*, had its viscosity increased 5-fold.

Further incubation of the recovered oil with bacterial cultures resulted in a 26-fold increase in viscosity

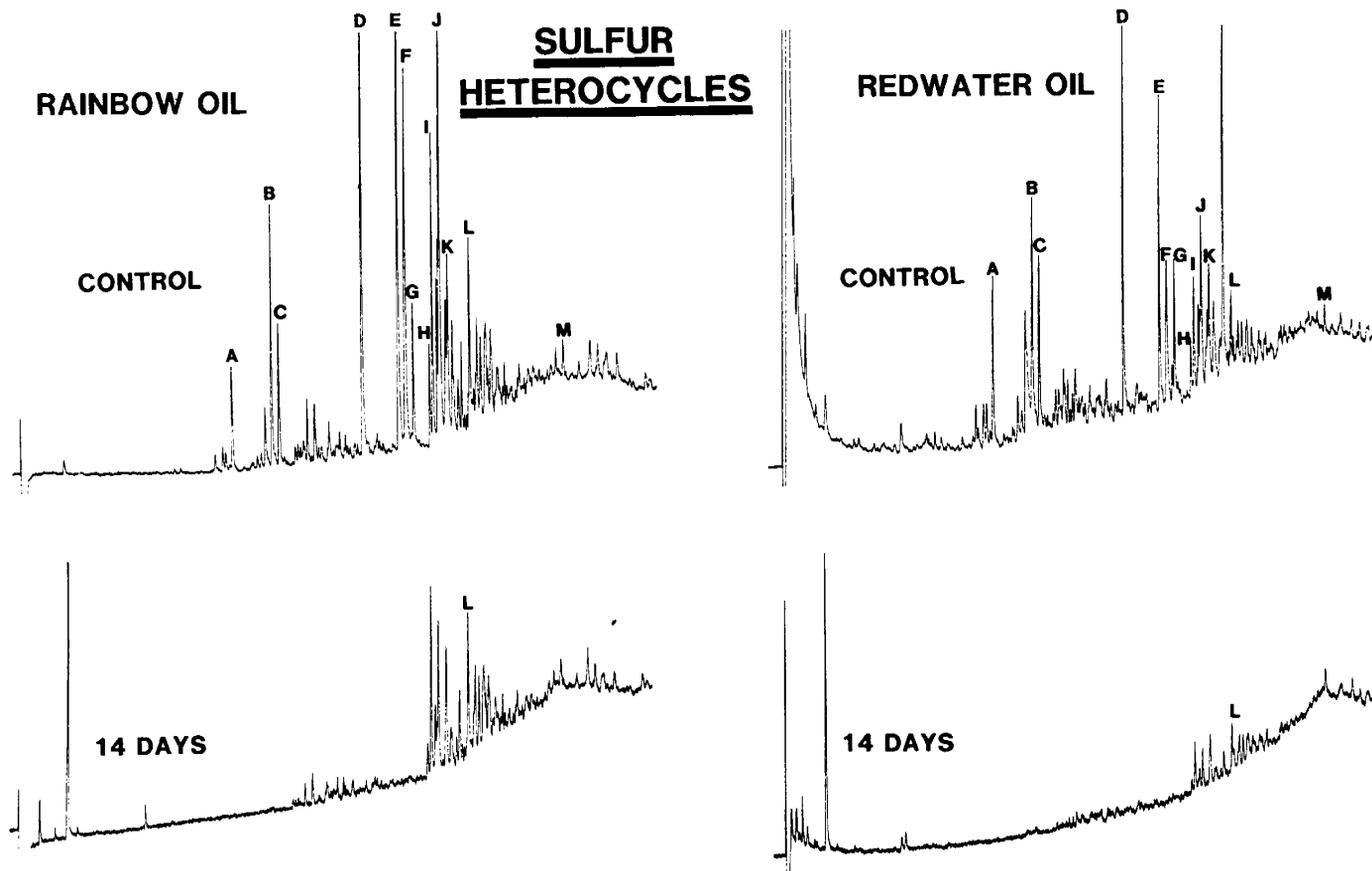


FIG. 3—Gas chromatographic profiles of the sulfur heterocycle components of the aromatic fractions (flame photometric detector) of Rainbow and Redwater oils.

and the formation of a tarry, viscous material with a high polar content. Biodegradation of Prudhoe Bay oil also resulted in a reduction of its A.P.I. gravity from 27° to that of water, 10°.

The optical activity of biodegraded oil was found to increase as a result of an increase in the concentration of the highly optically active but relatively non-biodegradable steranes and triterpenes and moderately optically active, but biodegradable acyclic isoprenoids.

The physical form in which oil exists in the liquid state has a marked effect on its biodegradability and is the most readily recognized physical character to be modified by microbes and their activities. Oil introduced to aquatic systems will disperse as a thin slick on the surface of the water and if sufficient mechanical energy is present, emulsions, either oil in water or water in oil can be formed. This latter type of emulsion is referred to as a "mousse," is very stable physically, and is resistant to microbiological attack (Atlas³). While these changes in state can be initiated by abiotic processes, microbial activity can accelerate both the dispersion and emulsion formation processes.

Many microorganisms produce surface-active compounds which facilitate the utilization of water insoluble materials and some produce them when growing

on hydrocarbons (Reisfield, Rosenberg and Gutnick²⁷; Guire, Friede and Ghulson¹⁷). Such compounds should increase the surface area of oil available for microbial colonization and hence the rate of biodegradation as well as the mobility of the oil by reducing surface tension and the viscosity of oil. In their review on the chemical nature of surface-active compounds by microorganisms Cooper and Zajic⁹ conclude that most of them are lipid in nature and range from simple molecules like fatty acids to complex heteropolymers containing carbohydrates, amino acids or phospholipids.

A patent has been recently granted (January 1982) for the industrial use of "emulsan," a protein-lipo-hetero-polysaccharide emulsifier produced by a strain of *Acinetobacter calcoaceticus*.* However, many microbes which readily emulsify oil do not bring about readily demonstrable changes in the chemical composition of the oil. It is probable however that the production of surface active compounds will accelerate the biodegradation of oil, although the ability to produce a surface active compound is not a prerequisite for growth on hydrocarbons.

Non-toxic chemical dispersants have been reported

*McGraw-Hill's Biotechnology Newswatch 2, No. 7, 1-2, 1982.

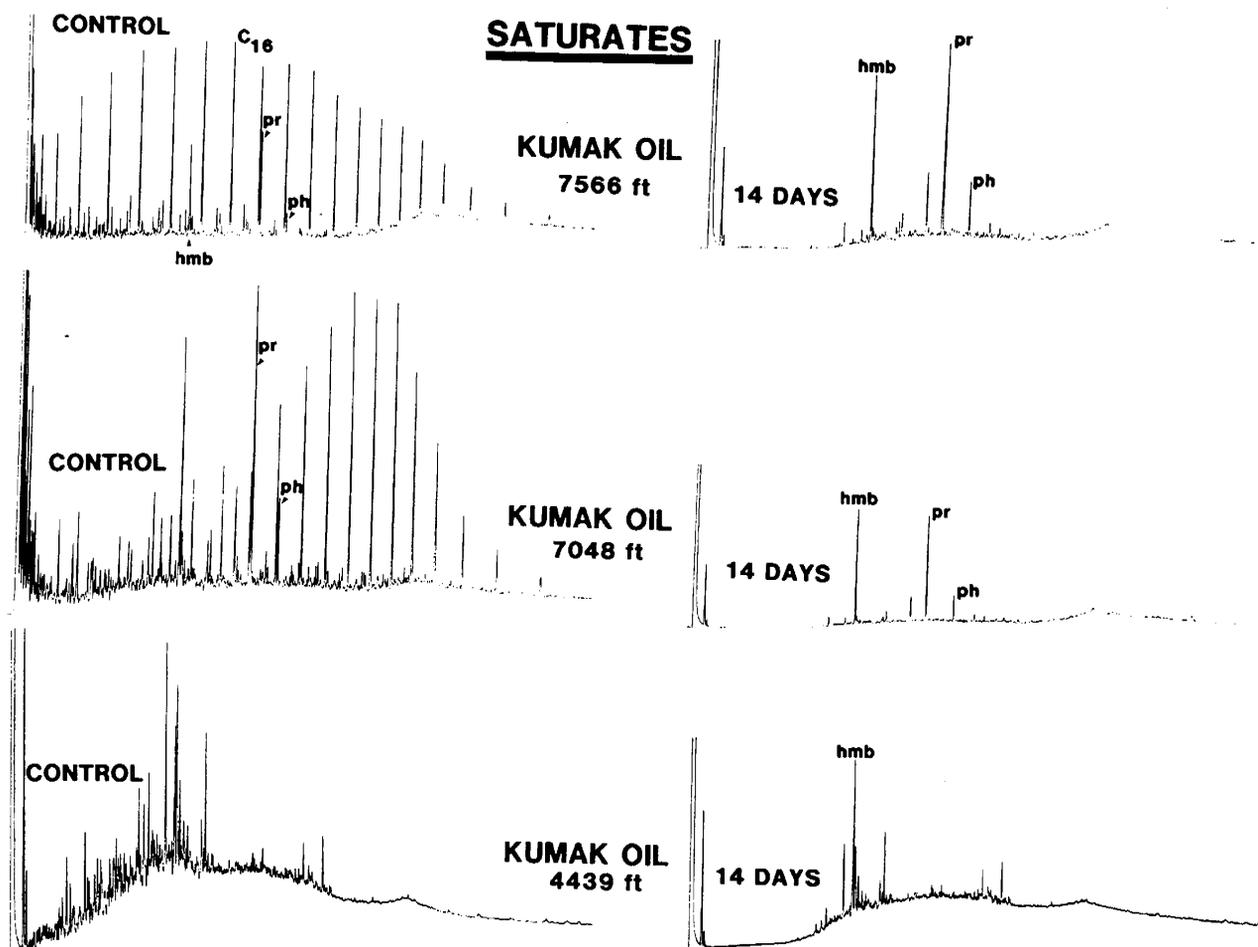


FIG. 4—Gas chromatographic profiles of the saturate fractions of Kumak oils using the methods of Fedorak and Westlake (1982).

(Mulkins-Phillips and Stewart,²⁴ Traxler and Bhattacharya³¹) to accelerate microbial metabolism of oils. However, Mulkins-Phillips and Stewart²⁴ reported that dispersant-oil combination caused population shifts and Foght and Westlake¹⁵ (1982) showed a selective sensitivity to the presence of a chemical dispersant in the utilization of compounds in Prudhoe Bay oil.

RESERVOIR DATA

Direct evidence for the microbial degradation of oil in sub-surface reservoirs is very limited: altered isotope ratios being one of the most widely used measurements. However there is considerable indirect evidence that it occurs. Such data are usually based on the recognition of chemical changes in the composition of reservoir oils (e.g. loss of *n*-alkanes and isoprenoids) similar to those obtained in the microbial degradation of such oils under laboratory conditions. Winters and Williams³⁶ provided such data for subsurface biodegradation in a Cretaceous field and Bailey et al⁴ for a series of Mississippian oil pools.

Bailey, Jobson and Rogers⁵ used an aerobic oil-degrading population under laboratory conditions to bring about chemical changes in the composition of the Mississippian oils (Williston Basin) such that they resembled those of "biodegraded" oils found *in situ* in this field. Burns, Hogarth and Milner,⁶ in a study of Beaufort Basin liquid hydrocarbons divided 20 oils into two groups based on their chemical-physical characteristics and in particular on the gas chromatographic profile of the C₁₁ to C₃₀ fraction of whole oils. Group I oils were recovered from deep reservoirs, had a normal *n*-alkane content, and higher A.P.I. gravities, pour points and lower viscosities than shallow Group II oils. These latter oils were hypothesized as being derived by bacterial degradation of Group I oils.

For example, three samples of Kumak oils were obtained from 4439, 7048 and 7566 ft. The shallow oil (4439 ft) did not have any *n*-alkanes and isoparaffins whereas the next deeper oil (7048 ft) showed only slight chemical modifications (a loss of *n*-alkanes up to C₁₆ and reduction in peak heights of those up to C₂₀ with the isoparaffins still present) compared with the deep oil (7566 ft). The deeper oil was in general similar in chemical composition and physical characters to the sample obtained at 7048 ft.

The capillary gas chromatographic profiles of the saturate, aromatic and sulfur heterocycle profiles of these Kumak oils are presented in Figs. 4, 5 and 6 respectively. The data on the saturate profiles (Fig. 4) confirm the observations of Burns, Hogarth and Milner.⁶ The oil from the shallow pool (i.e. 4439 ft) shows a saturate gas chromatographic profile associated with a biodegraded oil, i.e. the loss of *n*-alkanes and isoprenoid components. The *n*-alkanes in the deeper oils are susceptible to biodegradation as they, but not the isoprenoids, were completely removed by microbial action under laboratory conditions.

The data in Fig. 5 on aromatic profiles and Fig. 6 on sulfur heterocycle profiles also confirm the observation of Burns, Hogarth and Milner,⁶ that is, Kumak oil from the shallow well (4439 ft) has been subjected to microbial attack *in situ*. The gas chromatographic profiles of the microbially modified deep oils indicates that these components are also susceptible to microbial attack

but that the deeper oil (7566 ft) is slightly more recalcitrant than the oil from the pool at 7048 ft. However, both these oils could be converted to a chemical composition similar to the oil found in the shallow pool at 4439 ft by microbial action.

Changes in the physical properties of these Kumak oils can be related to their "biodegradation" state (Burns, Hogarth and Milner⁶). The A.P.I. gravity of the degraded oil (i.e. Group II from 4439 ft) had been lowered, the viscosity slightly increased and the pour point lowered from +25°F to -35°F. The change in this latter characteristic is a benefit for pipeline transportation of such an oil under arctic conditions. These authors further observed that the shallow oils which showed evidence of biodegradation had reservoir temperatures below 150°F. This suggests that, at least as far as Beaufort oils are concerned, this is a maximum temperature above which biodegradation will not take place.

It is hypothesized that the oxygen, nutrients and microorganisms responsible for biodegradation of oil in reservoirs would reach oil pools through meteoric water via faults, fractures and other conduits. Kuznetsova and Gorlenko²⁰ concluded that sulfate reduction via sulfate-reducing bacteria in reservoirs depended in part upon the presence of and activity of aerobic bacteria belonging to the genus *Pseudomonas*.

Jobson, Cook and Westlake¹⁹ showed that many other aerobes were able to carry out the aerobic attack on oil. The degree of sulfate reduction observed depended on the nature of the aerobic population, the chemical composition of the oil and whether the sulfate-reducing bacteria were added as pure or mixed cultures. The microbial production of sulfide in reservoirs can result in the conversion of a sweet to a sour crude (i.e. containing detectable amounts of H₂S and mercaptans). The interaction of aerobic and anaerobic bacteria in bringing about changes in the chemical-physical characteristics of oil in reservoirs is shown in Fig. 7.

The aerobic population initiates the attack on hydrocarbons present in oil and the by-products from their incomplete oxidation e.g. low molecular weight organic acids would be available for sulfate-reducing and sulfide-generating bacteria (Obuekwe, C. O.²⁵) to grow and produce H₂S. The production of such acids in reservoirs containing carbonates would also lead to CO₂ production which can affect the physical properties of the reservoir oil.

SUMMARY AND PERSPECTIVE

Microorganisms, in particular aerobic bacteria, readily bring about changes in the chemical and physical properties of oil under non-restrictive growth conditions. Such changes have been readily demonstrated under laboratory conditions, in surface terrestrial and marine oil spills and, based on comparative chemical-physical observations, implied to have taken place in some oil reservoirs.

Chemical changes demonstrated primarily by gas chromatography may include loss of *n*-alkanes, isoprenoids and the lower molecular weight aromatic and sulfur heterocyclic compounds. The loss of these compounds, some of them of high commercial value, are associated with changes in the physical properties of

oil such as pour points and viscosity. Some of these physical changes such as the lowering of pour points by microbial removal of *n*-alkanes are desirable as such an oil would retain fluid mobility at lower temperatures. However, prolonged microbial activity by mixed bacterial cultures, as would be observed under reservoir conditions, can result in the conversion of a fluid crude oil into a tar-like material such as is found in the Athabasca tar sands.

Pure bacterial cultures isolated from mixed populations of oil-degrading bacteria do show some specificity as to the types of hydrocarbons metabolized. If conditions are suitable for the growth of aerobic organisms—that is plenty of oxygen, nitrogen and phosphate—such changes can take place relatively rapidly.

Reservoir oils have been found that show all of the chemical-physical characteristics associated with oils subjected to microbial growth under laboratory conditions. Thus microbial activity can be assumed to take place in reservoirs if the temperature is not so high as to inhibit microbial growth. The microbes, oxygen and nutrients necessary to sustain growth are assumed to be provided initially via meteoric water. During the

processes of oil recovery, the drilling process and the use of secondary recovery procedures such as water flooding add to and alter the microbial flora found in oil reservoirs.

Theoretically it should be possible to seed oil wells (i.e. a tertiary recovery process) with microbial populations capable of bringing about changes in the physical properties of an oil, which should result in an enhanced rate of recovery. However, the oxygen and nutrients supplied to sustain the growth of the seeded population would also allow the growth of the indigenous population and it is doubtful that the chemical and physical changes would be limited to those of the seeded cultures and, for example, souring of the crude oil could occur.

Anaerobes, which produce CO₂, H₂ and solvents have been used in enhanced oil recovery studies with variable results (Cowey¹⁰). While such organisms do not require oxygen, and in fact their growth is inhibited by it, they do require the addition of a readily utilizable energy source since they are not able to use petroleum hydrocarbons as sources of cell carbon and energy. Thus they have the advantage over aerobes

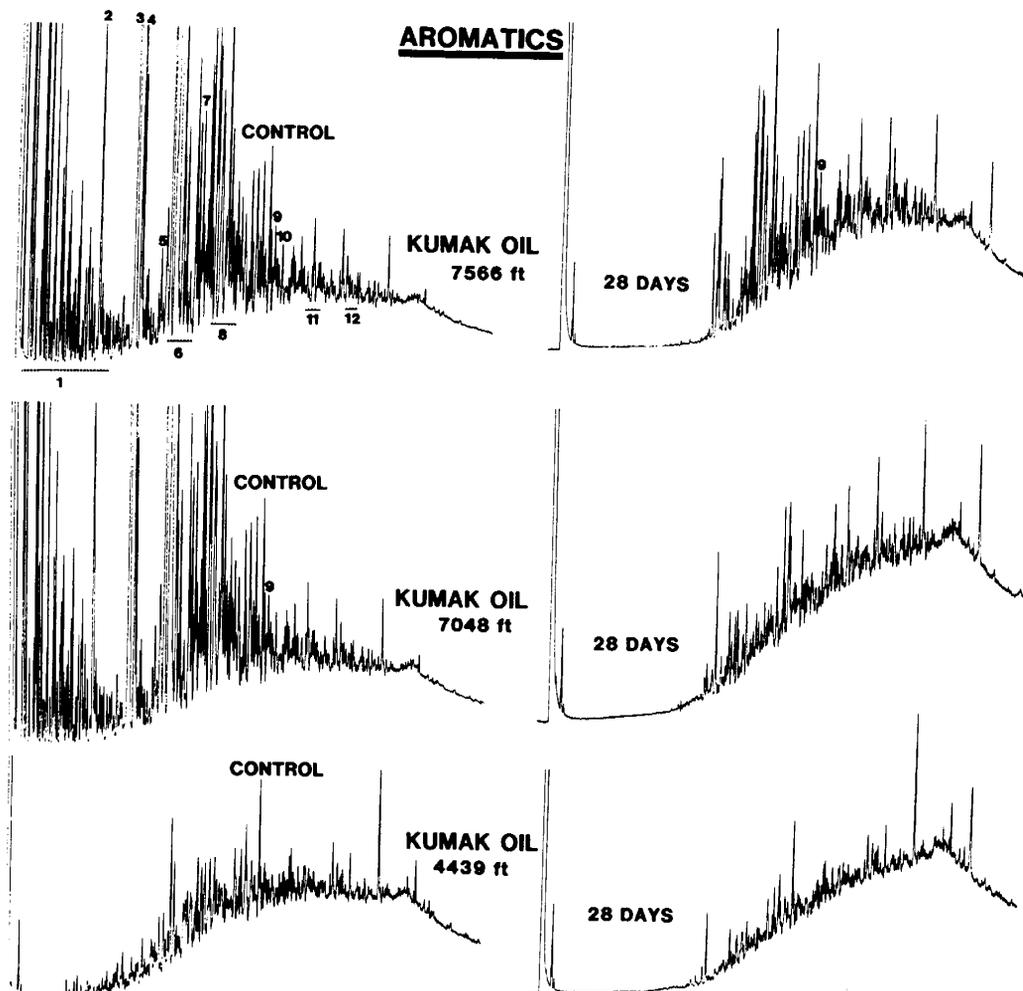


FIG. 5—Gas chromatographic profiles of the aromatic fractions (flame ionization detector) of Kumak oils using the methods of Fedorak and Westlake (1982).

found in oil reservoirs of not using valuable hydrocarbon components of oil but the disadvantage of requiring the addition of a utilizable energy source which might not always be readily available.

Surface active compounds are produced by many microorganisms when growing on hydrocarbons; these affect the flow properties of oil. It is possible that some of these represent new classes of surface active chemicals which could be produced by fermentation processes or by chemical synthesis and injected to facilitate oil recovery processes.

There is a potential for the use of microbes and their activities for tertiary oil recovery processes. However before this interaction can be efficiently exploited information is needed on the factors controlling microbial activities in the diverse environmental reservoir conditions where oil is found.

ACKNOWLEDGEMENT

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REFERENCES

1. Anonymous. 1972. Bacteria have destroyed 10 percent of world's crude. *World Oil*, Feb. 1, pp. 28-29.
2. Atlas, R. M. 1975. Effects of temperature and crude oil composition on petroleum biodegradation. *Appl. Microbiol.* 30, 396-403.
3. Atlas, R. M. 1981. Microbial degradation of petroleum hydrocarbons: an environmental perspective. *Microbiol. Revs.* 45, 180-209.
4. Bailey, N. S. L., H. R. Krouse, C. R. Evans and M. A. Rogers. 1973a. Alteration of crude oils by water and bacteria. Evidence from geochemical and isotope studies. *Amer. Assoc. Pet. Geol. Bull.* 57, 1276-1290.
5. Bailey, N. S. L., A. M. Jobson and M. A. Rogers. 1973b. Bacterial degradation of crude oil: Comparison of field and experimental data. *Chem. Geol.* 11, 203-211.
6. Burns, B. J., J. T. C. Hogarth and C. W. D. Milner. 1975. Properties of Beaufort Basin liquid hydrocarbons. *Bull. Can. Petrol. Geol.* 23, 295-303.
7. Clark, J. B., D. M. Munnecke and G. E. Jenne-man. 1981. "In situ" microbial enhancement of oil pro-

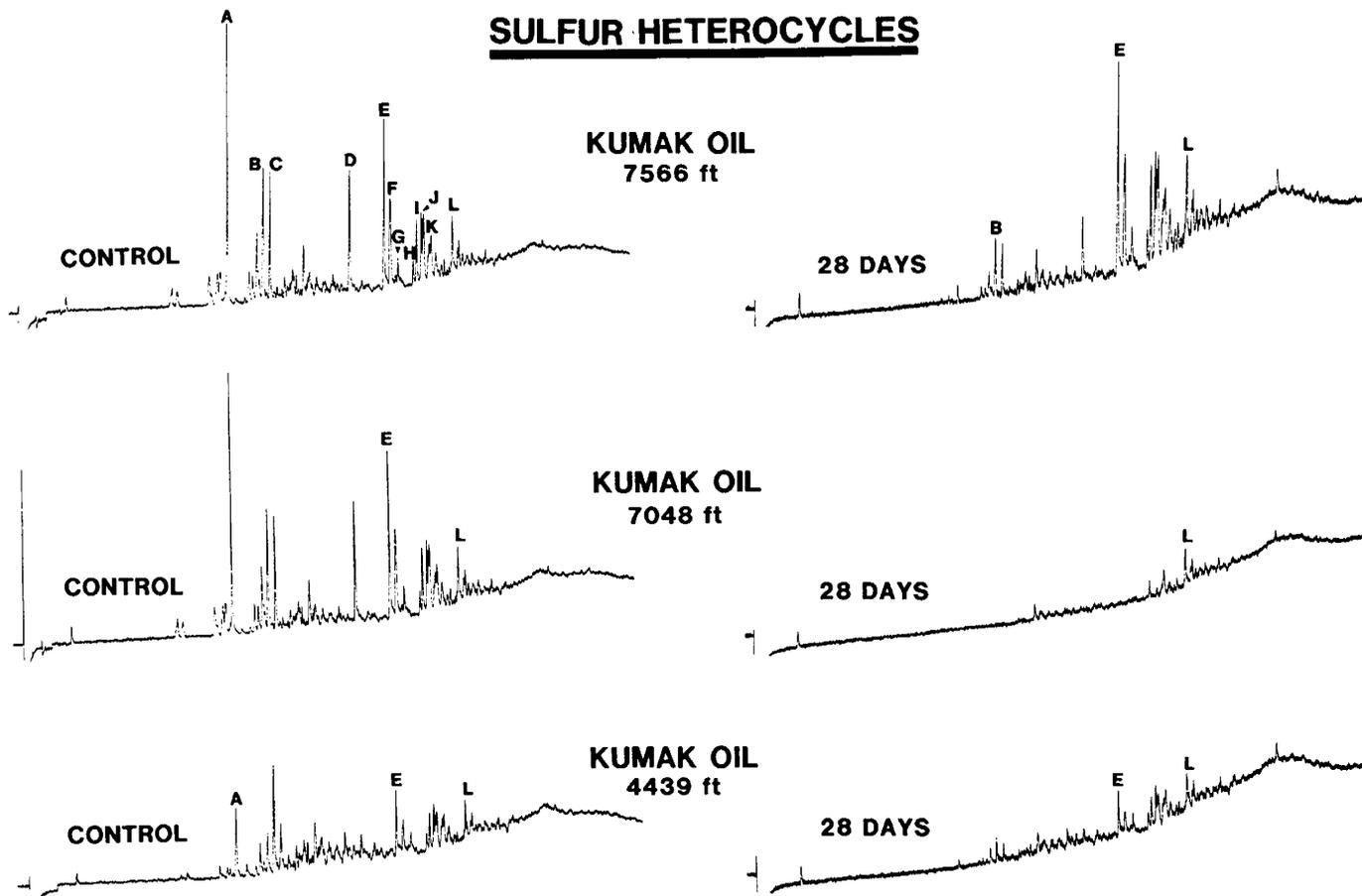


FIG. 6—Gas chromatographic profiles of the sulfur heterocycle components (flame photometric detector) of the aromatic components of Kumak oils using the methods of Fedorak and Westlake (1982).

duction. *Developments in Industrial Microbiology* 22, 695-701.

8. Colwell, R. R. and J. D. Walker. 1977. Ecological aspects of microbial degradation of petroleum in the marine environment. *Crit. Rev. Microbiol.* 5, 423-445.

9. Cooper, D. G. and Zajic, J. E. 1980. Surface active compounds from microorganisms. *Adv. Appl. Microbiol.* 26, 229-253.

10. Cowey, F. K. 1976. Enhanced recovery of petroleum using microorganisms—A literature review. pp. 57-75. In *Institute of Petroleum Conference on the Genesis of Petroleum and the Microbiological Means for its Recovery*. Birmingham, U.K. (Conference Proc. 57).

11. Crawford, R. J., C. Spyckerelle and D. W. S. Westlake. 1977. Biodegradation of oil reservoirs. pp. 163-176. In: *Oil Sand and Oil Shale Chemistry*. O. P. Strausz and E. M. Lown (eds.). Verlag Chemie.

12. Fedorak, P. M. and D. W. S. Westlake. 1981. Microbial degradation of aromatics and saturates in Prudhoe Bay crude oil as determined by glass capillary gas chromatography. *Can. J. Microbiol.* 27, 432-443.

13. Fedorak, P. M. and Donald W. S. Westlake. 1982. Microbial degradation of organic sulfur compounds in Prudhoe Bay crude oil. Submitted *Appl. and Environ. Microbiol.* April 1972.

14. Finnerty, W. R. 1981. Microbial desulfurization and denitrification Abst. BR88. 28th Congress International Union of Pure and Applied Chemistry. Vancouver, B.C., Canada. Aug. 16-21.

15. Foght, J. M. and D. W. S. Westlake. 1982. Effect of the dispersant Corexit 9527 on the microbial degradation of Prudhoe Bay oil. *Can. J. Microbiol.* 28, 117-122.

16. Forbes, A. D. 1980. Microorganisms in oil recovery. pp. 169-180. In *Hydrocarbons in Biotechnology*. D. E. F. Harrison, I. J. Higgins and R. Watkinson (eds.). Heyden and Son, Ltd.

17. Guire, P. E., J. D. Friede and R. K. Gholson. 1973. Production and characterization of emulsifying factors from hydrocarbonoclastic yeast and bacteria. pp. 229-231. In: D. G. Ehearn and S. P. Meyers (ed.), "The Microbial Degradation of Oil Pollutants." Pub. No. LSU-SG-73-01. Centre for Wetland Resources, Louisiana State University, Baton Rouge, LA.

18. Jobson, A. M., F. D. Cook, D. W. S. Westlake. 1972. Microbial utilization of crude oil. *Appl. Microbiol.* 23, 1082-1089.

19. Jobson, A. M., F. D. Cook and D. W. S. Westlake. 1977. Interaction of aerobic and anaerobic bacteria in petroleum biodegradation. *Chem. Geol.* 24, 355-365.

20. Kuznetsova, V. A. and V. M. Gorlenko. 1965. Effects of temperature on the development of microorganisms from flooded strata of the Romashinko oil field. *Microbiologiya* 34, 274-278.

21. MacLeod, W. D. Jr., D. W. Brown, R. G. Jenkins, L. S. Ramos and V. D. Henry. 1977. Petroleum hydrocarbons in the Northern Puget Sound area: a pilot design study. United States Environmental Protection Agency. Publication No. EPA-600-7-77-098.

22. McGill, W. B., M. J. Rowell and D. W. S. Westlake. 1981. Biochemistry, ecology and microbiology of petroleum components in soil. pp. 229-296. In: *Soil Biochemistry* Vol. 5. E. A. Paul and J. N. Ladd (eds.), Marcel Dekker, Inc.

23. Milner, C. W. D., M. A. Rogers and C. R. Evans. 1977. Petroleum transformation in reservoirs. *J. Geochem. Explor.* 7, 101-153.

24. Mulkins-Phillips, G. J. and J. E. Stewart. 1974. Effect of four dispersants on biodegradation and growth of bacteria on crude oil. *Appl. Microbiol.* 28, 547-552.

25. Obuekwe, C. O. 1980. Microbial corrosion of crude oil pipeline. Ph.D. Thesis, Dept. of Microbiology, University of Alberta, Edmonton, Alberta, Canada.

26. Philippi, G. T. 1977. On the depth, time and mechanism of origin of the heavy to medium gravity naphthenic crude oils. *Geochim. et Cosmochim. Acta.* 41, 33-52.

27. Reisfield, A., E. Rosenberg and D. Gutnick. 1972. Microbial degradation of oil: Factors affecting oil dispersion in seawater by mixed and pure cultures. *Appl. Microbiol.* 24, 363-368.

28. Rubinstein, I. and O. P. Strausz. 1977. The biodegradation of crude oils: The origin of the Alberta oil sands. pp. 177-190. In: *Oil Sands and Oil Shale Chem-*

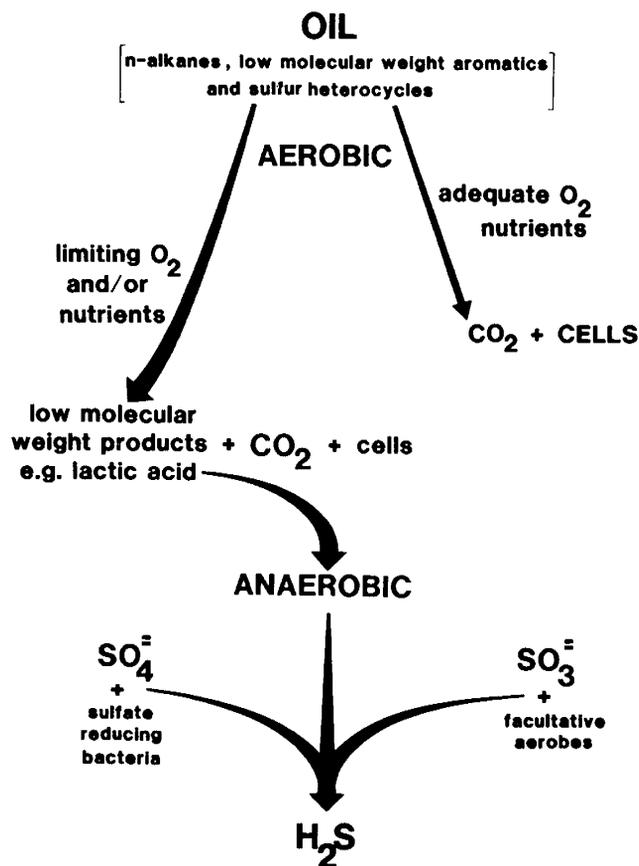


FIG. 7—Interaction of aerobic and anaerobic bacteria in the degradation of oil and the production of H₂S in oil reservoirs.

istry. O. P. Strausz and E. M. Lown (eds.). Verlag Chemie.

29. Rubinstein, I., O. P. Strausz, C. Spyckerelle, R. J. Crawford and D. W. S. Westlake. 1977. The origin of the oil sand bitumens of Alberta: A chemical and a microbiological simulation study. *Geochim et Cosmochim. Acta.* 41, 1341-1353.

30. Shockley, R. K., H. Attaway and W. R. Finnerty. 1982. Microbial modification of dibenzothiophene. p. 157. Abst. of the Annual Meeting of the Amer. Soc. for Microbiology, Atlanta, Georgia, U.S.A.

31. Traxler, R. W. and L. S. Bhattacharya. 1978. Effect of a chemical dispersant on microbial utilization of petroleum hydrocarbons. In: *Chemical Dispersants for the Control of Oil Spills*. Edited by L. T. McCarthy, Jr., G. P. Lindblom and H. F. Walters. American Society for Testing and Materials. ASTM STP659. pp. 181-187.

32. Walker, J. D., R. R. Colwell and L. Petrakis. 1975. Microbial petroleum degradation: Application of computerized mass spectrometry. *Can. J. Microbiol.* 21, 1760-1767.

33. Westlake, D. W. S., A. Jobson, R. Phillippe and F. D. Cook. 1974. Biodegradability and crude oil composition. *Can. J. Microbiol.* 20, 915-928.

34. Westlake, D. W. S. and F. D. Cook. 1980. Petroleum biodegradation potential of Northern Puget Sound and Strait of Juan de Fuca environments. U.S. Environmental Protection Agency Pub. No. E.P.A.-600/7-80-13.

35. Winters, J. C. 1975. Origin, generation and maturation of petroleum and the chemical nature of specific crude oil and other hydrocarbon deposits. In *Proc. 1976 Eng. Foundation Conf.—The Role of Microorganisms in the Recovery of Oil*. Nat'l Sci. Foundation (U.S.)

36. Winters, J. C. and J. A. Williams. 1969. Microbial alteration of crude oil in the reservoir. *Symp. of Petroleum Transformation in Geologic Environments*. Am. Chem. Soc. Meeting, New York, N.Y., Sept. 7-12, pp. E32-E41.

37. Wyndham, R. C. and J. W. Costerton. 1981. "In vitro" microbial degradation of bituminous hydrocarbons and "in situ" colonization of bitumen surfaces within the Athabasca oil sands deposit. *Appl. and Environ. Microbiol.* 41, 791-800.

38. Zajic, J. E., B. Supplisson and B. Volesky. 1974. Bacterial degradation and emulsification of No. 6 fuel oil. *Environ. Sci. Technol.* 8, 664-668.

Biosurfactants and Enhanced Oil Recovery

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INTRODUCTION

There are many possible uses of surfactants for enhanced oil recovery. This paper briefly considers the potential of using biosurfactants and related compounds for enhanced oil recovery. The phrase enhanced oil recovery is used here loosely to cover not only tertiary recovery but also bitumen release from tar sand and de-emulsification of the products from steam or fire flooding.

Microbial surface active agents

Microorganisms produce a wide range of surface active compounds.¹⁻¹⁴ The microbial surface active agents, which have been described, include surfactants, emulsifiers, de-emulsifiers, flocculating agents, foam stabilizers, etc. Most of these are poorly characterized. Biosurfactants, which have been characterized, are lipids. Lipids usually have the classical surfactant structure.

Most lipids have a hydrophobic hydrocarbon portion and a hydrophilic polar group. Many different hydrophilic groups are found in lipids which results in a very large range of potential biosurfactants.¹ These include neutral functional groups such as esters, alcohols, carbohydrates and ethers and ionic substituents such as amino acids, carboxylic acids, amines, phosphates and sulphates.

TAR SAND

A number of organisms from a variety of sources have been screened for the ability to release bitumen from tar sand. The initial test was an erlenmeyer flask with 5 g of tar sand and 50 ml of salt solution to which was added 0.1 or 1.0 ml of microbial whole broth. This was shaken for 48 hr and then analyzed for bitumen release.⁵

In this way several promising bacteria were selected.⁵⁻⁸ One of these was *Corynebacterium lepus* which was isolated from oil soaked ground at Oil Springs, Ontario. When grown on kerosene of other hydrocarbons this bacteria produced large amounts of a biosurfactant which was extremely effective at bitumen release.^{6,7} If the product was harvested at the end of fermentation, the active agent was discovered to be a mixture of lipids. More than 90 percent of this product was a mixture of at least two lipopeptides which could be isolated and shown to be effective for bitumen release.⁷

If the active agent was harvested earlier in the fer-

mentation, during exponential growth, the product was a mixture of corynomycolic acids.^{6,7,8} These varied in total carbon number from 25 to 40. The structure was a modified carboxylic acid with an alkyl branch on the α carbon and a hydroxyl substituent on the β carbon. Corynomycolic acid is as effective as the lipopeptides for bitumen release and is much more stable. This biosurfactant can be autoclaved without loss of activity. Its properties also appear to be insensitive to pH changes from 2 to 12.⁹

Some of the biosurfactants, including crude corynomycolic acid, were tested for the ability to improve bitumen release from a pilot scale "cold water" extraction apparatus.⁵ This was run for eight hr at a time with a feed rate of 500 kg/hr of tar sand and 22 l/hr of kerosene. After these were mixed with water at 19°C, the slurry was passed continuously through settling chambers where sand and clay were removed, the bitumen-kerosene froth was collected and the water taken to a settling tank before being recycled. Various effluents and streams were sampled periodically during several 8 hr tests. These included controls and biosurfactant additions to either the kerosene or water input.

The data from these tests had fairly high deviations due to the variability of the tar sand feed, but the general trends showed that the biosurfactants improved bitumen separation. The sand and clay residues had significantly less adhering bitumen, the water had less suspended particles and bitumen and the crude product stream contained substantially less water.

ENHANCED OIL RECOVERY

The concepts of MEOR are covered thoroughly in other papers in this publication. Microbes grown in reservoirs could enhance oil production by a combination of products including polymers, organic acids, alcohols and gases but this paper will concentrate only on surfactants. The problems encountered with *in situ* growth such as harsh growing conditions, nutrient availability and unwanted side products are covered by other contributors.

Many of these problems will be circumvented by strain selection programs. Biosurfactant production is one more parameter to maximize. The most important problem for biosurfactant production is the lack of oxygen. All of the organisms reported to give good

yields of extracellular biosurfactants were grown aerobically.¹⁻⁹ Usually, a hydrocarbon substrate is necessary for good surfactant production and, if anaerobic degradation of hydrocarbon is possible, it probably is not very efficient.

All anaerobic organisms do synthesize lipids which are all potential surfactants. Anaerobic bacteria can cause some lowering of surface tension¹⁰ and there are several examples of biosurfactant production with non-hydrocarbon substrates. It seems likely that bacteria will be found which can produce biosurfactants in good yield when grown anaerobically.

A possible solution to the above problems would be to grow the organisms on the surface under controlled conditions and then pump the biosurfactant down the well. For this to be economically feasible it is necessary to produce biosurfactants on cheaper, carbohydrate substrates and to significantly improve yields.

An example of the possibilities in this direction are shown by surfactin production by *Bacillus subtilis*.¹¹ Surfactin is a lipopeptide which can lower the surface tension of water to 17 mN/m. It is produced extracellularly in media with glucose or nutrient broth as the carbon source. The yield can be increased over 100 times by the addition of a trace amount (10^{-6} moles) of Mn(II). Research is underway to determine if the effects of trace elements on other biosurfactants are this dramatic.

DEMULSIFICATION

The recovery of heavy oils by steam flooding or fire flooding results in thick water in oil emulsions which must be de-watered (less than 1 percent water) before processing. Recently a number of bacteria have been shown to be able to coalesce, almost instantaneously, hydrocarbon and water emulsions.^{2,3,12,13,14} These test emulsions were made with water or an aqueous salt solution, kerosene or another hydrocarbon and a synthetic surfactant. Bacterial broths or isolated products were added to these very stable emulsions and the rate of decay monitored.

Several *Corynebacterium*, *Nocardia* and related species were found to be very effective at emulsion coalescence. Initial studies show that the active agents are insensitive to salt addition and pH changes. The broth can also be autoclaved or extracted with organic solvents without significant loss of activity.

The studies with these test emulsions do not necessarily prove that these bacteria will be useful in the field. Preliminary testing with heavy-oil water emulsions gave positive results.¹³ However, the test used involved thinning the emulsions with a significant amount of toluene^{13,15} and thus the results may not be realistic.

SUMMARY

There are an unlimited number of microbial surface active agents providing a large range of different properties. There are also many possible applications of these compounds in the oil industry.

Biosurfactants can be used to release bitumen from tar sand at ambient temperatures. They can significantly enhance the yield of bitumen in a pilot scale

“cold water” separation process.

Biosurfactants could be used for *in situ* oil recovery—either by inoculating the reservoir or by producing the compound on the surface and putting it down the well. If the surfactant is to be produced in place it is necessary to select for organisms which produce good yields of biosurfactants must be improved and the fermentations must use cheaper substrates than hydrocarbons.

Another potential use of microbes is for the coalescence of oil and water emulsions. Preliminary tests have shown that several bacteria can cause almost instantaneous de-emulsification. The active agents show activity at various salt concentrations and pH values and are stable to heat.

REFERENCES

1. D. G. Cooper and J. E. Zajic. 1980. Surface-active compounds from microorganisms. *Adv. Appl. Microbiol.*, 26, 229-253.
2. J. Akit, D. G. Cooper, K. I. Manninen and J. E. Zajic. 1981. Investigation of potential biosurfactant production among phytopathogenic corynebacteria and related soil microbes. *Current Microbiol.*, 6, 145-150.
3. D. G. Cooper, S. N. Liss, R. Longay and J. E. Zajic. 1981. Surface activity of *Mycobacterium* and *Pseudomonas*. *J. Ferment. Technol.*, 59, 97-101.
4. C. R. Macdonald, D. G. Cooper and J. E. Zajic. 1981. Surface-active lipids from *Nocardia erythropolis* grown on hydrocarbons. *Appl. Environ. Microbiol.*, 41, 117-123.
5. D. F. Gerson, J. E. Zajic, D. G. Cooper and M. D. Ouchi. 1977. Microbial separation of bitumen from Athabasca tar sand. *Biochem. Eng. Res. Report*, 3.
6. D. F. Gerson, J. E. Zajic and D. G. Cooper. 1979. Microbial separation of bitumen from Athabasca tar sand. *Biochem. Eng. Res. Report*, 5.
7. D. G. Cooper, J. E. Zajic and D. F. Gerson. 1979. Production of surface-active lipids by *Corynebacterium lepus*. *Appl. Environ. Microbiol.*, 37, 4-10.
8. D. G. Cooper, J. E. Zajic and D. E. F. Gracey. 1979. Analysis of corynomycolic acids and other fatty acids produced by *Corynebacterium lepus* grown on kerosene. *J. Bacteriol.*, 137, 795-801.
9. D. G. Cooper, J. E. Zajic and C. Denis. 1981. Surface active properties of a biosurfactant from *Corynebacterium lepus*, *J. Amer. Oil Chem. Soc.*, 58, 77-80.
10. D. G. Cooper, J. E. Zajic, D. F. Gerson and K. I. Manninen. 1980. *J. Ferment. Technol.*, 58, 83-86.
11. D. G. Cooper, C. R. Macdonald, S. J. B. Duff and N. Kosaric. 1981. Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. *Appl. Environ. Microbiol.*, 42, 408-412.
12. D. G. Cooper, J. Akit and N. Kosaric. 1982. Surface activity of the cells and extracellular lipids of *Corynebacterium fascians* CF 15. *J. Ferment. Technol.*, 60, 19-24.

13. D. G. Cooper, J. E. Zajic, W. L. Cairns and N. Kosaric. 1980. De-emulsification and microbes. Biochem. Eng. Res. Report, 6.

14. W. L. Cairns, D. G. Cooper, J. E. Zajic, J. M. Wood and N. Kosaric. 1982. Characterization of *Nocardia*

amarae as a potent biological coalescing agent of water-oil emulsions. Appl. Environ. Microbiol., 43, 362-366.

15. D. G. Cooper, J. E. Zajic, E. J. Cannel and J. W. Wood. 1980. The relevance of "HLB" to de-emulsification of a mixture of heavy oil, water and clay. Can. J. Chem. Eng., 58, 576-579.

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The practical application of microbial cultures to subsurface oil reservoirs imposes several severe restrictions on the microbial cultures. They must be able to migrate, or be transported, deep within the reservoir for any applications to be of practical significance, and the microbes must be able to multiply in the subsurface environment over which the engineer has no control. Papers were presented at the Conference on laboratory investigations of these unique problems and field applications of mixed cultures that exhibited potentially beneficial properties under laboratory conditions. Finally, one paper reviewed and summarized a large number of field experiments that have been reported in the literature.

Bubela examined the effects of simulated reservoir environmental conditions on microbial cultures. He found that the toxicity to heavy metals increases at elevated temperature; and, since heavy metals are frequently present in reservoir waters, the increase of temperature in the subsurface ($25^\circ + 18^\circ\text{C}/\text{km}$ of depth) might prevent the effective application of an otherwise suitable microbial culture. Therefore, microbes intended for use in a petroleum reservoir should be tested with the reservoir fluids at simulated subsurface environmental conditions.

Bubela observed changes in the morphology of *Bacillus stearothermophilus* when exposed to pressures up to 20,000 kPa in combination with a temperature increase up to 60°C . The bacteria changed from rod-shaped to a spherical form. This change makes the organism more suitable for EOR since the coccoidal forms pass through pore throats more readily.

Marquis remarked that a considerable amount of pilot testing of bacteria selected for EOR is necessary to establish barotolerance and the effects of pressure on the limits of tolerance of bacteria for temperature, salinity, and on growth rate and formation of endospores. He based this on the lack of research on specific species to determine the influence of these environmental conditions that will certainly be imposed on bacteria used for EOR.

In agreement with many others, Marquis noted that some sulfate reducers have been found to be more detrimental to oil field operations than helpful; however, a concentrated study to develop more information on the numbers and types of microorganisms indigenous to oil reservoirs may

result in the discovery of bacteria that are capable of releasing oil when activated by proper nutrients. According to Marquis, these bacteria may have already developed mechanisms to cope with the extremely harsh environments which are normally biologically limited factors.

Marquis noted that low pressure generally enhances the growth rate of bacterial cultures at their optimal growth temperature, but higher pressures reduce the growth rate at all temperatures. Barotolerance has generally been found to be greatest at the optimal growth temperature. However, growth is a very complicated function that is commonly more barosensitive than simpler metabolic functions: for example, sulfate reducers carried out the reduction of sulfate to sulfide at pressures up to 1.5 mPa even though their growth was completely inhibited at one half that pressure. Marquis concluded that growth is retarded or even stopped by extremely high pressure, but simpler metabolic activities continue, although at a slower rate.

M. Gula and Sewell isolated a facultative bacterium along with *Desulfovibrio* from crude oil and brine samples from a depth of 1,000 meters in the Wilmington oil field, Long Beach, Calif. The facultative organism is a Gram-negative rod, motile, non-spore forming, and produces a non-diffusible biopolymer. It showed a peculiar synergistic relationship to *Desulfovibrio*; when grown in close proximity to the facultative organisms, *Desulfovibrio* exhibited a more rapid growth rate and enhanced production of hydrogen sulfide. The implications of the association of this microbe with *Desulfovibrio* are very significant to petroleum recovery. It may result in an increase of corrosion, destruction of EOR chemicals, and resistance to biocides.

M. Gula and Sewell reported that the *Desulfovibrio* bacteria are stimulated in growth by polyacrylamide polymers used for EOR and the bacteria cause a significant reduction of the viscosity of polymer solutions. The mechanism by which the molecular size of the polymer solution viscosity is reduced has not been elucidated.

Certain strains of *Pseudomonas* also were found by M. Gula and Sewell to release measurable amounts of ammonia from solutions of polymer. This could be due to enzyme action, but the enzyme involved was shown not to be the low molecular weight aliphatic amidase found in

many wild-type strains of *Pseudomonas aeruginosa*. Another strain of *Pseudomonas* isolated from an oil field sand-polyacrylamide mixture was shown to be markedly stimulated in rate of growth during the early phases of the growth cycle by the presence of polyacrylamide.

Zhang and Qin stated that because the oil fields that have thus far been discovered in China are very heterogeneous and contain viscous oils, the approach to MEOR in China has been the development of microbial cultures that can utilize crude oil hydrocarbons to make bioproducts which may aid in recovery of oil as waterflood additives, preparation of drilling fluids, and other oil field operations.

They have found that aerobic fermentation of crude oil yields a biosurfactant which readily forms a microemulsion with crude oil and water which has a greatly reduced viscosity compared to the crude oil. When the products of fermentation are mixed with crude oil having a viscosity of 2,500 centistokes, in a 1:1 ratio, the viscosity of the resulting mixture is between 12 and 46 centistokes. Zhang and Qin postulated that biosurfactants of this type will aid in recovery of residual oil when added to injected water which will be the emphasis in the future. The properties of the biopolymer discussed by Zhang and Qin are very similar to those of the glycolipid discussed by Singer et al.

Zhang and Qin briefly described a second microbial product which they called a thickening agent because its intended use is to plug high permeability zones by injection of this product. It is a viscous polysaccharide obtained from a Gram-positive, non-spore forming, rod with a tendency toward V-shaped association. They have tentatively named the microbe *Cornynebacterium gummi-ferm*. The bacteria produce copious yields of polymer from mixed paraffins as their sole carbon source, but show a considerably reduced yield when grown with a crude oil substrate.

The thrust of future work in MEOR in China is to develop technology for the production of the biosurfactant and biopolymer in the oil field where they will be used as additives to injection water.

MEOR research in Romania was carried from the laboratory stage directly to field trials with work starting in 1972 by Lazar et al. The work began with a concentrated effort to isolate bacteria that were adapted to reservoir conditions. This began by isolation of bacteria from produced water, but the search was widened to include well bottom-hole mud, soils around oil wells, food processing wastes, and others.

Oil displacement experiments showed conclusively that the microbial cultures obtained from

produced waters and sugar processing plants were more effective. The mixed cultures were adapted to reservoir conditions and increased to concentrations of 10^7 - 10^9 bacteria/ml. Species identified in these mixed cultures were: *Pseudomonas*, *Escherichia*, *Arthrobacter*, *Mycobacterium*, *Micrococcus*, *Peptococcus*, *Bacillus* and *Clostridium*. These mixed cultures were more efficient in releasing oil than the pure strains or bacteria obtained from other sources.

Seven oil fields in Romania were inoculated, but only two of the oil fields responded with an increase of production ranging from 16-200 percent. The well with the 200 percent increase produced 6.7 m³/month over a period of several years and then rose to 20.0 m³/month average production after inoculation of the field. The increased oil production continued for more than one year and is currently rising. Lazar attributes the failure of the other five reservoirs to respond to microbial treatment to heterogeneity, low permeability, and high salt concentration.

Monitoring of the microbial population in the produced waters after inoculation showed that about six months after inoculation the microbial count began to increase from 10^4 /ml to 10^6 - 10^9 /ml while a nutrient solution of 2 percent molasses was being injected constantly. One year after the nutrient injection stopped, the microbial counts in the produced waters dropped to 10^3 - 10^4 /ml. The results indicate that the inoculated microbial culture multiplied in the reservoir and migrated from the injectors to the producing wells.

Yarbrough et al described a successful field test using *Clostridium acetobutylicum* in a two-spot pattern with 120 m between wells. The formation is a loosely consolidated sand of high permeability containing up to 16 percent carbonates at a depth of 700 meters. Core tests indicated a maximum residual oil saturation of 8.5 percent and the test well production averaged 3 m³/month prior to the inoculation.

Injection of a 2 percent molasses solution and bacteria commenced in July 1954 and was continued until November at an average rate of 25 m³/D; a total of 800 liters of bacteria culture was injected in this period. In October, 3 months after the initial injection, significant changes occurred at the production well. The oil recovery increased from 3 to 10 m³/month and continued through the duration of the test (May 1955). Production of acids, carbon dioxide and methane also occurred at the production well in October 1954. The cumulative production of acid and CO₂ over the test period was 35,000 kg of acids and 5,700 m³ (11,000 kg) of carbon dioxide. No quantitative measurement of the methane production was made. Hydrogen was not detected, and the authors believe that it may have been used by other

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bacteria in the reservoir and was involved in the production of much of the methane.

In considering the application of MEOR to North Sea reservoirs, Moses et al and Springham et al conducted experiments to determine the feasibility of using the crude oil itself as the substrate for anaerobic bacterial growth. The North Sea wells are widely spaced (1 km or more) from isolated platforms where the wells fan out radially from the platform. Thus, according to Moses, the injection of nutrients to support a microbial flora that could penetrate from injectors to the widely spaced producers is not possible since the bacteria would consume the substrate long before it could migrate far enough to be effective.

Therefore, they have conducted numerous experiments under anaerobic conditions with crude oil as the sole carbon source. Microbial cultures were obtained from a variety of industrial and natural locations and they were maintained for many months at 28°C under an inert gas with a mineral medium and crude oil. Gas and protein production was monitored. Carbon dioxide and protein concentrations rose slowly in all cases; thus these experiments show that anaerobic microbial growth on crude oil is possible and it may be possible to develop a technology for MEOR in the North Sea. Moses will concentrate on finding cultures that produce metabolic products at higher rates and elucidation of the mechanisms and microbial taxonomy.

Hitzman reviewed all other published literature on field application of microbial enhancement of oil recovery. More than 200 field tests have been conducted since 1954, but only about 50 are documented in sufficient detail for good comparison of the results. In most cases, the wells that were inoculated with bacteria for MEOR were of very poor quality because of the conservative attitude of most oil companies toward the injection of bacteria which have a known potential for plugging wells and for causing entire oil fields to go sour, due to microbial production of hydrogen sulfide *in situ*.

Hitzman noted that as experience was gained in field applications of microorganisms, the techniques became more complex and technically advanced. They began with simple inoculation of single wells without prior preparation to more complex injection of a low salinity water, injection of various nutrients, establishing a fermentation period for cell growth, etc. In some cases the MEOR was reduced to a well clean-out procedure and in others the objective was the long term stimulation of a large portion of the reservoir.

Hitzman developed 12 general conclusions from his review, and perhaps the most important are that (1) in several reservoirs a positive response to MEOR was obtained, (2) *in situ* microbial growth results in chemical and petrophysical changes in the reservoir, and (3) temperature and pressure conditions are not as restrictive to MEOR as laboratory experiments indicate.

Combined Effects of Temperature and Other Environmental Stresses on Microbiologically Enhanced Oil Recovery

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INTRODUCTION

The program of our Laboratory, associated with microbiologically enhanced oil recovery, is concerned with the operative conditions present in reservoirs. Any organism to be effective *in situ* in Microbiologically Enhanced Recovery (MEOR) has to be able to produce surfactants and/or viscosifiers at pressures and temperatures encountered in reservoirs. Practical biologically acceptable temperature limits are about 80°C and the hydrostatic pressures in Australian reservoirs are about 20,000 kPa.

Two major factors are involved in the techniques of MEOR:

1. Biological production of surfactants and/or viscosifiers.
2. Distribution of the organisms and their metabolic products through the reservoir.

Surfactants and viscosifiers capable of increasing significantly crude oil recovery may be biologically produced:

1. In industrial installations on the surface.
2. *In situ* in the reservoir.

We considered a surface installation less likely to be suitable for microbiologically enhanced oil recovery because of extensive financial and energy expenditure, limited volume capacity relative to the size of the reservoir, difficulties in distributing dense bacterial population uniformly through a reservoir and problems of distributing a bacterial culture and its products from an inflexibly located installation.

In considering the *in situ* technique which requires the biological activity to take place within the reservoir, a number of factors affecting the organism have to be taken into account. The organisms have to be active at biologically acceptable high temperatures up to 80°C, pressures of 20,000–30,000 kPa, at high electrolyte concentrations, in the presence of heavy metals, at low or zero atmospheric oxygen concentration, and to be able to utilize a readily available substrate while producing one or more substances having surface activity and/or being capable to affect favorably the mobility ratio of the oil/water system.

The total effect of the factors influencing biological activity in reservoirs is not equivalent to the sum of the individual effects caused by a variety of factors. Many of the factors interact and their activities may be mutually enhanced or hindered.

Biologically high temperature environments are those with temperatures higher than 50°C. In some cases microorganisms living at such temperatures are classified as:

1. Caldoactive with a maximum growth temperature above 70°C, optimum above 65°C, and minimum above 40°C.
2. Thermophilic with a maximum growth temperature above 60°C, optimum above 50°C and minimum above 30°C.

A number of oil reservoirs would provide the environmental temperature as indicated for the caldoactive range. A number of reviews have been published regarding thermophilic and caldoactive organisms.^{1,14} In the majority of cases the literature cited refers to the effects of elevated temperatures only, without taking any other parameters into consideration. The combined effects of heavy metal toxicity and elevated temperatures is not discussed frequently. An increase in thermotolerance with an increase of pressure is discussed by Morita et al¹¹ who studied the heat inactivation of malic dehydrogenase and inorganic pyrophosphatase at elevated pressures in cell free system.

Marquis⁹ observed that microorganisms usually show their maximum barotolerance at temperatures just above their optimum growth temperature. Bubela (unpublished results) observed a 10 fold increase in the production of H₂S by *Desulfotomaculum denigrificans* when the organism was grown at 10,000 kPa and 70°C than when it was grown at 55°C and 100 kPa. The increase in H₂S production was observed even when a cell-free preparation was used. The enhancement was 3 times higher than during an experiment where the organism was grown at 60°C at atmosphere pressure.

The observation of an increase in growth as detected by monitoring the turbidity of the growth media as reported by Marquis and Maturma¹⁰ has to be

evaluated cautiously as Bubela (unpublished results) observed considerable effect of pressure on the morphology of microorganisms resulting in a different light dispersion pattern at the same bacterial concentration. This paper describes some of the interactions between biologically high temperatures and other factors likely to be encountered in reservoirs.

METHODS AND RESULTS

Effect of temperature, metal toxicity

The toxic effect of heavy metals on microorganisms, in particular by copper has been known and studied for more than 50 years. The mechanism of the toxicity is not known to its full extent even today. Morphological changes, increase in osmotic fragility, decrease in cellular cytochrome and magnesium content, production of previously undetected metabolites, changes in the amino acid composition of bacterial cell walls and changes in X-ray diffraction patterns of cell-wall macropolymers are some of the changes reported.^{2,8,12,13,15}

To evaluate some of the aspects of heavy metals—temperature effect on the growth of microorganisms, *Bacillus stearothermophilus* strain NCA 1503-4 was examined with copper present in the growth medium. The organism was propagated in an apparatus described previously by Bubela and Oberhauser⁷ in a liquid medium containing amino acids, inorganic salts and glucose.⁵ Copper was added as cupric acetate. The growth temperature varied depending on the experiment.

When *B. stearothermophilus* was grown at 53°C a growth pattern presented in Fig. 1A was observed. The mean generation time was 33 min. When the temperature of growth was elevated to 63°C (Fig. 1A2) the mean generation time was decreased to 24 min. If copper was added to the growth medium during early log phase, the growth pattern was interrupted for several hours but the organisms eventually recovered and resumed their normal growth with the same mean generation time of 33 min (Fig. 1B1). At a growth temperature of 63°C the addition of copper inhibited the growth of the organisms completely (Fig. 1B2).

If magnesium was present in the medium prior to the addition of copper, the bacterial growth at 53°C was not delayed as in B1, but proceeded immediately at a reduced rate, the mean generation time increasing to 57 min (Fig. 1C1). At 63°C in the presence of magnesium the copper inhibited the growth for several hours. The organisms recovered eventually but its mean generation time has increased to 99 min. The small but sharp apparent increase of optical density on addition of copper was due to the slight increase in color of the medium.

It is evident that the copper was much more toxic to the organism at the higher temperature. The antagonistic effect of the magnesium on the copper toxicity was much less pronounced at 63°C than at 53°C. No significant differences in the copper and magnesium contents of the whole washed freeze dried and over P₂O₅ stored cell was detected, when the organisms were grown in the standard or copper/magnesium enriched medium.

Irrespective of the growth temperature the average copper content in the copper poor medium was 30 μ/g

of dried biomass; in the copper spiked medium the content was 3800 μ/g, while the corresponding figures for magnesium were 270 μ/g and 750 μ/g respectively. This observation shows that the growth temperature has no significant effect on the metal accumulation in the cell and therefore that the higher toxicity at 63°C must be due to parameters other than metal accumulation.

Bacterial growth in the presence of copper depended significantly on the concentration of available oxygen. The lower the oxygen tension, the higher was the copper toxicity. This phenomenon has been discussed before by Bubela.³

A number of propositions may be offered to explain the increased copper toxicity at 63°C:

1. Lowering of oxygen due to its decreased solubility at higher temperatures. The solubility of O₂ at 63°C is only 20 percent less than that at 53°C. The organism was able to grow at 50 percent of the original oxygen saturation in the presence of copper. Therefore it is improbable that the toxicity increase was due to a lack of oxygen.

2. It was shown by Bubela and Holdworth⁵ that some of the metabolites in thermophilic organisms have an extremely high turnover rate. Therefore an interference with mechanisms responsible for such a turnover could result in an increase in toxicity at higher temperatures.

The actual copper distribution through the bacterial cell is hard to estimate as the copper binding sites are difficult to determine, since upon the disruption of the cell the copper becomes rapidly redistributed amongst the cellular fractions.¹³

The possibility of the copper being preferentially present as Cu⁺ or Cu⁺⁺ depending on the temperature was eliminated as electron spin resonance technique indicated that at least 99.9 percent of the total copper is at all the time in the Cu⁺⁺ form. The possibility that at least traces of the copper are present as Cu⁺ cannot be excluded. As metal toxicity varies frequently with the oxidative state of the metal and a partial reduction of copper at the elevated temperatures could occur due to the presence of oxidizable organic matter, this possibility cannot be eliminated.

Bacterial response to temperature variation

B. stearothermophilus was grown on a simple medium composed of salts, glycerol, sodium glycerophosphate and glucose (Bubela²) and on the complex medium described in the previous section at 53°C and a variety of elevated temperatures. The duration of the lag and log phases, and the biomass expressed in mg dry weight at the end of the log phase were measured.

Mean generation time, number of generations per log phase, and biomass per generation per millilitre medium at the end of log phase (expressed in mgm dry weight) were calculated from such measurements. The relationship between the temperature and the duration of the lag and log phases respectively are presented in Fig. 2A. The minimum lag value corresponds to a temperature of about 50°C and the minimum log phase value was obtained at about 56°C. The calculated values for mean generation times as plotted against temperature are presented in Fig. 2B.

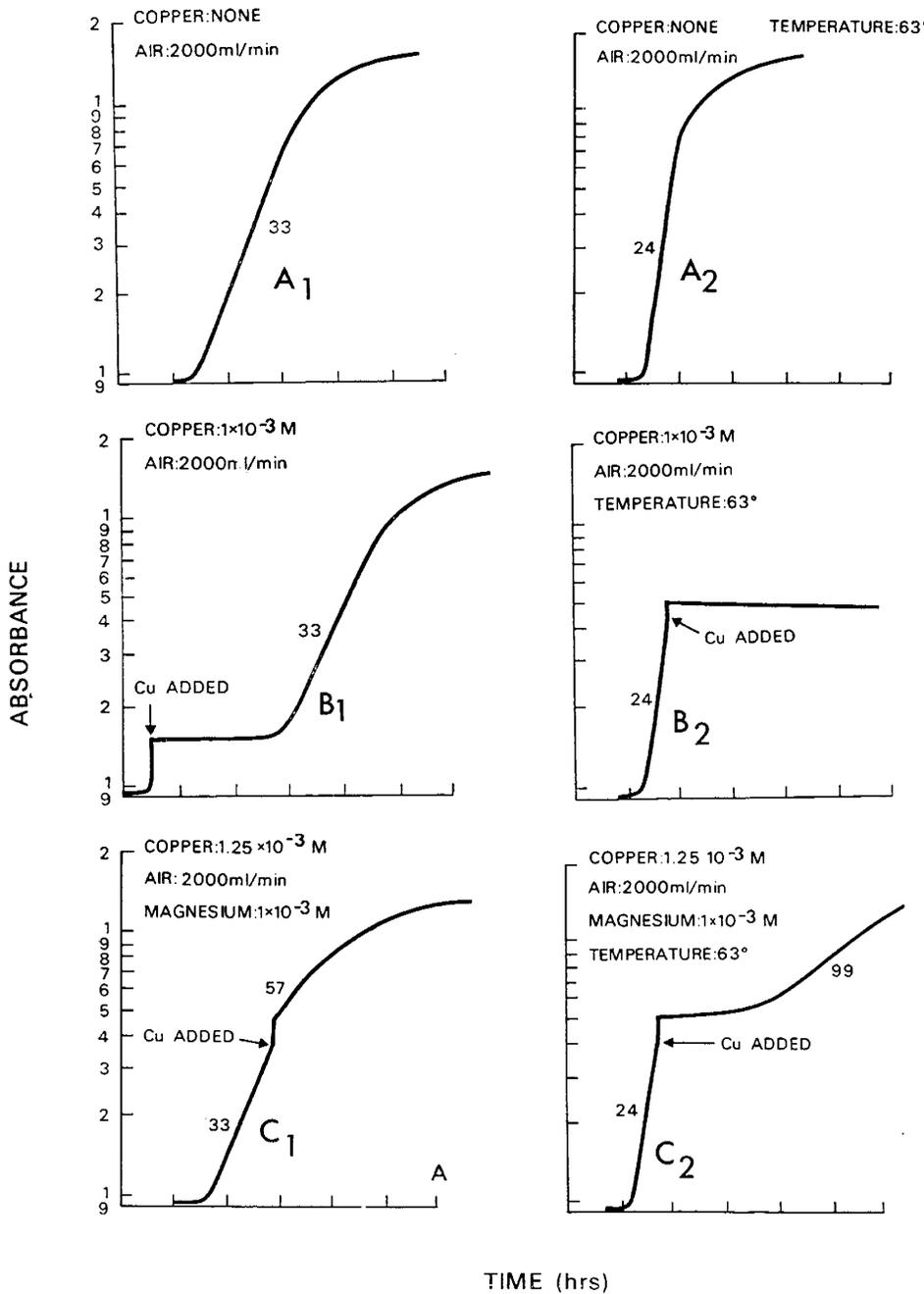


FIG. 1—Effect of temperature on Cu-Toxicity to *B. stearotherophilus*: A₁; A₂: Growth curves at 55°C and 63°C respectively. B₁; B₂: with copper added. C₁; C₂: with copper and magnesium.

The number of generations was plotted against the temperature, against dry weight per millilitre of medium at the end of log phase, as well as against the apparent dry weight per generation per millilitre of medium. The corresponding graphs are presented in Fig. 2C. The number of generations and the dry weight decreased with the experimental temperature but the weight per millilitre per generation was constant.

An inhibition test where the organisms were grown at temperatures ranging from 42°C up to 69°C, the organisms removed by centrifugation and the medium sterilized by a double filtration through a millipore

filter (0.45 μ), spiked with its components to their original concentrations and freshly inoculated, has shown that no inhibitory factor was produced during the growth at temperatures up to 69°C.

The organisms grew well on agar plates containing the complex or the simple medium or where silica gel was used instead of the agar at temperatures of 53°C. The organisms did not grow on the simple medium above 59°C but colonies were observed up to 69°C with the complex medium.

The results indicate that this organism can grow in the absence of relatively complex organic molecules.

The possibility that the organisms assimilate some components present in agar was eliminated by the observed growth on silica gel.

The possibility that a thermosensitive mutant was developed during the experimental work was eliminated by the observation that when replicas of the organisms were made from the simple medium in combination with agar or silica gel onto the complex medium agar plates, the same number at corresponding locations was observed at temperatures above 60°C.

It is evident from the above observations that despite the fact that the organism was able to live on a relatively simple substrate, some specific material has to be supplemented at higher temperatures.

It was shown by Bubela and Holdsworth (1966b⁶) that a high turnover of proteins and nucleic acids takes place in *B. stearotherophilus* requiring a high balanced rate of catabolic and anabolic processes. Should the rate of catabolic processes taking place at high temperatures exceed the anabolic processes responsible for the synthesis, a synthesis of a particular metabolite usually present in sufficient concentration, may be decreased. Then the concentration of such a metabolite may become a factor limiting the bacterial growth.

The enzymes or enzymic systems involved in the metabolisms of the cell may be labile at the elevated temperatures. Therefore at extreme temperatures the cell would lack an endogenous supply of components, usually produced by such enzymes unless supplied from the medium. It is possible that both mechanisms are present and operative concurrently.

Combined effect of temperature and pressure

As the average hydrostatic pressure of Australian reservoirs is in the vicinity of 20,000 kPa, an apparatus capable of sustaining a continuous growth of organisms under anaerobic conditions, operative at pressures up to 25,000 kPa and temperatures up to 150°C based on the principles of a chemostat was constructed (Bubela 1981⁴).

To investigate simultaneous effects of pressure and temperature, an anaerobic, rod shaped organism 6–8 μ long and 3–4 μ wide, producing a metabolite capable of reducing interfacial tension of the oil/water system, was isolated from Melbourne sewage plant's anaerobic digestors. Under atmospheric pressure the optimum temperature of this organism was 50°C with a mean generation time of 17 hr.

When a pressure of 20,000 kPa was introduced dur-

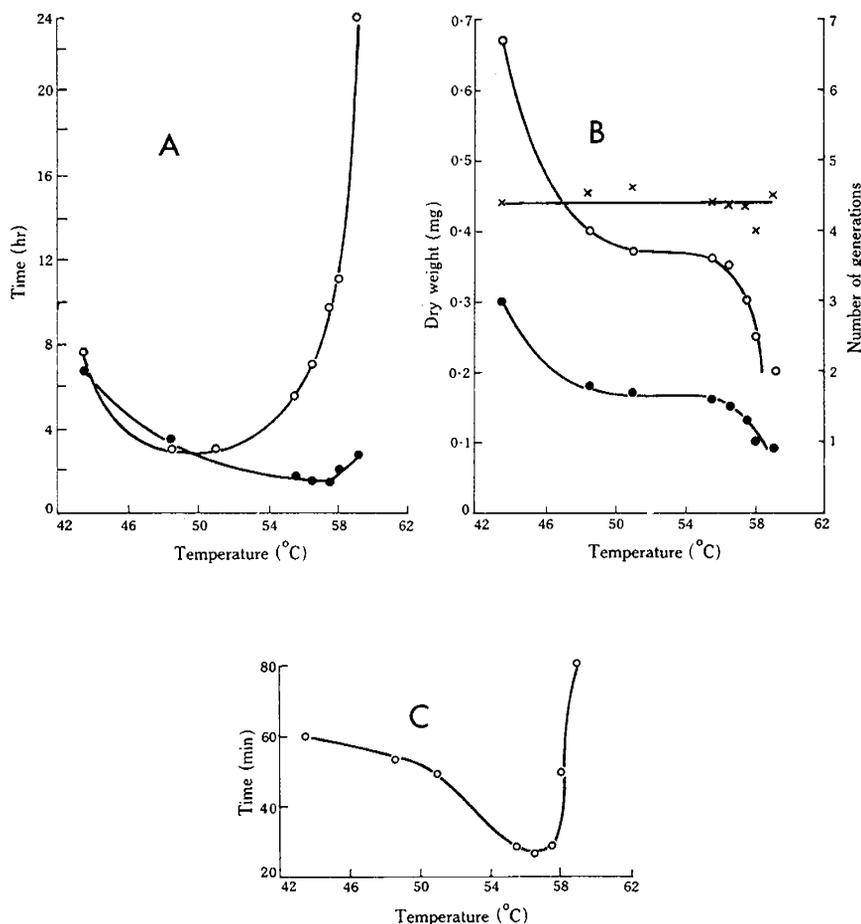


FIG. 2—Effect of temperature on growth characteristics of *B. stearotherophilus*: A: duration of the lag (○) and log (●) phases plotted against the experimental temperature. B: number of generations (○), the maximum dry weight at the end of the log

period in mg/l (●), and the dry weight at the end of the log in mg/l (○), and the dry weight per generation/ml (x), plotted against the experimental temperature. C: calculated mean generation times plotted against the experimental temperature.

ing the growth period, a maximum growth was obtained at 65°C with MGT of 12 hr. As the organisms were grown on a complex medium (Bubela⁵), no additional nutritional requirements at high pressures were observed. On exposure to the high pressure the morphology of the organism changed to a coccoidal form of about 5 μ in diameter.

When depressurized suddenly, the organisms disintegrated. If the pressure was released gradually over a period of 24 hr the organisms regained their original rod shape. No gas vacuoles which could have been responsible for the rupture of the cell were observed any time during the growth period. This organism capable of growing apparently normally at sodium chloride concentrations up to 3 percent, did not grow at this salt concentration at pressures above 15,000 kPa.

Effect of temperature on biological alteration of crude oil

Literature reports on the effects of biological activity on the composition of crude oil are frequent. Practically all the cases report changes occurring at ambient temperatures, and in the presence of atmospheric oxygen. The conditions in oil reservoirs differ from the surface environment by e.g. elevated temperatures, pressure and limited amount of available oxygen.

When samples of Australian crude oil were exposed for 3 months at 30°C to biological activity by an anaerobic microbial consortium, no significant differences were observed in the content and composition of aliphatic and aromatic hydrocarbons. Neither were changes observed when an imported heavy oil with an asphaltene content of 5 percent was used despite the fact that a viable microbial population was detected at the end of the experimental period.

When the experimental temperature was elevated to 60°C, the results obtained with the low asphaltene oils remained the same, but the amount of material isolated as asphaltenes from the imported oil rose on average up to 8 percent. Analytical results indicated a number of components in the 2,000-2,500 Daltons range with frequent ester bonds. No such a material was detected in the absence of the biological activity at 60°C. No significant changes were observed in the absence of the organisms.

DISCUSSION

The results of the experimental work described in this paper indicate the necessity of evaluating a microorganism for its suitability for MEOR by exposing it simultaneously to as many environmental stresses likely to be encountered in a reservoir under consideration, as possible.

An elevated temperature, which may otherwise be quite tolerable to the organisms may become a limiting parameter in the presence of heavy metals. In cases where the toxic effect of a metal may be counteracted by an increase in the concentration of another element, an example being the antagonistic couples of copper-magnesium and copper-manganese (Bubela²), such an antagonistic interaction may become ineffective at elevated temperatures.

As heavy metals are frequently present in reservoir waters, biologically high temperatures, in combination with the presence of heavy metals, may prevent the

effective application of an otherwise suitable organism.

An increase in environmental stresses e.g. temperature may affect the nutritional requirements of an organism. Propagation of a suitable organism in a reservoir may require the addition of economically and technologically acceptable substrates.

We have observed that some substrates become deficient in supporting bacterial growth, when the growth temperature of the medium is elevated. Additional supplements have to be provided.

In some cases (e.g. with whey) the substrate may produce a significant amount of solids at elevated temperatures and pressures encountered in a reservoir, thus decreasing the permeability of the reservoir and interfering not only with the distribution of the organism through the reservoir rock, but hindering the movement of reservoir fluids as well.

The morphology of an organism to be used in MEOR plays a significant role in its suitability. Our work with simulated systems shows that the average pore size/bacterial cell size ratio is very important in producing pore plugging.

The bacterial shape is important as well. Rod-shaped organisms have the tendency to accumulate at pore throats and by interlocking at this location they produce plugs difficult to remove even by an increase of pressure of several thousands of kPa. Coccoidal forms, on the contrary, pass through the pore throats more readily and should plugging occur, it may be usually cleared by a moderate increase in pressure.

The morphology of an organism is liable to change significantly when such an organism is exposed to environmental stresses. Changes due to the presence of heavy metals have been described by Bubela.² Our work indicates that in some cases the combination of elevated temperatures and pressure may cause a change from a rod shape to a spherical form, thus making a given organism more suitable to MEOR, than in its original form.

It is evident from the examples cited that a satisfactory production of a surfactant and/or viscosifier under laboratory conditions does not necessarily make an organism a suitable candidate for MEOR.

CONCLUSION

The final evaluation of the suitability of a microorganism for MEOR has to be based on experimental work conducted as closely as possible under reservoir conditions, in order to obtain a meaningful assessment of the organism under stresses likely to occur in selected reservoirs.

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REFERENCES

1. Ameluxen, R. E., and Murdoc, L. A., 1978, in D. J. Kushner (ed.) *Microbial Life at Extreme Environments*. Acad. Press London, New York, and San Francisco, pp. 217-278.
2. Bubela, B., 1970, Chemical and Morphological Changes in *Bacillus stearothermophilus* induced by Copper. *Chem.-Biol. Interactions* 2 107-116.
3. Bubela, B., 1973, Effect of Copper on the Growth of *Bacillus stearothermophilus*. *Zbl. Bact. Abt. 11, 128*, 274-284.
4. Bubela, B., 1981, An apparatus for continuous growth of microorganisms under oil reservoir conditions. *Bull. Am. Assoc. Petroleum Geol.* (in press).
5. Bubela, B., and Holdsworth, E. S., 1966,^a Amino acids uptake, protein, and nucleic acids synthesis and turnover in *B. stearothermophilus*. *Biochim. biophys. Acta*, 123, 364-371.
6. Bubela, B., and Holdsworth, E. S., 1966,^b Protein synthesis in *B. stearothermophilus*. *Biochim. biophys. Acta*, 123, 372-376.
7. Bubela, B., and Oberhauser, D. F., 1966, An automatic apparatus for inoculation, growing and harvesting of microorganisms. *Biotech. bioeng.*, 8, 453-455.
8. Bubela, B., and Powell, T. G., 1973, Effect of copper on the composition of bacterial cell wall peptides. *Zbl. Bakt. Abt. II, 128*, 457-466.
9. Marquis, R. E., 1976, High pressure microbiological physiology. *Adv. Microbiol. Physiol.* 14, 159-241.
10. Marquis, R. E., and Matsamura, P., 1978, in D. J. Kushner (ed.) *Microbial Life at Extreme Environments*. Acad. Press, London, New York, and San Francisco, pp. 105-158.
11. Morita, R. Y., and Mathemeier, P. F., 1964, *J. Bacteriol.* 88, 1667.
12. Moronch, G., 1958, Untersuchungen Ueber die Physiologische und Morphologische Wirkung von Kupfer auf *Mucor advenitus* var. *aurantiacus*. *Arch. Mikrobiol.*, 30, 231-255.
13. Sadler, W. R., and Trudinger, P. A., 1967, The inhibition of organisms by heavy metals. *Mineralium Deposita*, 2, 158-168.
14. Tensey, M. R., and Brock, T. D., 1978, in D. J. Kushner (ed) *Microbial Life at Extreme Environments*. Acad. Press, London, New York, and San Francisco, pp. 159-216.
15. Weed, L. L., and Longfellow, D., 1954, Morphological and biochemical changes induced in a population of *E. coli*. *J. Bacteriol.* 67, 27-33.

Barobiology of Deep Oil Formations

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Hydrostatic pressure is considered to be a cardinal ecological factor in two major regions of the biosphere—the deep ocean and the deep earth. Of course, we should realize that hydrostatic pressure is a cardinal environmental factor for all living organisms, including the human species, and all of our enzymes and physiological systems are specifically designed to function efficiently at a pressure of about one atmosphere. (One atmosphere = $14.70 \text{ lb/in.}^2 = 1.033 \text{ kg/cm}^2 = 101.3 \text{ kPascals}$.)

The first signs of pressure problems with humans subjected to compression occur in the form of the high pressure nervous syndrome at about 50 atm. The syndrome can be alleviated, at least partially, by adding narcotic gases to the compressed helium-oxygen mixtures used to apply pressure without at the same time collapsing the lungs.

Study of the biology of the deep ocean has progressed steadily for more than a century and has included a voyage by J. Piccard and D. Walsh in the bathyscaph *Trieste* to the bottom of the Challenger Deep in the Pacific Ocean (Piccard and Dietz¹⁸), where the depth is some 11,600 m, and the pressure due to the weight of the water column is some 1,160 atm.

Even these hadal depths of the ocean have a flora and a fauna adapted to life at very high pressures and the low temperature of only about 2°C. The combination of high pressure and low temperature in the deep ocean makes for a highly restrictive environment biologically and a need for compromises in biomolecular design that lead to inherently slow reaction rates. Life in the deep ocean moves slowly.

The microbial flora of the deep earth has been only very sketchily studied, and there is clearly need for a prolonged, systematic characterization of the organisms in this frontier of the biosphere. The restrictive elements of the deep earth include high pressure and high temperature.

The mean pressure gradient is somewhat in excess of 1 atm per 10 m of depth, although in overpressurized strata the gradient may be as great as 2.3 atm per 10 m. The depth for maximum recovery of oil currently is about 2,000 m and is expected to increase to at least 2,400 m or even 3,000 m.¹¹ Therefore, the pressures most pertinent in terms of the petroleum microbiology of presently productive zones are in the range of 200 to 300 atm. Of course, in terms of enhanced recovery, we must consider a range from only slightly greater than

one atm to as high as 500 atm, at the predicted limiting depth for liquid hydrocarbons.

Certainly, pressures of 200 to 300 atm have significant effects on the activities of microbes. When other environmental conditions, especially temperature and pH, are not optimal, microbes become hypersensitive to pressure, and pressures as low as 50 atm can completely stop growth.¹⁴

Perhaps we can put some of these generalities into more specific terms by considering the activities of the group of microbes often considered to be the bane of the petroleum industry—the sulfate reducers. Certainly, it seems that these anaerobes are among the least desirable bacteria associated with oil formation, although attempts were made to use them for enhanced recovery, and one of the first demonstrations in the laboratory that microbes actually liberate oil from sand was that of ZoBell with sulfate reducers.

Some of the sulfate reducers appear to be among the most barotolerant and thermotolerant bacteria studied to date. ZoBell²³ reported growth of an isolate from the deep earth at a pressure of some 1,000 atm and a temperature of 104°C. A recent report by Heinen and Lauwers¹⁰ indicates that other thermophiles can grow at 105°C at a pressure of only a few atm. In all, it seems that we must consider 105 to 110°C as maximal temperatures for bacterial growth, and 1,000 to 1,400 atm as maximal pressures for growth.

As Postgate¹⁹ has pointed out, the thermophilic sulfate reducers are generally strains of *Desulfotomaculum nigrificans*, which usually grows best at about 55°C. We have in the past determined the barotolerance of the type strain of this organism and found that it had moderate barotolerance, i.e., it was capable of growing at pressures of 300 to 400 atm at its optimal growth temperature. Recently, Rozanova and Nazina²⁰ have described a thermophilic, nonsporulating *Desulfovibrio thermophilus* isolated from the Apsheron Peninsula oil deposits at a depth of 3,100 to 3,300 m and a temperature of 84°C.

They also isolated *D. nigrificans* from gas and oil bearing strata of Western Siberian deposits. From one of the gas bearing strata, at a depth of 1,600 to 1,620 m and a temperature of 59°C, they obtained a salt tolerant subspecies designated *salinus* that could produce H₂S at NaCl concentrations as high as 4 percent (ca. 0.7 M). This salt tolerance is not great in relation to the salt concentrations of reservoir brines, which generally

have NaCl concentrations in the range 0.5 to 1.5 M with maxima approaching 6M.⁴ Rozanova and Nazina²⁰ concluded, however, that high temperature limited the occurrence and activities of these species.

The adaptations of thermophiles and thermotolerant bacteria to high temperature do not also make them more pressure tolerant than mesophilic or psychrophilic bacteria. In fact, it appears that temperature adaptation has little influence on barotolerance, as one might conclude from a review of the effects of temperature and pressure on the chemical bonds important for biopolymer stabilization.¹⁴ Figure 1. presents representative data on the temperature ranges for growth of the thermophile *Bacillus stearothermophilus*. The pattern seen here is a typical one for bacterial growth. Low pressures, here some 132 atm, generally enhance growth at the optimal temperature and shift the optimum by a few degrees to higher temperatures. Still higher pressures inhibit growth at all temperatures and tend to narrow the range of temperatures over which growth occurs.

It is also generally true that barotolerance is greatest at the optimal growth temperature, or a few degrees above it, and that higher and lower temperatures reduce barotolerance. Basically, the ideal gas law does not apply to bacterial growth in condensed systems, and one can predict that bacteria functioning at high temperatures in deep oil wells would be hypersensitive to pressure.

Kuznetsova and Pentskhava^{12,13} concluded that salinity or the balance of divalent to monovalent cations limited the activities of sulfate reducing bacteria in oil formations. Indeed, Dostalek and Kvet⁶ have even

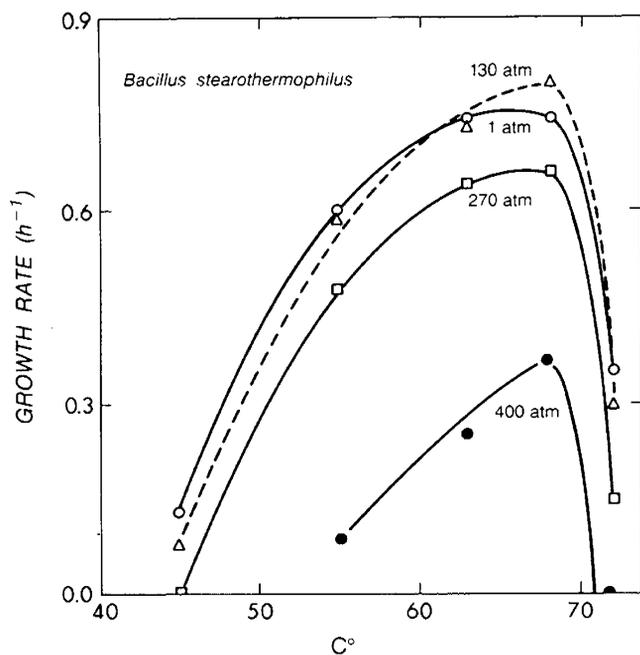


FIG. 1—Effects of pressure and temperature on the growth of *Bacillus stearothermophilus* in tryptone-glucose-Marmite broth plus 1 percent (w/v) KNO_3 . Growth was assessed in terms of culture optical densities for light of 700 nm wavelength. Procedures for pressurization of cultures have been described previously (Marquis, 1976).

suggested that it might be possible to distinguish indigenous from introduced organisms in reservoirs on the basis of salt tolerance.

However, bacteria can become physiologically adapted to growth in saline environments. In other words, there are facultatively halotolerant bacteria, especially among the gram-positive bacteria. Moreover, tolerance may actually be increased by hydrostatic pressure, as we have shown for *Bacillus licheniformis*.¹⁵

One of the less desirable activities of sulfate reducing bacteria is in the corrosion of metals. Willingham and Quinby²² have assessed the effects of increased pressure on attack rates and amounts of corrosion of iron, stainless steel and aluminum caused by three strains of marine sulphate reducers. They found enhanced corrosion of ingot steel coupons at 200 atm, compared with 1 atm, and greatly reduced corrosion at 600 atm.

The bacteria did not grow at 600 atm at the experimental temperature of 20°C. At a higher temperature of 40°C, corrosion without apparent growth of the bacteria occurred at 600 atm, but the corrosion was less extensive than that at 200 or 1 atm, at which both corrosion and growth occurred. Corrosion of aluminum under that same conditions was enhanced at 200 atm but reduced at 600 atm. Stainless steel, 316 type, did not corrode appreciably at any of the pressures. Unfortunately, therefore, it appears that the pressures of deep oil wells could be expected to enhance corrosion rather than retard it.

The finding of corrosion at pressures above the maximum growth pressure can be readily interpreted. Growth is a very complicated function that is commonly more barosensitive than other simpler functions such as sulfate reduction. ZoBell²⁴ reported that washed suspensions of sulfate reducers could carry out reduction of sulfate to sulfide at pressures as high as 1,400 atm, even though their growth was completely inhibited by pressures well below this level.

In fact, sulfate reduction was maximal at 1,000 atm, well above the maximum growth pressure. In general, catabolic functions, including glycolysis, nitrate reduction, urease activity, dehydrogenase activity and oxygen reduction, all are less sensitive to pressure in a wide variety of bacteria than is growth.

In contrast, some functions are more sensitive to pressure than is growth. For example, the production of flagella by *Escherichia coli* can be completely suppressed by pressures of only a little over 100 atm at 37°C. At this temperature, the bacterium is able to grow at pressures as high as 550 atm. The maximum pressure for motility with previously formed flagella was found to be about 400 atm.¹⁷ With this same organism, we have found¹⁶ that derepression of the *lac* operon also is highly barosensitive and can be completely suppressed by pressures of about 300 atm at temperatures at which growth can occur at some 500 atm. In general, various regulatory systems show a range of sensitivities to pressure.

The formation of bacterial endospores appears also to be generally more barosensitive than is growth but still can occur in many *Bacillus* species at pressures of some hundreds of atm.⁹ As far as I am aware, there is

no information concerning sporulation by bacteria such as *Desulfotomaculum* in deep oil formations or on the roles of these resistant forms in the life of bacteria in the deep earth.

Bacterial spores have been found by a number of investigators to have enhanced resistance to the killing actions of kilobar pressures.⁸ Surprisingly, they are generally very sensitive to lower pressures of 1,000 atm or less. This apparent anomaly is due to the germinating action of low pressures and the pressure sensitivities of the germinated forms. Pressure has become a useful agent for studies of spore germination because germination can be induced by compression to a pressure of, say, 800 atm, without the need to add chemical germinants.

Fortunately, in terms of the possible use of spores as inocula for seeding wells, there is generally a threshold pressure below which germination does not occur to any appreciable extent. For example, at 25°C, the threshold for germination of spores of *Bacillus megaterium* ATCC 19213 is ca. 250 atm.¹ However, the optimal temperature for pressure induced germination is generally some 40 to 60°C, and the threshold is very much reduced at these higher temperatures.

There has been very little exploration of the interactions between pressure and oxidation-reduction potentials (Eh) affecting microbial growth. There is a claim²⁵ that hydrostatic pressure greatly sensitizes bacteria to oxygen toxicity. However we have not been able to detect this sensitization, even with extensive and repeated experiments with a variety of bacteria. We have found recently that the Eh of growth media, poised with standard Eh buffers, can affect markedly the barosensitivities of some bacteria but not others.

Examples are presented in Table 1. *B. licheniformis* is a facultatively anaerobic bacterium which can grow in stab culture in complex tryptone-glucose-Marmite medium at Eh values as low as -571 mV. The data presented indicate that *B. licheniformis* is moderately

barotolerant and can grow at 37°C at a pressure of 500 atm but not at 700 atm. Pressure appears to have no effect on the capacity of the bacterium to grow over the Eh range tested here, nor does the Eh value of the growth medium appear to affect barotolerance.

In contrast, the data presented for *Streptococcus faecalis* indicate that the barotolerance of this facultatively anaerobic bacterium is greatly enhanced at low Eh values. Again, the bacterium is moderately barotolerant with a maximum growth pressure of some 500 atm at 37°C in tryptone-glucose-Marmite agar. However, if the initial Eh value of the growth medium is reduced with cysteine to -210 mV, the organism can grow at a pressure of 700 atm. Then, if the initial Eh value is reduced further to -330 or -571 mV, the bacterium is able to grow at the remarkably high pressure of 1,100 atm.

Therefore, it seems that the anaerobic conditions that develop in deep oil reservoirs would not adversely affect barotolerance and may even enhance it. The rise in Eh values associated with flooding and secondary recovery may greatly increase barosensitivities of at least some organisms. Clearly, there is a need for much more experimental work on this subject.

Yet another factor in oil reservoirs which may modify barotolerance is the presence of dissolved gases at high partial pressures, especially in saturated, gas-capped reservoirs, Buckley et al, 1958.² Figure 2 presents an extensive collection of data obtained by Stephen R. Thom²¹ on the effects of compressed gases on growth of *Escherichia coli* B in complex medium. In brief, the data indicate that there are two general classes of gases. He, N₂ and Ar had little or no effect on growth at pressures as high as 100 atm.

In contrast, N₂O, Xe and Kr were highly inhibitory and could stop growth completely at pressures of about 30 atm. Even though He, N₂ and Ar did not appear to affect growth, they could modify barotolerance as shown by the data presented in Fig. 3 for the yeast

TABLE 1—Effect of Growth Medium on the Barosensitivities of *Bacillus licheniformis* and *Streptococcus faecalis*

Organism and Eh Buffer Added to TGM Medium ^a	E ₀ ' of Buffer (mv)	Growth at Pressure (atm)					
		1	300	500	700	900	1100
<i>Bacillus licheniformis</i>							
None		+ ^b	+	+	-	-	-
0.05 percent Na thioglycolate	-100	+	+	+	-	-	-
0.025 percent cysteine	-210	+	+	+	-	-	-
0.05 percent dithiothreitol	-330	+	+	+	-	-	-
0.025 percent cysteine plus 0.025 percent Na ₂ S	-571	+	+	+	-	-	-
<i>Streptococcus faecalis</i>							
None	-100	+	+	+	-	-	-
0.05 percent Na thioglycolate	-100	+	+	+	+	-	-
0.025 percent cysteine	-210	+	+	+	+	-	-
0.05 percent dithiothreitol	-330	+	+	+	+	+	+
0.025 percent cysteine plus 0.025 percent Na ₂ S	-571	+	+	+	+	+	+

^aTGM medium is Tryptone-Glucose-Marmite medium. Eh buffers were added following the procedures recommended by Costilow (1981).

^bThe symbol "+" refers to growth along the stab line in the agar media used. Growth was readily visible by eye. The symbol "-" refers to lack of growth visible with a 10× hand lens. The incubation temperature was 37°C, and cultures were incubated for at least 72 h. Vials with flexible seals were used for all cultures, and each agar medium was topped with broth medium of the same composition. All inoculations and manipulations were performed in an anaerobic glove box.

Saccharomyces cerevisiae, which is a relatively baro-sensitive organism. The lower curve shows the inhibitory action of hydrostatic pressure alone established without a gas phase. The upper curve is a composite one showing the effects of pressures established with the gases helium, nitrogen and argon.

At pressures below 50 atm, these gases stimulated growth somewhat. They were inhibitory at higher pressures, but the inhibitory effects were always less than those due to hydrostatic pressure alone. In other words, they enhanced the barotolerance of this facultatively anaerobic organism.

Could the gases dissolved in reservoir waters have significant effects on the microbes growing there? Generally, the dissolved gas contents of reservoir water increase with depth, and for example, Buckley et al. (1958) reported that waters in the Woodbine Formation collected from depths ranging from 900 to 2,700 m contained per liter from 500 to 2,100 ml of dissolved gas. The specific gases present vary among sites, but usually, the predominant gas is methane with smaller fractions of ethane, propane and higher hydrocarbon gases.

If in the example above, the dissolved gas was mainly methane, then the CH₄ concentrations in the waters would be 22.3 to 93.8 mM. At 25°C, these amounts would be equivalent to about 15 to 62 atm on the assumption that the Ostwald coefficient of 0.03395 volumes of gas per volume of solution does not change greatly with pressure. These pressures of methane should affect cells. The anesthetic dose for methane for the rat is 101 atm.³ However, pressures of only 40 atm have been found (Ferguson et al., 1950⁷) to arrest mitosis of *Allium cepa* root cells.

Unfortunately, at this time, we can only speculate on the microbiological effects of methane at elevated pressures in reservoirs. Methane has about the same narcotic potency as argon. If it were to affect microbes in the same ways that argon does, then one would expect that methane would enhance barotolerance but would also increase oxygen sensitivity.

In this presentation, I have attempted to emphasize the importance of hydrostatic pressure as a biological

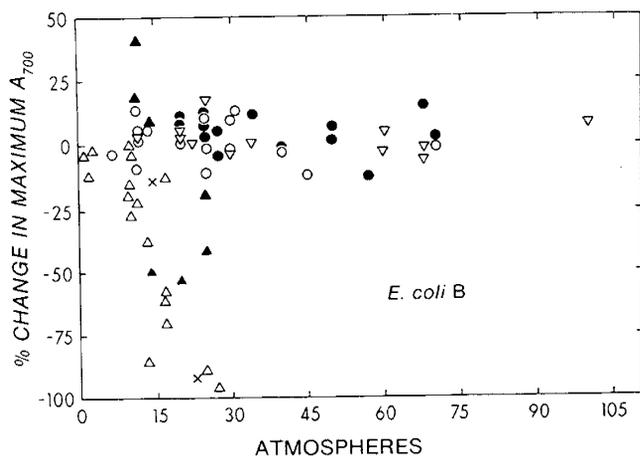


FIG. 2—Effects of helium (●), nitrogen (▽), argon (○), nitrous oxide (Δ), krypton (▲) and xenon (×) on the extent of growth of *Escherichia coli* B at 37°C in tryptic-soy broth plus 1 percent (w/v) KNO₃.

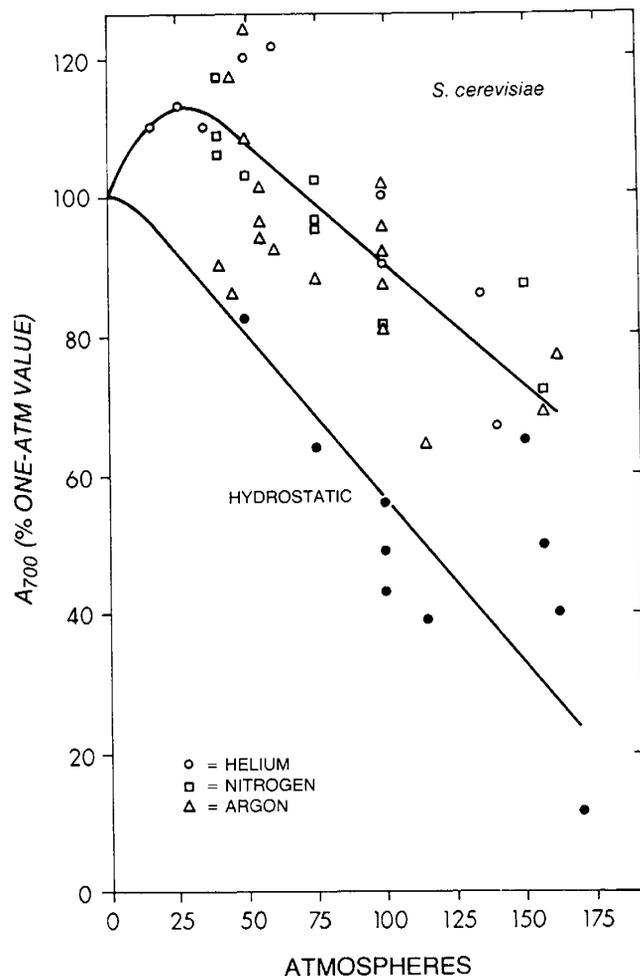


FIG. 3—Comparative effects of hydrostatic pressure (●) and gas pressures established with helium (○), nitrogen (▽) or argon (Δ) on growth of *Saccharomyces cerevisiae* in tryptone-glucose-Marmite broth plus 162 μg ampicillin per ml at 24°C. The figure is from the Ph.D. thesis of S. R. Thom (1981).

cally pertinent factor in the deep earth. Certainly, it seems that pilot operations for enhancement of oil recovery through use of microbes need to be conducted with the test organisms at *in situ* pressures if they are going to be useful for predicting microbial behavior in reservoirs.

Clearly, we would like to have more information on the numbers and types of organisms indigenous to oil reservoirs so that determinations could be made of the mechanisms they have to cope with extremely harsh environments and of the biologically limiting environmental factors for the deep earth flora. The limits could possibly be extended through genetic engineering.

However, the flora already adapted to live at the extremes certainly should provide good starting material for engineering projects, especially if the desired characteristics for freeing oil from reservoir rock could be superimposed on inherent high levels of environmental tolerance.

REFERENCES

1. Bender, G. R. and R. E. Marquis. 1982. Sensitivity of various salt forms of *Bacillus megaterium* spores to

the germinating action of hydrostatic pressure. *Can. J. Microbiol. in press.*

2. Buckley, S. E., C. R. Hocott and M. S. Taggart, Jr. 1958. Distribution of dissolved hydrocarbons in subsurface waters, p. 850-1882. *In* L. G. Weeks (ed.) *Habitat of Oil: A Symposium*. Amer. Assoc. Petrol. Geol., Tulsa, Oklahoma.

3. Carpenter, F. G. 1954. Anesthetic action of inert and unreactive gases on intact animals and isolated tissues. *Amer. J. Physiol.* 178: 505-509.

4. Collins, A. G. 1975. *Geochemistry of Oil Field Waters*. Elsevier Publishing Co., Amsterdam.

5. Costiliow, R. N. 1981. Biophysical factors in growth, p. 66-78. *In* P. Gerhardt (ed.) *Manual of Methods for General Bacteriology*. American Society for Microbiology, Washington, D.C. 20006.

6. Dostalek, M. and R. Kvet. 1964. Utilization of osmotolerance of sulfate-reducing bacteria in a study of the origin of subterranean waters. *Folia Microbiol.* 9: 103-114.

7. Ferguson, J., S. W. Hawkins and D. Doxey. 1950. c-Mitotic action of some simple gases. *Nature* 165: 1021-1022.

8. Gould, G. W. and A. J. H. Sale. 1972. Role of pressure in the stabilization and destabilization of bacterial spores, p. 147-157. *In* M. A. Sleight and A. G. Macdonald (ed.) *The Effects of Pressure on Organisms*. Academic Press, New York.

9. Hauxhurst, J. 1976. Lytic enzymes and the production and stability of bacterial endospores at increased hydrostatic pressure. Ph.D. Thesis, University of California at San Diego.

10. Heinen, W. and A. M. Lauwers. 1981. Growth of bacteria at 100°C and beyond. *Arch. Microbiol.* 129: 127-128.

11. Hunt, J. M. 1979. *Petroleum Geochemistry and Geology*. W. H. Freeman and Co., San Francisco.

12. Kuznetsova, V. A. 1960. Occurrence of sulfate-reducing organisms in oil-bearing formations of the Kuibyshev region with reference to salt composition of layer waters. *Mikrobiologiya* 29:T298-301.

13. Kuznetsova, V. A. and E. S. Pantskhava. 1962. Effect of freshening of stratal waters on development of halophilic sulfate-reducing bacteria. *Mikrobiologiya* 31:T103-106.

14. Marquis, R. E. 1976. High-pressure microbial physiology. *Adv. Microbial Physiol.* 14:159-241.

15. Marquis, R. E. 1982. Environmental extremes and microbial enhancement of oil recovery. *In* J. E. Zajic, N. Kosavic, T. Jack and D. Cooper (ed.), *Microbial Enhanced Oil Recovery, in press.*

16. Marquis, R. E. and D. M. Keller. 1975. Enzymatic adaptation by bacteria under pressure. *J. Bacteriol.* 122:575-584.

17. Meganthan, R. and R. E. Marquis. 1973. Loss of bacterial motility under pressure. *Nature* 246:525-527.

18. Piccard, J. and R. S. Dietz. 1961. *Seven Miles Down*. Longmans Publishers, London.

19. Postgate, J. R. 1977. *The Sulphate-reducing Bacteria*. Cambridge Univ. Press.

20. Rozanova, E. P. and T. N. Nazina. 1979. Occurrence of thermophilic sulfate-reducing bacteria in oil-bearing strata of Apsheron and Western Siberia. *Mikrobiologiya* 48:T907-911.

21. Thom, S. R. 1981. Non-narcotic actions of nitrogen, oxygen, nitrous oxide and noble gases on microbial growth. Ph.D. Thesis, The University of Rochester.

22. Willingham, C. A. and H. L. Quinby. 1971. Effects of hydrostatic pressures on anaerobic corrosion of various metals and alloys by sulfate-reducing marine bacteria. *Devel. Indust. Microbiol.* 12:278-284.

23. ZoBell, C. E. 1958. Ecology of sulfate reducing bacteria. *Producers Monthly* 22:12-19.

24. ZoBell, C. E. 1964. Hydrostatic pressure as a factor affecting the activities of marine microbes, p. 83-116. *In* Y2 Miyake and T. Koyana (ed.), *Recent Researches in the Fields of Hydrosphere, Atmosphere and Nuclear Geochemistry*. Maruzen Co., Ltd., Tokyo.

25. ZoBell, C. E. and L. L. Hittle. 1976. Some effects of hyperbaric oxygenation on bacteria at increased hydrostatic pressure. *Can. J. Microbiol.* 13:1311-1319.

Microbial Interactions with Polyacrylamide Polymers

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ABSTRACT

Polyacrylamides, as they are manufactured for use in oil recovery projects involving polymer floods, are capable of stimulating the multiplication of populations of aerobic soil microorganisms. Aerobic plate counts of soil enrichment cultures in the presence of polymer, using 15 soil samples, were 10-100 fold greater than in the absence of polymer. One possible reason for this is the release of ammonia from polymer. Several polymers, with molecular weights ranging from $3-4 \times 10^6$ daltons to $9-10 \times 10^6$ daltons, were shown to be capable of supporting growth of several strains of soil pseudomonads as a sole nitrogen source in a chemically defined medium.

Extent of growth was a function of concentration over the range 0.05-0.5 percent. Shear degradation of polymer did not affect its ability to support growth, nor did stated degree of hydrolysis (ranging from 1-4 percent to 25-35 percent). Washed cells of certain strains of *Pseudomonas* were able, under the right conditions, to release measurable amounts of ammonia from solutions of polymer. This may be the result of an enzyme action. The enzyme involved is not the low molecular weight aliphatic amidase found in many wild-type strains of *Pseudomonas aeruginosa*.

A strain of *Pseudomonas* isolated from a mixture of polyacrylamide and sand (sample obtained from an oil reservoir where a polymer flood was in progress) was shown to be markedly stimulated in rate of growth during the early phases of the culture cycle by the presence of polyacrylamide (0.05-0.5 percent) in a chemically defined medium. This effect could be partially duplicated by the monomer acrylamide.

Growth and sulfate reduction by strains of *Desulfovibrio* are stimulated under certain conditions by the presence of polyacrylamide in the growth medium. There seems to be a relationship between possible sulfate deficiency in the medium and extent of stimulation by the polymer. Polymer in the medium lowers the O/R potential; this may be a factor in stimulation of growth.

There is evidence that growth of either aerobic (pseudomonads) or anaerobic (*Desulfovibrio*) microorganisms in a medium containing polyacrylamide lowers the screen factor of the solution more than the decrease that occurs in an equivalent control (uninoculated) medium.

We have no evidence that any of the microbial cultures we have used are able to metabolically degrade the carbon chain of the polyacrylamide.

INTRODUCTION

Since polyacrylamides are xenobiotic (i.e., not synthesized by a biological agent), it is presumed that they are not metabolized by microorganisms and are essentially non-biodegradable. Furthermore, the backbone consists of a chain of $-CH_2-$ groups linked by carbon-carbon covalent bonds which, unlike the anhydro bonds linking the monomeric units of most biopolymers, are not subject to hydrolytic cleavage. The molecular size should also render biodegradability unlikely for two reasons:

a) the longest $-CH_2-$ chain subject to attack by hydrocarbon-degrading microorganisms is approximately only a little over 40 carbons in length.

Hydrocarbon-degrading microorganisms oxidize a terminal carbon first to a primary alcohol, then an aldehyde, and finally to a carboxylic acid, the latter being then subject to a process of β -oxidation which cleaves off two carbon atoms at a time. Such a process has never been observed with polyacrylamides, whose chain may be thousands of carbon atoms in length;

b) the molecular size precludes passage of the molecule through the cell membrane—no transport systems or extracellular enzymes attacking polyacrylamide are known to exist.

TABLE 1—Polyacrylamides Used
in Biodegradation Studies

Designation	Molecular Weight	Degree of Hydrolysis
J332	$9-10 \times 10^6$	25-35 percent
J333	$6-7 \times 10^6$	25-35 percent
J334	$3-4 \times 10^6$	25-35 percent
A (J279)	3×10^6	1-4 percent
D	4×10^6	8-10 percent
Non-hydrolyzed		
PAA (obtained as solution—22.3 percent solids)	Unknown	
Cationic PAA	4×10^6	Reacted PAA (none)

Field observations by workers who handle these polymers indicate, however, that they somehow enhance microbial growth. We have undertaken studies to find out how they are able to cause this stimulation, and what effect, if any, the interaction with microbes has on the polymer.

MATERIALS AND METHODS

Polymers were obtained through the courtesy of Dowell Division, Dow Chemical Company, Tulsa, Oklahoma. Average molecular weight and degree of hydrolysis are given in Table 1. These polymers were prepared in such a way that they had a monomer content of less than 0.1 percent. Except for the unhydrolyzed and cationic polymers they were in a powder form, obtained by methanol precipitation which removes impurities such as inorganic ions.

For aerobic organisms, a chemically defined medium with the composition given in Table 2 was used. Sulfate-reducing bacteria were cultivated in the Allred medium,¹ or the American Petroleum Institute medium,² or Postgate's media B, C, and sometimes E.⁷ Anaerobic incubation was accomplished using an ANEE anaerobic system flushed with a gas mixture consisting of 85 percent N₂, 10 percent CO₂, and 5 percent H₂.

Microbial growth of aerobes was monitored by the aerobic plate count, or by determining absorbance (optical density) of cultures. Growth of *Desulfovibrio* was monitored by visual observation of blackening, or by determining absorbance. Enumeration of sulfate-reducing bacteria was done using a 5-tube Most Probable Number test, with blackening of medium taken as a positive indication of growth. Medium used for the MPN enumerations was Postgate's Medium E.

Absolute viscosity in centipoises was determined using a Brookfield Viscosimeter, and screen factor was determined using a screen viscometer as described by Foshee et al.³ All of these determinations were done after removing bacteria and ferrous sulfide by filtration or centrifugation.

All procedures involving preparation of polymer solutions, sterilization of polymers, and adding to media were carried out so as to minimize thermal, oxidative, or shear degradation of polymer.⁵ Control media, in which the polymer was handled in exactly the same manner as in experimental media, except that the former were uninoculated, were always done. When sheared polyacrylamide was desired, shearing was done by a five-minute treatment at high speed in the Waring Blender.

RESULTS AND DISCUSSION

Types of microbial interactions with polymers may be divided into two categories for convenience, aerobic and anaerobic. These are carried out by entirely different microorganisms, by different mechanisms, and have different effects on the polymer. The aerobic interactions will be considered first.

Early in our biodegradability screening studies⁴ we noted that serial transfer enrichment cultures prepared using the chemically defined medium (Table 2) with a certain polyacrylamide as a sole carbon source and inoculated with soil showed a marked enhancement of

TABLE 2—Composition of Basal Chemically Defined Medium Used in Enrichment Cultures

Substance	Amount per 100 ml
NH ₄ Cl	400 mg
MgSO ₄ ·7 H ₂ O	3 mg
KH ₂ PO ₄	136 mg
K ₂ HPO ₄	174 mg
Trace Minerals:	
H ₃ BO ₃	0.5 μg
CaCO ₃	10.0 μg
CuSO ₄ ·5 H ₂ O	1.0 μg
FeSO ₄ ·(NH ₄) ₂ SO ₄ ·6 H ₂ O	50 μg
KI	2.0 μg
MnSO ₄ ·H ₂ O	2.0 μg
MoO ₃	1.0 μg
ZnSO ₄ ·7 H ₂ O	5.0 μg

growth of populations of soil microbes, as compared with the control (no carbon source) (Fig. 1). These results were obtained in the fourth serial culture, in which carryover of soil nutrients should have been minimal.

In all cases, viable counts obtained with polymer in the medium were 100-fold to 10⁵ times as high as in the control. Similar results were obtained with the same 15 soils samples at 25°C, but a smaller proportion (9 of 15) were positive at 55°. A positive result was taken as one in which the viable count was at least 10 times higher than in the control.

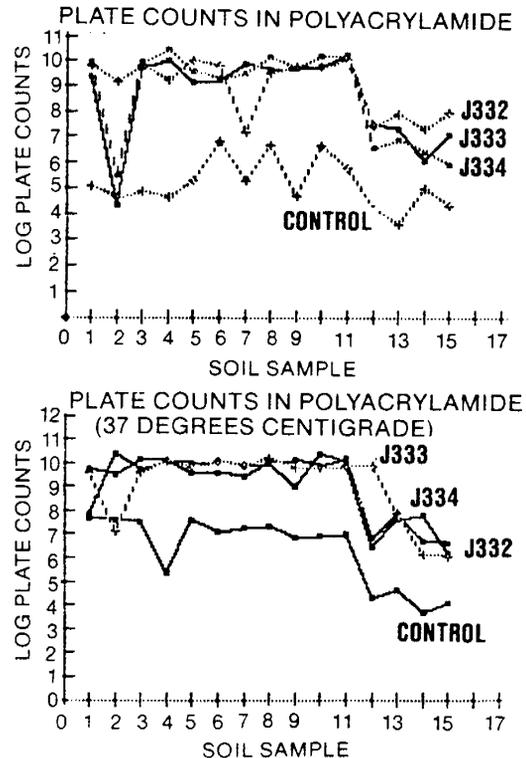


FIG. 1—Enhancement of growth of soil microorganisms by three polyacrylamides. Numbers on horizontal axis represent different soil samples (total of 15). Plate counts are those obtained in the fourth serial enrichment culture. Data in top graph were obtained at 25°C, in bottom graph, at 37°C.

Similar tests with the polymers J₃₃₃, J₃₃₄, and J₂₇₉ gave very similar results. However, polymers labeled "unhydrolyzed" and "cationic" were completely negative using the same test, i.e., there was no increase in microbial population in presence of polymer.

From this we inferred that the reason the other polymers stimulated growth was that they were able to serve as a source of utilizable nitrogen for the soil microorganisms. This proved to be the case. Using strains isolated from polyacrylamide enrichment cultures (these were always pseudomonads), the polymers J₃₃₂, J₃₃₃, J₃₃₄, and J₂₇₉ each could serve as a nitrogen source in the presence of any of three carbon sources. Extent of growth was a function of concentration in the range 0.05 percent to 0.5 percent.

The most important factor in this stimulation is undoubtedly the ammonia released by spontaneous hydrolysis of the polymer. Dialysis of the polymer solution removed this growth-supporting capacity. Neither the unhydrolyzed nor the cationic polymer was able to support growth in this fashion. We have no explanation for the fact that in carbon-limited cultures strains utilizing the polymer as a source of nitrogen were selected. Several tests have shown that aerobic growth of pseudomonads utilizing the polymer as a source of nitrogen does not decrease screen factor of polymer solutions.

A strain of *Pseudomonas* isolated from a mixture of polyacrylamide and sand (sample obtained from an oil reservoir where a polymer flood was in progress) was shown to be markedly stimulated in rate of growth during the early phases of the culture cycle by the presence of polyacrylamide (0.05-0.5 percent) in a chemically defined medium. Ultimate growth attained was not increased by presence of the polymer. This effect could be partially duplicated by the monomer acrylamide (Table 3).

Interactions with anaerobic microorganisms—

These are possibly of greater significance in the recovery of oil using polyacrylamides than the aerobic interactions. We obtained samples of crude oil and samples of produced water from a number of wells in the Wilmington Field near Long Beach, Calif., after this field had undergone a polymer flood in which Dow polyacrylamides ("pushers") were used. Previously this field also had been flooded with sea water.

Only certain wells ("T series") had actually been involved in the polymer flood. We had samples of crude oil and water from wells that had been involved in the polymer flood, and from wells that had not.

TABLE 3—Stimulation of Early Growth by Polyacrylamide of a Pseudomonad Isolated from a Polymer-Sand Mixture from an Oil Field in Illinois

Medium*	Absorbance (O.D. ₅₄₀)		
	6 hr**	7.5 hr	9 hr
Control (no PAA).....	0.21	0.48	0.82
0.1 percent J279***.....	0.37	0.83	1.20
0.4 percent J279.....	0.48	0.96	1.15
1.0 mg/liter acrylamide.....	0.29	0.73	1.08

*Basal defined medium (Table 2)—complete

**Time after inoculation

***Dialyzed polymer used

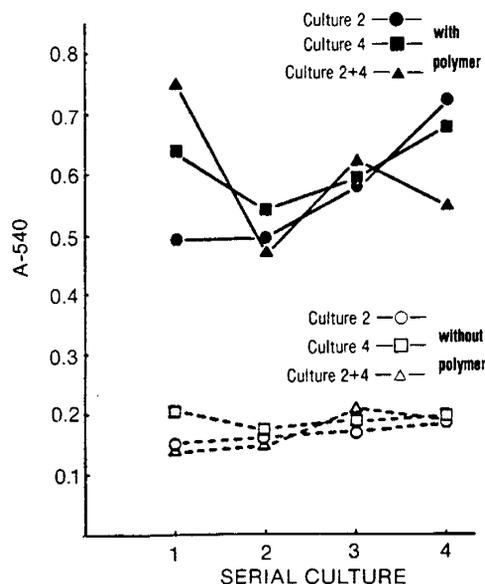


FIG. 2—Stimulation of growth of mixed cultures of *Desulfovibrio* by polyacrylamide in the Allred medium.

Four crude oil samples, two from T-series wells and two from other wells, were received first. These were cultured in the Allred Medium¹ for sulfate-reducing bacteria. The two samples from the T-wells yielded sulfate-reducing bacteria at 37°C (not at 55°C), whereas the others did not.

The produced water samples were received about five months later. Sulfate-reducing bacteria in all were enumerated using Postgate's Medium E⁷ in a five-tube Most Probable Number test. Results are given in Table 4. The high counts of sulfate reducers in the T-wells are in striking contrast to the negative or very low counts of the others.

Several strains of sulfate-reducing bacteria were isolated from these cultures. They were all mesophilic, non-spore forming, Gram-negative curved rods, and were motile by means of a single polar flagellum. Cultural and biochemical characteristics (except for failure to grow with choline minus sulfate) corresponded to those given for *Desulfovibrio desulfuricans* by Postgate⁷.

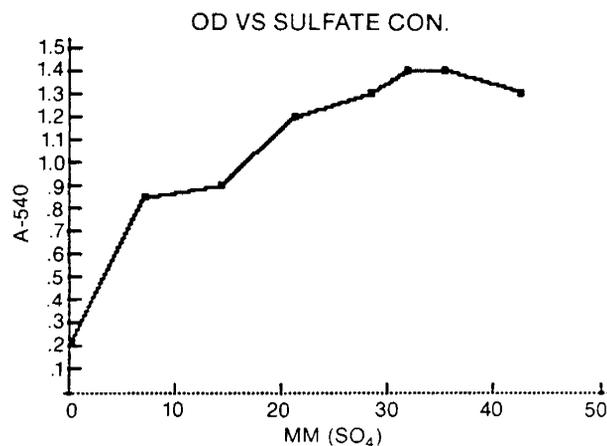


FIG. 3—Growth of *Desulfovibrio* (T-104 isolate) in Postgate's Medium C as a function of sulfate concentration. Cultures were anaerobically incubated 48 hr at 35°C.

Effect of polyacrylamides on growth of *Desulfovibrio*—Mixed cultures of *Desulfovibrio* obtained from the crude oil samples showed a high degree of growth stimulation by polyacrylamide in the Allred medium. This effect persisted over three serial transfers (Fig. 2). It was shown not to result from the ammonia content of the polymer.

Since the Allred medium was found to be deficient in sulfate (it contains 1.4 mM/per liter, or less than one-tenth the optimum level of sulfate for growth of *Desulfovibrio*) (Fig. 3), we next tried the effect of polyacrylamide in Postgate's Media B and C. Growth in the complete medium C was stimulated only very slightly by the polymer (Fig. 4). Polyacrylamide was not able to substitute for lactate in this medium. Preliminary data indicate that in Medium C suboptimal in sulfate concentration, polyacrylamide stimulates more than it does in the complete medium. Growth stimulation and an enhancement of the rate of blackening occur in Medium B, but we do not have quantitative data at present to document relationship of growth stimulation to degree of sulfate deficiency in this medium.

The mechanism by which the polymer stimulates growth of *Desulfovibrio* is unknown. Presence of polyacrylamide in Medium C lowers the oxidation-reduction potential of the medium (Fig. 5). This may be a factor in stimulation, but it would not account for stimulation in the Allred medium, which contains two reducing agents (ascorbic acid and sodium thioglycollate). The polymer evidently is not serving as a source of energy, since it cannot substitute for lactate in Medium C. Enhancement of sulfate reduction, as indicated by blackening in Medium B, and growth stimulation as well, may be an indirect effect of the polymer, occurring because of its ability to alter certain physico-chemical parameters of the medium (among them O/R potential), rather than a direct effect on the microorganism.

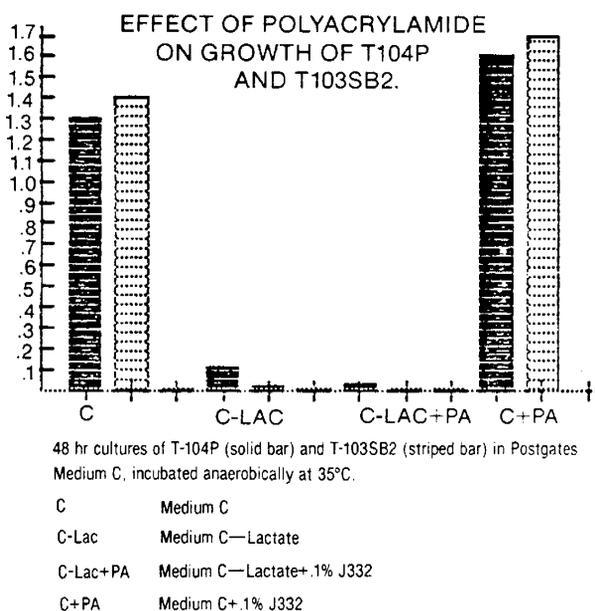


FIG. 4—Effect of lactate, polyacrylamide, and polyacrylamide plus lactate on growth of two strains of *Desulfovibrio* (T-104—solid bar) and T-103SB2—striped bar) in Postgate's Medium C. Cultures were incubated 48 hr at 35°C.

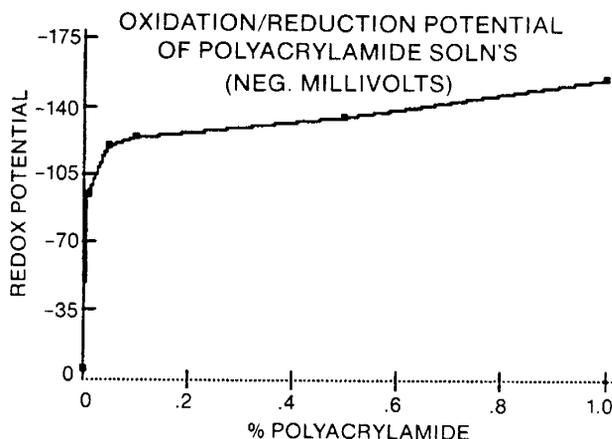


FIG. 5—Effect of polyacrylamide concentration on oxidation-reduction potential of uninoculated Postgate's Medium C after autoclaving and 5 days anaerobic incubation at 37°C.

Xanthan gum stimulates the growth of *Desulfovibrio* (Fig. 6). Fig. 6 also shows that sheared polyacrylamide (J332, molecular weight $9-10 \times 10^6$, 25-35 percent hydrolyzed) stimulated growth as well as the unsheread polymer. A polyacrylamide of medium molecular weight and only 1-4 percent hydrolyzed showed only minimal stimulation. Polyacrylamide (no hydrolysis) showed slight inhibition of growth while polyacrylic acid and the cationic polyacrylamide were quite markedly inhibitory.

These observations indicate that growth stimulation is not simply a property of this general type of polymer, and also that a marked lowering of the average molecular weight of a 25-35 percent hydrolyzed polymer does not destroy its growth stimulating properties.

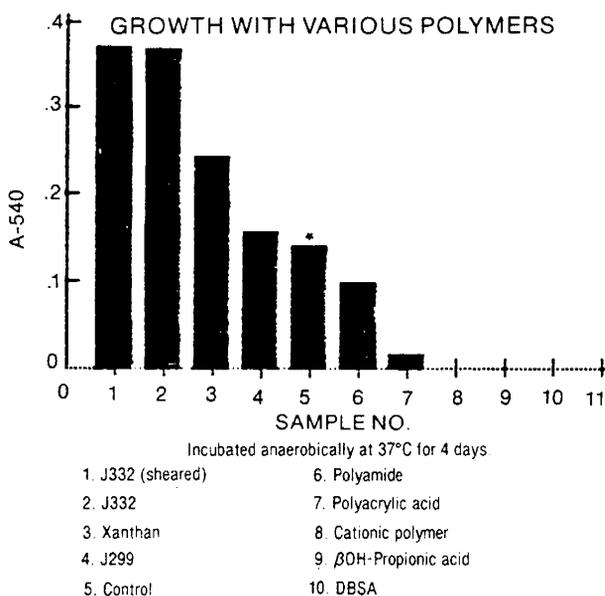


FIG. 6—Effect of various polymers, β -OH propionic acid, and n-dodecylbenzene sulfonic acid (DBSA) on growth of *Desulfovibrio* strain SB-2-A in Postgate's Medium C with a deficient level of sulfate. 0.1 percent polymer. Incubated anaerobically for 4 days at 37°C.

TABLE 4—Total Most Probable Number Counts of Sulfate-Reducing Bacteria in Water Samples from Oil Wells in the Wilmington Field

Well	MPN/ml Produced Water
T-100	4.9×10^2
T-101	2.2×10^6
T-102	7.9×10^1
T-103	4.9×10^5
T-104	3.5×10^4
T-105	4.9×10^3
M 486	0
M 526	0
M 711 E	24

Effect of growth of *Desulfovibrio*—According to Foshee et al³ screen factor, in the case of polyacrylamide polymers, has been of considerable value as an easily measured quantity reflecting the activity of polyacrylamide solutions more reliably than solution viscosity. The standard screen factor instrument or screen viscometer, and its method of operation have been described.⁵

In contrast to the lack of effect on screen factor by aerobic pseudomonads, we found that growth of *Desulfovibrio desulfuricans* in presence of polyacrylamide often results in a significant reduction in screen factor (Fig. 7). A parallel reduction in absolute viscosity in centipoises, as measured with a Brookfield viscosimeter, also occurred (Fig. 8). In all these tests, a control medium was always prepared in which the polymer solution was handled in exactly the same way as in the inoculated cultures; therefore a reduction in screen factor or absolute viscosity could be attributed to growth of the organism. Different strains reduced screen factor to varying extents (Fig. 8).

We do not know the mechanism of this reduction in screen factor. Although a reduction in molecular size has occurred, it probably is not the result of a direct enzymatic attack on the $-\text{CH}_2-\text{CH}-$ chain of the polymer. It may, as we suggested in the case of growth stimulation, be an indirect effect of physicochemical changes brought about in the medium by growth of the organism.

Obviously, a loss of screen factor of the magnitude we have observed with some of our cultures, if it occurred in an oil reservoir, would seriously affect if not destroy the functioning of the polymer.

Role of other bacteria—In natural ecosystems, very few, if any, organisms exist in isolation. Nearly always a variety of microorganisms that affect and interact with each other are present in a given natural environment. This is true of oil reservoirs as well as of other environments, although under the generally harsh conditions of most oil reservoirs the total number, and number of kinds of microorganisms may be less than in more favorable environments.

In every case, with samples of crude oil and of produced water from the Wilmington Field in which we found *Desulfovibrio*, we also found a facultative aerobe* that produces a non-diffusible pigment. Some

*We use this term to mean an organism which prefers aerobic conditions, but will also grow anaerobically.

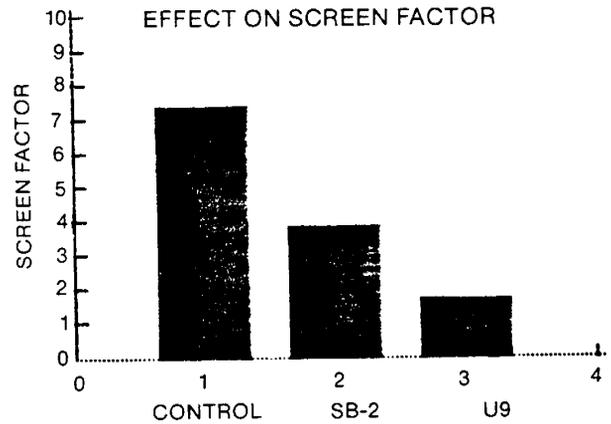


FIG. 7—Effect of growth on two strains of *Desulfovibrio* (U9—from an oil field in Robinson, Ill., and SB-2—from an oil field in Long Beach, Calif.) for 5 days in Postgate's Medium C on screen factor of polymer (0.1 percent J332). Control was handled in the same way as the cultures except that it was uninoculated.

physiological and biochemical properties of this organism are given in Table 5.

Five strains, with very similar properties, were isolated. All are medium-sized Gram-negative rods, motile, and non-spore forming. Best growth occurs in the mesophilic temperature range. Growth under aerobic conditions is much more abundant than anaerobic growth. Addition of 1 percent peptone to NH_4^+ -glucose medium allows growth of all strains. All strains grow well aerobically in Postgate's Media B and C, and show varying degrees of blackening of Medium B.

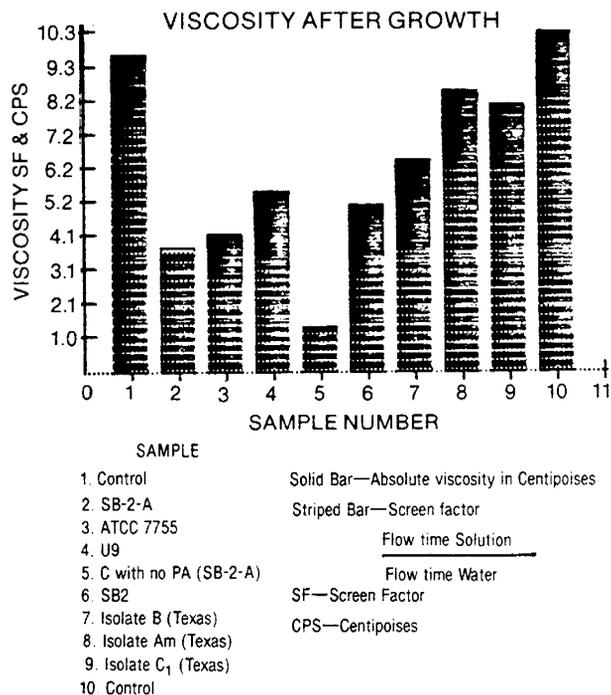


FIG. 8—Reduction in screen factor and in absolute viscosity (centipoises) by several strains of *Desulfovibrio*. Bars 1 and 10 represent uninoculated media. Incubated anaerobically with 0.3 percent J332 in medium for one week.

TABLE 5—Properties of Aerobic Bacterial Strain Isolated from Samples of Crude Oil and Produced Water from the Wilmington Field

Cetalse+
Oxidase+
GelatinLiquefaction
AgarNo liquefaction
GlucoseNo fermentation
LactoseNo fermentation
SucroseNo fermentation
NaClGrows over a range of 0-5 percent; optimum growth at 2.5 percent
KIAAlkaline/Neutral H ₂ S produced; no detectable gas
NH ₄ Cl-glucose minimal mediumNo growth

These strains and *Desulfovibrio desulfuricans* show a peculiar synergistic relationship when grown in close proximity. Plates containing solidified Medium F⁷ were streaked down the middle with *Desulfovibrio* Strain T-103 BB (isolated from Wilmington Field well T-103), and then streaked at right angles with strains of the aerobic isolate. Incubation was anaerobic for 3-5 days at 37°C. Growth and H₂S production by T-103 BB were enhanced in the presence of the aerobic organism, as shown by larger colony size and considerably more blackening of the medium. The medium was clear (no FeS) immediately under growth of the aerobic strains, possibly because of some oxidation process. Growth of the aerobes also seemed to be enhanced by the presence of *Desulfovibrio*. These effects could not be duplicated by *E. coli*, *E. aerogenes*, *B. subtilis*, or *P. aeruginosa*.

The implications of the presence of these aerobic organisms in oil reservoirs** are important for the oil industry, especially in connection with polymer floods. The enhancement of growth of *Desulfovibrio* might well occur in the reservoir as it does in laboratory media. Increased corrosion problems, loss of polymer,

and increased resistance of sulfate-reducing bacteria to biocide could all be associated with the presence of these aerobic bacteria. It is apparent that the solutions of these problems will require further research.

ACKNOWLEDGEMENTS

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REFERENCES

1. Allred, R. C., T. A. Miles, and H. B. Fisher. Bacteriological Techniques Applicable to the Control of Sulfate-Reducing Bacteria in Waterflooding Operations. Producers Monthly, pp. 31-32, December 1954.
2. Applied Petroleum Microbiology, 1965, Chapter 3.
3. Foshee, W. C., R. R. Jennings, and T. J. West. 1976. Preparation and Testing of Partially Hydrolyzed Polyacrylamide Solutions. Paper Number SPE 6202, Society of Petroleum Engineers of AIME, 6200 N. Central Expressway, Dallas, Texas 75206.
4. Gula, M. M., and E. A. Gula. 1979. First Annual Report, Contract DE-AT19-78BC-30201, U.S. Department of Energy, BETC. Biodegradation of Materials Used in Enhanced Oil Recovery.
5. Jennings, R. R., J. H. Rogers, and T. J. West. Factors Influencing Mobility Control by Polymer Solution. 1971. Journal of Petroleum Technology (March 1971), 341-401.
6. Mac Williams, D. C., J. H. Rogers, and T. J. West. 1973. In: Bikales, T. M. (Ed): *Water-Soluble Polymers*, Plenum Publishing Corp., New York, N.Y. pp. 105-126.
7. Postgate, J. R. 1979. *The Sulphate-Reducing Bacteria*. Cambridge University Press, Cambridge, London, New York, Melbourne.

**Recently (since the above results were obtained) we have found similar bacteria in produced water from oil wells in Illinois and West Texas.

A Survey of Research on the Application of Microbial Techniques to the Petroleum Production in China

Zhang Zhaochen and Qin Tongluo

ABSTRACT

Petroleum microbial techniques have been investigated in a number of oil fields, research institutes, and universities in China. Most of the work has been done in laboratories. A few pilot tests have been conducted but no definite conclusions have resulted from the tests. Screening of microorganisms and the properties of their products are described in this paper. The results of in-house modeling are also presented.

INTRODUCTION

Microbial techniques were first applied in the Chinese petroleum industry in the late 1950's. Petroleum microbes were used as indicators of the presence of oil in geochemical exploration. Since then a number of microbial techniques have been used in different fields of endeavor:

- Microbial dewaxing is used to improve the quality, and decrease the pour point, of petroleum white oils.
- Laboratory experiments were conducted on high sulfur petroleum from the Middle East to develop microbial desulfurization.
- Measurement of drilling fluid invasion into cores using bacteria as indicators.⁷
- The distribution of different petroleum microorganisms in oil fields has been studied.^{2,3}
- Conditions of the development of sulfate-reducing bacteria in reservoir rock near the well bore of injection wells and their plugging action have been determined.
- The manufacture of petroleum proteins has been studied in laboratories.
- The use of enzymes in fracturing fluids as degrading agents has been tested.
- Iron bacteria are utilized to indicate the water producing horizon of water injection wells.

Some of these have been field tested extensively, while for some of the others only screening of bacteria and laboratory tests have been performed. These studies have resulted in better preparation of both personnel and technology for the study of microbial enhancement of oil recovery.

GEOLOGICAL AND ENGINEERING CONDITIONS

Most of the oil fields in China, especially those in the Baohai Bay Area, are characterized by lake deposited reservoir formations and strong tectonic movements; consequently, they are complicated both structurally and stratigraphically. The depths of reservoirs in many oil fields are greater than 2,000 meters and they are very heterogeneous, that is: short of continuity of pay sands, many shale breaks, a great number of faults, and great variation in permeability both horizontally and vertically.

Over 90 percent of China's oil fields are developed by pressure maintenance through water injection. The reservoir conditions are complicated by:

- High temperature, 80°C, or more.
- High pressure, 200 atmospheres, or more.
- The composition of reservoir fluids, especially that of water, changes continuously as water injection proceeds.
- The structure of the pore spaces of the reservoir rocks change with time as water is injected. Both the porosity and the permeability are increased, but the pore-size distribution becomes less uniform. Observations of cores with a scanning electron microscope after waterflooding show that clay minerals (usually kaolinite) adhering to the sand particle surfaces are removed and some pore throats are clogged.
- In the Daqing oil field, after vigorous waterflooding for 20 years, a decrease in temperature at the bottom of the injection wells of 5°C has been observed. A mathematical model describing the behavior of the temperature change in the reservoir, using the principle of conservation of energy, with injection and producing wells as sources and sinks has been constructed. The calculated results match with measured results closely. This shows that reservoir temperature distribution changes continuously during water injection.

All of these show that physical and chemical properties of the reservoir rocks and reservoir fluids are dynamic ones: that is, they are time-dependent rather

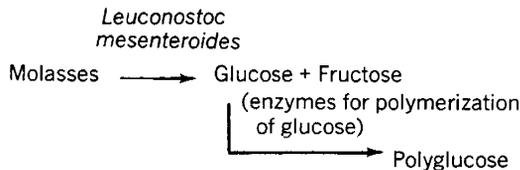
than constant. Taking this into consideration, the *in situ* injection of microorganisms to enhance the oil recovery will be very difficult to control at the ground surface.

In addition there are two points to be mentioned further: (1) with the vast population in China, the high demand for good food makes the use of carbohydrates as nutrients for the usual *in situ* microorganism process impractical. Thus using petroleum hydrocarbons as the sole carbon source for microbial cultivation is attractive; (2) since the petroleum reservoirs are in a strong reducing environment, the cultivation of aerobic bacteria (they are essential for manufacturing biosurfactants) is not possible in the reservoir itself. This also makes the *in situ* injection process very difficult.

With the above considerations, we have focused our attention mainly on the surface bioengineering processes using petroleum hydrocarbons as the sole carbon source, instead of any *in situ* injection processes.

DISCUSSION OF RESEARCH

In the middle of the 1960-70 decade, the Umen Petroleum Administration in the Gansu Province, with the cooperation of the Research Institute of Microbiology of the Academia Sinica of China, carried out in-house experiments on the cultivation of microorganisms to synthesize a polyglucose thickening agent:



The products were pilot tested for reduction of water breakthrough. No definite results were reported.

The microbial processes have been carried out mainly in three categories:

1. Manufacture of mobility control agents
2. Manufacture of water blocking agents (the plugging of high permeability zones in the reservoir).
3. Manufacture of biosurfactants

MANUFACTURE OF MOBILITY CONTROL AGENTS

Research groups of Shengli oil field, Dakang oil field, and Nankai University worked together to study

the manufacture of mobility control agents by microorganism fermentation with petroleum. Tests using reservoir models have been carried out.

Screening of the types of bacteria—133 samples were taken from oil-contaminated clays, crude oils, and produced water. Petroleum microorganisms were separated and purified from these samples. The various strains of bacteria were cultivated with a base fluid containing petroleum and inorganic salts, and were isolated and purified by the direct plate method. Those used for the manufacture of thickening agents were screened primarily by observation of the mobility of the resultant fluid when oscillated in a shaken bed, or by their clogging effect in alcohol or acetone solutions. Forty strains have been screened. After further cultivation in a base fluid containing 15 percent (by volume) petroleum as the sole carbon source, eleven better cultures were obtained.

It was discovered that some of the strains may yield thickening agents, when cultivated in a base fluid with liquid paraffin as a sole carbon source, but they will not yield (or will yield only a small amount of thickening agents) when petroleum is used instead of the liquid paraffin. Only 4 strains of bacteria were found that could yield thickening agents when petroleum was used for cultivation of the base fluid. The color of all of these colonies is orange-red.

Identification of the bacteria groups—As an example, take the best one of the above mentioned 11 strains (S-114). Identification by the Research Institute of Microbiology and Nankai University⁵ showed that this strain which was separated from the oil contaminated clay in the Shengli oil field, is positive in the Gram stain test, and is a non-spore forming aerobic bacteria with no flagellum. The colony of bacteria, with beef juice as the base fluid, appears circular with a finely toothed border, and is red in color. Each single bacterium is in the form of a rod and arranged either as isolated rods or in a V-shape. The nitrogen source may be taken from inorganic nitrogen compounds and the carbon from petroleum, to produce viscous polysaccharides. Identification of S-114 has proved that this is a new type of bacterium, different from all types mentioned in Bergey's Manual of Determinative Bacteriology, and has been named "*Corynebacterium gummi-ferm* Nov. sp. Wang et Yang." Its conservation number is ASI-944. S-114 is not harmful to white mice.

Fermentation tests—Research groups in the Shengli oil field, Nankai University and Research Institute of Forrest and Soil at Shenyang performed a series of tests to study such items as: growing factors,

TABLE 1—Composition of the Base Fluid Used with Culture S-114

Crude Oil	CaCO ₃	NH ₄ NO ₃	KH ₂ PO ₄	Na ₂ HPO ₄ ·12 H ₂ O	Corn Paste	MgSO ₄ ·7 H ₂ O	Fe ⁺⁺
10 percent (V)	0.5 percent	0.3 percent	0.2 percent	0.2 percent	0.043 percent	0.1 percent	20 mmg/l

TABLE 2—Properties of Crude Oil from Well 7-66, Dakang Oil Field

Sp. Gr. (d ₄ 20)	Viscosity (50°C, cs)	Pour Point (°C)	Water Content, Percent	S Content, Percent	Asphaltene Content, Percent	Paraffin Content, Percent
0.8222	5.85	32	7.0	0.078	4.37	17.68

optimum nitrogen sources, the amount of petroleum to use, requirements of tracers, and the optimum volume of fluid in the agitated bottles. Tests were also conducted with 50–500 liter medium fermentors. Some of the developments were:

- Corn paste is not a necessary requirement for production of thickening agents, but addition of a suitable amount may increase the amount of thickening agents produced.

- Different nitrogen sources and different pH's of the base fluid affect both the quantity and the viscosity of the thickening agents. The viscosity is doubled when ammonium nitrate is used instead of ammonium sulfate.

- Tracer tests showed that Fe^{++} is necessary for the growth of culture S-114, and that its optimum amount is 20 mg/liter.

- Composition of the base fluid was determined on the basis of 60 batch and bottle tests and is listed in Table 1. Table 2 lists the properties of the oil.

- The optimum amount of fluid was 20–30 ml per run. Greater or lesser amounts than this give poorer results.

- Chromatographic analysis shows that nearly all normal paraffinic hydrocarbons are completely consumed, but only a part of the iso-hydrocarbons are consumed. For example, in a 50-liter fermentor with a period of 80–90 hours, the resultant fluid, at 80°C, still retains a viscosity of 20 centistokes even when diluted with an equal volume of water. It is a gum at room temperature.

- Compositional analysis showed that the components in the base fluid of the fermentation by S-114 are utilized in different ways through metabolism. Some of these are transformed into nucleic acids, proteins, etc., and result in growth of the bacteria with production of some metabolites such as thickening agents. The transformation efficiency of the petroleum into the thickening agents is calculated as only 19.2 percent on the basis of reducing saccharides. It is important therefore, to pay close attention to selection of microorganisms with a high transformation efficiency.

- Modification of the proteins by the bacteria remaining in separated thickening agents is the main reason for degradation of the polymer. Separation of the bacteria from the biopolymer, or addition of formaldehyde as an inhibitor, stabilizes the thickening agents, but the viscosity may be reduced by a third.

- The thermal stability and corrosiveness of the thickening agent solutions were studied.

Laboratory-scale oil-displacement tests

Using sand-packed models and the following conditions: water saturation = 25–30 percent, viscosity of the synthesized oil (a mixture of degassed crude oil and refined oil) = 33 c.s., temperature = 55–60°C, viscosity of the thickened water (thickening agents added) = 3–5 c.s., rate of injection = 15–68 ml/hour.

Results from 69 runs (with 64 successful runs) showed that with the addition of biochemically synthesized thickening agents, the recovery before water breakthrough was increased by 18.8–8.4 percent (compared with conventional water displacement). The time

of water-free oil production was increased by 1.5–4.3 times, in the case of Dakang crude oil. In the case of Shengli crude oil, the amount of water-free oil increase was 3.9–7.2 percent. The ultimate recovery for the Dakang crude and Shengli crude oil when displaced with this thickened water was increased by 9.5–29.4 percent and 8.3–27.6 percent respectively.

A displacement test using this thickened water was run after a conventional waterflood on the model to a water cut of 95 percent, and an increase in the ultimate recovery of 11–23 percent resulted.

If the thickened water is not treated, the permeability of the sand is decreased by 75 percent. The reduction of permeability will be 15–35 percent, similar to the injection of other polymer solutions, when proper treatment has been made.

Another research group with scientists and engineers from the Daqing oil field and the Research Institute of Microbiology of the Academia Sinica of China selectively obtained a new strain of bacteria from 700 strains which were separated from 110 samples of oil contaminated clays, crude oils, and oilfield water. The bacterium was named *Brevibacterium viscogenes* Nov. sp. 74-230 obtained from oil contaminated clays from the Naking refinery. This bacterium produces a viscous extracellular polysaccharide from a base fluid containing petroleum as the carbon source. This biopolymer is described in detail in the paper by Mr. Wang Xieyuan in these proceedings.

The dimensions of the cells are 0.5×1.3 – 4.5 mm (after 16–24 hours of growth) and 0.5×0.8 – 1.7 mm after 72 hours. This strain of bacteria is present in the S-114 colony and it is positive in Gram's stain test.

In the initial stage of cultivation, the petroleum is first emulsified. The degree of emulsification is increased with respect to time as fermentation continues, and it is thickened apparently after 36–48 hours. After 3–5 days (the end of the fermentation period) a brown colored, viscous, fluid is obtained. Its viscosity is 42–74 c.s. at 45°C, when diluted 1:1 with water.

It was also discovered that better results may be obtained with the crude produced from region II in Daqing oil field (saturated hydrocarbon content: 80.1 percent) with a transformation efficiency of the saturated hydrocarbons of 95.83 percent, and that of crude oil, 28.57 percent. Crude polysaccharides obtained from 150 samples by precipitation in alcohol amounted to 8.0 grams/liter. The recovery efficiency, calculated on the basis of total amount of crude oil used, is 6.7 percent.

Oil displacement tests with this thickened water injected after the sand packed model was flooded to an end point of 98 percent water cut, showed an increase in ultimate recovery of 5.59–9.12 percent when a slug size of 0.1–0.2 pore volume was used. The displacement pressure increased, the water to oil producing ratio decreased and oil production increased in this process.

Manufacture of water-blocking agents—In the Shengli oil field, a cooperative research group has screened out a kind of pseudomonosporrin bacterium designated as Yuan-A-144. The fermentation fluid has the peculiar property of viscosity increase with temperature rise, and forms a gum-like substance at the end of the fermentation period. A preliminary analysis showed that it contains saccharides, proteins and ashes.

Tests to block high permeability zones were conducted in laboratories at 65°C. Thermal stability tests showed that some of the gum-like substance could be kept unchanged as long as 60 days, and at least a 10-day stability was obtained. It is recognized that this is more suitable to the Shengli oilfield which is characterized by greater depth and heterogeneity of the reservoir.

Field pilot tests were carried out, but interpretation of the data has not been definitive; therefore, no conclusions can be drawn from the field tests.

Similar experiments have been carried out in the Daqing oil field.

Manufacture of biosurfactants—Research crews of the Peking Petroleum Institute and the Research Institute of Microbiology Academia Sinica held a meeting in 1968 to discuss this subject. In 1970, research groups from these organizations, with the cooperation of research scientists of the Shengli oil field, carried out a series of in-house and field pilot tests.

In the early 70's it was discovered that fermented hydrocarbon fluids of certain petroleum bacteria, when added into a viscous crude oil, disperse the crude into the fermented fluid creating a dispersed fluid system similar to an oil/water emulsion, or microemulsion system.

Samples taken from the oil-contaminated clay were cultivated in a base fluid with the petroleum from Yuen-ancheng oil field as the sole carbon source, and oscillated on a shaking bed 4 times successively. Seven milliliters of the fermented fluid was removed and mixed with 10 grams of viscous crude oil (viscosity = 2344 c.s. at 50°C) and then stirred mechanically for a long time. If the viscous oil was dispersed, the fermented hydrocarbon fluid was considered an effective viscosity reducing agent, and vice versa.

Using this standard procedure, ten strains were screened out from a total of 60 samples as those having viscosity-reducing properties. Among them, cultures

TABLE 3—Viscosity of Fermented Oils

Type of Bacteria	Activated Fermented Hydrocarbon		Maximum Viscous Crude Oil Volume Dispersed
	Volume (ml)	pH	
4B105g	10	8.5 (4-9)	29-35
4-13	10	6 (5-6)	29-31

labeled 4B105g and 4-13 have been further tested on a larger scale in a 500 liter fermentor.

The fermentation experiments showed that the fermented hydrocarbon fluid separated into 2 layers. The upper layer contained metabolites and a densely segregated bacteria group, while the lower layer was a turbid aqueous mixture. The interface disappears immediately when shaken and appears again after settling. Viscous oil dispersion is mainly caused by the activated upper layer. When examined under a microscope hydrocarbon fluids are adhering to the surfaces of viscous oil droplets. It can be postulated that this fermented hydrocarbon fluid is a kind of activated material with a certain HLB value and has the ability to disperse the viscous crude to form oil/water emulsions which modify the mobility of such a system.

The maximum amount of viscous oil dispersed is given in Table 3. The pH noted in the table was determined by titration with standard NaOH or H₂PO₃ after 1:1 dilution with distilled water.

The time of stabilization of the system changes with the pH and the relative amount of the fermented fluid added. For 4B105g fermented fluid, with the addition of 10-25 grams of viscous crude (10 ml fermented fluid) at a pH = 8.5, the system is stabilized for a period of 23-168 hours.

Fermented fluid from culture 4B105g can retain its activity after exposure to the atmosphere for 132 days.

The effect of viscosity reduction is illustrated by taking 4B105g fermented fluid as an example. When the fluid is mixed with viscous crude in a ratio of 1:1 or 2:1, the viscosity of the system is reduced from 2344 c.s. to 12.0 and 46.0 c.s., respectively.

It has been found that the activity of the fermented fluid differs to a great extent when NaNO₃ or NH₄⁺ salt is used as the nitrogen source in fermentation.

No field pilot tests have been conducted. Further study on this product will be carried out.

CONCLUSIONS

1. It is attractive and desirable to manufacture chemical additives and water blocking agents in the oil fields by fermentation of crude oil, especially in Chinese oil fields.
2. There are many advantages to extensive screening of strains of bacteria for different purposes using crude oil as the sole carbon source.
3. Great attention should be paid to the suitability of the type of crude oil which is available for the bacterial culture screening process. Screening should be carried out in every isolated petroliferous area.
4. Efficient bioreactors should be designed and installed to produce sufficient fermented products for further field pilot tests.

REFERENCES

(All references are published in Chinese)

1. "Distribution of Microflora in the Crude and Water in Yumen Oil Field." Wang Xieyuan et al., *Acta Microbiologica Sinica*, Vol. 11, No. 4, Nov., 1965.
2. "The Growth of Microorganisms in L Formation in Laochunmiao Oil Field in the Course of Water Injection." Wang Xieyuan et al., *Acta Ecologica Sinica* Vol. 1, No. 1, Mar., 1981.
3. "The Incubation of *Candida Lipolytica* by Inspection with Ultra-thin Section Techniques." Tan Baoying et al., *Acta Microbiologica Sinica*, Vol. 19, No. 49, 1979.
4. "A Study on the Identification of Gum-producing Strain S114 in the Manufacturing of Visco-polysaccharides, Part I, Identification of the Strain." *Acta Microbiologica Sinica* Vol. 19, No. 4, 1979.
5. "Manufacture of Polysaccharides by Microorganisms Using Crude Oil as Sole Carbon Source, and a Discussion on Their Possible Use in Secondary Oil Recovery." Wang Xieyuan et al., *Acta Petrolei Sinica*, Vol. 1, No. 4, Oct., 1980.

MEOR Field Applications

6. "A New Petroleum Dewaxing Strain—Its Fermentation Properties." Kao Hongtong et al., *Acta Microbiologica Sinica*, Vol. 19, No. 4, Dec., 1979.

7. "Application of a Strain of Bacterium as an Indicator in Determining the Depth of Invasion of Mud Filtrate on Drilled Cores." Wang Xieyuan et al., *Acta Microbiologica Sinica*, Vol. 16, No. 2, May, 1964.

Microbial Enhancement of Oil Recovery in Romania

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ABSTRACT

The paper is a review of the investigations carried out in Romania, during the last 10 years, on the use of bacteria for the stimulation of oil release from reservoirs. The research was based on the idea of using bacterial populations adapted to the reservoir's conditions.

The main sources of bacteria for obtaining such adapted populations were the formation water of the reservoir submitted to microbiological treatment and the fermented scum from sugar processing plants.

Under laboratory conditions, the "adapted" bacterial populations obtained, cause 19.5–48.8 percent of oil release in a collector. Under the field conditions, the same "adapted" bacterial populations produce an increase of oil flow, from some wells, up to 200 percent for 1–5 years. Such an increase of oil production was recorded only in 2 out of the 7 reservoirs injected with "adapted" bacterial populations and nutrient support on molasses basis.

BRIEF HISTORY

After finishing primary energy, an important amount of crude oil (about 60–70 percent) remains in the reservoirs, partly blocked in the rock pores in the form of discontinuous globules. As mentioned by Guy de Lamballerie,²³ a 1000 times difference of pressure than the usual one between upstream and downstream, is necessary for this oil recovery. Part of this immense quantity of crude oil blocked or retained—usually called "dead" crude oil—can be extracted by secondary or tertiary recovery techniques. The microbiological technique is one of several others which received special attention after the oil crisis of 1973.

Since Beckman's idea in 1926 concerning the use of bacteria in oil release (cited by Beerstecher¹) several stages were covered as follows: the period 1943–1952 was characterized by the special research project API conducted by ZoBell; the period 1950–1960 characterized by intensification of investigations and development of ZoBell's methods; period 1960–1970 characterized by field trials in countries such as Czechoslovakia, USA, Hungary, Poland, Soviet Union; period 1970–1980 characterized by renewed, and intensified, research in countries such as Romania, USA, Great Britain, Australia, Canada, France, Switzerland and West Germany.

In the framework of microbial enhanced oil recovery

processes the bacterial treatment of wells played an important role. As it developed so far in Czechoslovakia (Dostalek and Spurny^{4,5}), USA (Updegraff,⁴⁸ Hitzman,^{14,16} Coty,² Johnson¹⁹), Hungary (Dienes and Jaranyi,³ Jaranyi,¹⁷ Jaranyi et al¹⁸), Poland (Karaskiewicz^{20,21}), Soviet Union (Ekzertsev,⁸ Kuznetsov et al,²² Senyukov et al⁴⁷) and Romania (Lazar 1976,²⁴ Lazar et al 1978,^{25–28} 1982a,³⁵ 1982b³⁶) it consists of injecting bacterial inoculum and nutrient support, generally based on molasses, into the formation.

At the formation level a series of bacterial products results such as gases, organic and mineral acids, biopolymers, biosurfactants, etc., which act on the one hand on the oil retaining rock, and on the other hand on the oil and water, facilitating the oil release from the rock pores and its migration to the production wells. The results of the field trials, carried out in the last 10–15 years in the countries mentioned above, indicated increased oil productions between 20–200 percent or even up to 300–360 percent for periods of a few weeks to 8 years.

The perspective of the period 1980–1990, as underlined in the Final Report of the International Conference, San Diego,⁹ an intensification of research is necessary at least along the following lines:

1. to develop the aspects of bioengineering with special reference to obtaining bacteria by mainly using genetic engineering technique, that after injecting into formation act as we desire;
2. to produce and to use the bioproducts of the biopolymers and biosurfactants type in the processes of oil recovery;
3. to know the ecology of reservoirs so that after injection with bacteria these must behave like large bioreactors or fermenters;
4. to know all transformations taking place in reservoirs at the phases rock-oil-water-gases under the action of bacteria.

STATUS OF INVESTIGATIONS IN ROMANIA

General considerations

In Romania, the investigations regarding the use of microorganisms for enhanced oil recovery have an organized character after 1971. Such investigations are

focused both on using the bacteria as such as some of their products such as biopolymers and biosurfactants. At the same time, aspects related to control some phenomena generated by bacteria such as corrosion and plugging, frequently met in the oil fields where methods of secondary or tertiary recovery with water injection or other fluids are applied.

Such phenomena have negative implications on the continuity of the technological flux of oil recovery. In the period 1971-1981 special attention was paid to inject "adapted" bacterial population as well as nutrient support based on molasses into formation in order to stimulate the release of retained oil. The other aspects were a small part of our research; these will be extended in the period 1981-1985. This is the reason for which the present paper refers exclusively to the problem of well injection with bacterial inoculum and nutrient support based on molasses. The investigations were carried out in three phases by a group of microbiologists of the Institute of Biological Sciences, Bucharest, in collaboration with the Research Institute for Oil and Gas "Cimpina" and a number of economic enterprises for oil extraction.

The first phase refers to the characterization of the bacterial flora of the formation water with special reference to that of the reservoirs selected for microbiological treatment; the second refers to obtaining bacterial populations adapted to the conditions of the reservoirs submitted to microbiological treatment; and the third one refers to the field trials. The results of our investigations in the first two stages are presented in several papers published in the period 1976-1980,^{6,7,13,25-30,32,37-39,41,42,43,50,51} and those obtained in the last stage in two recently elaborated papers.^{35,36}

We present further, in a synthetical form, some aspects widely presented in the papers mentioned above. Special attention will be paid to the aspects related to obtaining and characterizing the bacterial inoculum injected into formation together with the nutrient support based on molasses, as well as to the technology of well injection and the results obtained so far in the field trials carried out in several enterprises of oil extraction.

Bacterial inoculum injected into reservoirs

As presented in detail by Lazar,³¹ the bacterial inoculum used for injecting the wells was represented by bacterial populations adapted to the conditions in the oil reservoir, using a simple methodology. Obtaining such populations implies activities regarding natural sources or environments from which the bacteria are isolated, the equipment or systems for the adaptation of bacteria, isolated from various sources, to the reservoir's conditions, the method of adapting and obtaining the adapted bacterial populations in the quantities necessary for well injection.

Sources of bacteria: Using the Hungarian experience^{17,18} as well as the Polish one²⁰ in our country^{25-31,35,37,38,41} special attention was paid to the sources for isolating bacteria to be adapted to the reservoir's conditions, selected for microbiological treatment.

Two categories of sources for bacterial isolation were used. In the first one are included the formation water from the reservoirs that must be injected, the well mud resulting as the cleaning of the bottom of the

well, the soil around the well, sewage mud from purification stations for formation waters; the second category of sources refers to residual waters especially in food industry, anaerobically fermented sludge from biogas stations, sewage collected from the municipal or petrochemical treatment plants, residual water or scum from sugar processing plants, as well as sapropelic muds from the bottom of therapeutic lakes.

As for the microbiological aspects of reservoir water even if it was collected directly from wells as coproduced water or from formation water treatment plants as water for injection processes, many investigations were carried out.^{10-12,25,29,33,34,40,45,46,49,52}

The formation water collected as coproduced water from wells opened at the depth up to 1000-1200 m was populated by a rich mesophilic flora, both facultatively anaerobic and aerobic, as well as strictly anaerobic and also heterotrophic and chemoautotrophic. The same flora was found in water from formation water treatment plants, which is used both for evacuation or enhanced oil recovery processes.

Such waters are double or triple contaminated with bacteria if they are treated with such substances as multi-metal polyphosphates or polymers without biocide protection. Based on the investigations mentioned above^{29,33,37-39,42} it is evident that the formation water is an important source for isolating bacteria to be included in the adapted bacterial populations for well injection.

The study of various residual muds or sludge from treatment plants for formation waters, town drainage systems (sewage) or from petrochemical plants, biogas stations, therapeutic lakes and sugar processing plants, permitted us to affirm that the fermented scum collected from sugar processing plants is the best source of bacteria, having at the same time a stimulating action on bacterial growth on media with molasses.^{29,37,38,42}

The data acquired during experiments to obtain "adapted" bacterial populations for injection, indicated two sources to be used during the processes of adaptation and multiplication, namely: (1) the formation water collected as coproduced water from the reservoir to be subjected to microbiological treatment and (2) the fermented scum collected from sugar processing plant deposits.

Installations for bacterial adaptation: The method previously applied in Hungary and Poland, based on well injection with "adapted" bacterial populations was used as well during the investigation in Romania. Both in Hungary and Poland such populations were obtained by means of an installation presented by Karaskiewicz²⁰ and Lazar^{27,45} in their papers.

The bacterial inoculum prepared in Romania between 1975-1980 and injected into reservoirs with various physicochemical and petrophysical peculiarities was obtained simultaneously from 4 installations. These installations were called A, B, C, and D.^{27,29,37-39,45} Installation D provides anaerobic conditions and is the one previously used in Hungary and Poland.²⁰

The other three were designed by us, and among these, installation C offers anaerobic conditions, A offers partial anaerobic and B offers aerobic conditions. All these installations were obtained in variants

of different capacities or in several copies according to the quantity of required bacterial inoculum. For preparation of inoculum for injection wells, the maximum capacities of each installation are as follow: 10 liters 7-10 samples for installation A, 2-4 rotary shakers with 60-80 places of Erlenmeyer flasks of 750 ml for installation B, 25 liters in 5-7 samples for installation C, and 50-100 liters for installation D. They all were used only when inoculum was prepared for well injection.

Method of bacterial adaptation: The experience obtained in Hungary and Poland^{17,18, 20} mainly supported all our investigations in bacterial adaptation.

The adaptation of some bacteria present in sources as formation water and scum from sugar processing plants, was carried out by a single method of adaptation, without implying the mutagenesis or other special technique of selection. Both pure cultures and bacterial populations were isolated and adapted to the oil reservoir conditions. The results of laboratory experiments on collector models demonstrated that the bacterial populations are more active in releasing oil than bacterial strains or mixed strains. Therefore, the bacterial inoculum used for well injection was represented only by populations isolated from the formation water in the reservoir to be subjected to microbiological treatment and from the scum from sugar processing plants.

These populations were adapted to the conditions of the respective reservoir according to the method described in our papers.^{31,37} By this method, carried in three stages, bacterial populations with concentrations 10⁷-10⁹ bacteria/ml were obtained. In these populations the following genera were prevailing: *Pseudomonas*, *Escherichia*, *Arthrobacter*, *Mycobacterium*, *Micrococcus*, *Peptococcus*, *Bacillus* and *Clostridium*. Besides these, occasionally bacteria of the genera *Aerobacter*, *Flavobacterium*, *Brevibacterium*, *Cellulomonas*, *Corynebacterium*, *Nocardia* and also some yeasts were also noticed.

After the adaptation process, these populations are able to metabolize the molasses with production of gas, organic or mineral acids, biopolymers or biosurfactants in the presence of formation water, oil and at reservoir temperature. Such populations as well as the bacteria in the formation water or isolated from it, have the capacity to partially degradate oil, but unfortunately not always using the heavy fractions.^{6,13,30,50,51}

The bacterial populations resulting from all the four systems of adaptation are characterized from the point

of view of bacterial concentration/ml, main types of bacteria, capacity to release oil from a collector and to use oil. Only the populations that released the largest quantity of oil in collector models were retained and multiplied for well injection.^{37,39,41,43} The mixture of populations obtained from the four installations is much more active in oil release than each population used separately.^{25,29} Therefore it was useful to consider the mixture of "adapted" bacterial populations obtained simultaneously by the four systems of adaptation for field trials.³¹

Inoculum preparation for well injection: The bacterial populations selected during the adaptation process as the most active ones in oil release from a collector are multiplied in the quantities necessary for well injection. Before injection, the inoculum containing a mixture of bacterial populations from the four installations is tested again (on each installation both separately and in mixture form) in order to characterize it: bacteria/ml concentration, the main types of bacteria, and the capacity to use and release oil from collectors.

As two recently published papers explain,^{31,35} the bacterial inoculum (Table 1) obtained in the period 1975-1979 and injected in 7 wells of different reservoirs (from petrophysical and physicochemical point of view) had the following characteristics: cell concentration/ml of the range 10⁷-10⁹ and a structure in bacterial types as mentioned before; the capacity to cause the release of 19.5-48.8 percent oil from collectors (in 5-10 days) and the capacity to use oil in ideal laboratory conditions from 2 to 51 percent.^{30,31,35}

Nutrient support injected into the reservoirs

The nutrient support used for well injection was based on molasses and several bioproducts obtained by its fermentation. As mentioned in previous papers^{28,42} the nutrient used in the field trials in the period 1975-1977 had a similar composition to the one used in Hungary,^{3,17,18} that is, besides 2 percent molasses, little quantities of sugar and mineral substances as additional nitrogen and phosphorus sources.

After 1977, the nutrient support injected into reservoirs together with the bacterial inoculum was based only on 2 percent molasses that is very similar to the one used in Poland.²⁰ This change is based on the laboratory experiments which demonstrated that Romanian molasses together with mineral salts in the formation water is a complex nutrient support for the

TABLE 1—Some Characteristics of the Inoculum Injected into the Formation (Lazar et al, 1982a)

Reservoir	Number of Bacteria/ml ^a	Crude Oil Released from a Collector (Percent)	Crude Oil Utilized in Laboratory Conditions (Percent)
Baicoi	4 × 10 ⁸ -5.6 × 10 ⁹	36.6-48.5	4-27
Vata	8.6 × 10 ⁸ -1.6 × 10 ⁹	19.5-29.6	7-51
Suta Seaca	7.2 × 10 ⁶ -2.0 × 10 ⁹	27.2-34.8	8-36
Bragadiru	3.5 × 10 ⁹ -5.7 × 10 ⁹	30.7-48.8	7-31
Tintea	2 × 10 ⁸ - 4 × 10 ⁹	31.2-36.5	2-35
Beciu	1.2 × 10 ⁹ - 9 × 10 ⁹	27.3-44.3	2-49
Moreni	6.5 × 10 ⁸ -8.2 × 10 ⁹	23.5-30.5	3-29

^aThe main identified genera: *Pseudomonas*, *Escherichia*, *Arthrobacter*, *Mycobacterium*, *Peptococcus*, *Bacillus*, *Clostridium*. Sporadically: *Aerobacter*, *Flavobacterium*, *Brevibacterium*, *Cellulomonas*, *Micrococcus*, *Sarcina*, *Spirillum*, *Desulfovibrio* and some yeasts.

bacteria to be injected as inoculum. Two tons molasses, 90 cubic meters of formation water, and 300 liters of bacterial inoculum, were used for an injection. The nutrient support is 90 cubic meters of formation water with 2 percent molasses.

Equipment required for bacterial injection

One of the advantages of the microbiological method is that no special equipment, materials or investment are required.^{17-19,20} Our own experience^{36,42} demonstrated that the method can be applied by means of the usual equipment and materials that are commonly in any field. These materials and equipment are:

1. 110-120 cubic meters of formation water from wells to be subjected to microbiological treatment.
2. 10-cubic-meter tank where the molasses concentrated solution is prepared. In this tank, 2 tons of molasses, 8 cubic meters of formation water and 300 liters of bacterial inoculum are introduced.
3. 1-3 30-cubic-meter tanks where the final solution for well injection is prepared. About 3.3 cubic meters of concentrated solution from the 10 cubic meter tank are transferred in each tank of 30 cubic meters containing 27 cubic meters of water.
4. 1 truck for cementing, a usual tool in any derrick; used to mix the nutrient support and bacteria in the 10 cubic meter and 30 cubic meter tanks and to inject the nutrient support and bacteria into formation.

Conditions fulfilled by the reservoir

The field trial results so far showed that every reservoir cannot be subjected to microbiological treatment.^{17,18,20} The method is only successful when the physiochemical, petrophysical and biological peculiarities of the formation to be injected with nutrient support and "adapted" bacterial populations permit the reservoir to behave as a large scale bioreactor, or fermenter.

Therefore the reservoir to be subjected to the microbiological treatment based on using heterotrophic, mainly mesophilic, bacteria must have at least the following properties: permeability greater than 300 md; temperature as much as 50-55°C and a pH close to the neutral; moderate degree of water mineralization especially in chlorides; stratum continuity between the injection well and the production wells; best technical parameters of the injection well—it should open in the

formation lower or at least at the oil/water level in comparison with the production wells; the coproduced water produced by reservoir wells should be more than 30-50 percent; the reservoir fluids should not be toxic to the injected bacteria; the rock oil saturation should be between 60-80 percent; the microflora of the respective reservoir should be known and to what extent it can influence the nutrient support and the "adapted" bacterial populations.

The reservoirs with such properties are also suitable to other methods of secondary or tertiary recovery, with well known efficiency. This could be a disadvantage for the microbial enhanced recovery as the oil field enterprises prefer to use the well known methods. For this reason not all 7 reservoirs under experiment in the period 1975-1979 fulfilled the conditions required by the method (Table 2). In fact, the microbiological treatment was applied to reservoirs in an advanced stage of depletion.

TECHNOLOGY OF WELL INJECTION

As mentioned in other papers^{29,36} the wells were subjected to injection in the period 1975-1979 according to two methods:

1. To the special chosen well (with daily oil flow of 0.2-1 ton and with high percentage of coproduced water) 4 injections with nutrient support and bacterial inoculum, at the interval of 2 months, were applied. After 4 months, a new injection with nutrient support and bacterial inoculum was applied. After about 4-5 months from the fifth injection, another 1-3 injections with nutrient support and bacterial inoculum—if necessary—are performed only if positive results are recorded in the production wells. Between injections the well is closed and the effects are investigated in the production wells.
2. The application of bacterial inoculum and nutrient support in wells where previously water injection was applied as a method of enhanced oil recovery. Such wells, after injection with bacterial populations and nutrient support based on molasses, are kept closed for 1-2 months, then the water injection is given again for 1-2 months. This cycle is repeated 5 times. After 4 months break this cycle is repeated only if positive results are noticed in the production wells.

THE EFFECTS OF TREATMENT

The production evolution as well as the production decline data were recorded for 2-3 years from each well

TABLE 2—Some Characteristics of the Reservoirs Submitted to Microbiological Treatment (Lazar et al, 1982a)

Reservoir	Characteristics					
	Depth (m)	Temperature (°C)	Permeability (mD)	NaCl/l (grams)	Oil Saturation (percent)	Coproduced Water (percent)
Baicoi	663-554	27	600-1500	60-80	80	40-70
Vata	1027-971	40	700	76-83	72	15-95
Suta Seaca	1559-1484	52	400	170-190	75	98
Bragadiru	785-772	29	100-1000	5-10	95	70-90
Beciu	619-371	25	150	48-50	71	30-93
Tintea	505-336	15-32	600-1200	40-90	80	15-80
Moreni	825-788	35	800	26	71-81	83-92

of the reservoir before starting the treatment.

In this way reference data regarding the well's production before applying the injection with bacteria are obtained. At the same time, microbiological analysis as well as physicochemical analysis of fluids extracted from each well are carried out. Then, periodically (monthly or every 3 months) after the starting of the microbiological treatment, microbiological and physicochemical analysis of extracted fluids are performed. Also daily, or at least at every tenth day, measurements of extracted fluid flow through each production well is recorded.

Results of field trials

Spreading of injected bacteria into formation: The results presented in a recent paper³⁶ showed that 6-8 months after microbiological treatment, an evident increase of bacterial number was registered in the coproduced water of the reaction wells situated at a distance of about 100 meters from the injection well. The higher number of bacteria was maintained in the formation water as long as the nutrient support and bacterial inoculum were periodically introduced through the injection well. After about 6 months from the last injection with nutrient support, the number of bacteria/ml in coproduced water of the reaction wells returned to the initial value or even lower than this (Table 3).

Such results were obtained from the same reservoirs where increased flow was registered. For instance, the bacterial number increased from 10⁴/ml to 10⁶-10⁹/ml after injection with inoculum. But it reduced to 10³

after 1 year from the last injection of nutrient support. But, for some reservoirs, a full concordance is not established between the increased bacterial concentration in coproduced water of the production wells and the oil increase.³⁶ This nonconcordance could be explained due to some shortcomings of the measuring system for extracted fluids flow or due to the methods of estimation of the oil production of wells from respective reservoirs. For instance, in the 3 reservoirs from which an increased oil flow is not reported there are some reaction wells from which both increase of oil flow and concentration of bacteria per ml were reported. Such kinds of reservoirs were not considered as with positive oil production, since the registered plus oil production from some production wells is less than the minus oil production from the other production wells (Table 6), which initially it was appreciated could be under the influence of microbiological treatment.

The results presented in a recent paper³⁶ showed that the identified bacterial types in coproduced water of the production wells are much more numerous as compared to the pre-microbiological treatment period (Tables 3, 4, 5). This demonstrates that the injected bacteria multiplied and spread up to the production wells. A stimulation of growth of some specific reservoir bacteria, which were lost or were sometimes very sporadically present in the injected "adapted" bacterial populations was observed.

Physicochemical alterations of the extracted fluids: The physicochemical analysis carried out periodically on the production wells fluids showed, especially in the

TABLE 3—The Evolution of Aerobic Heterotrophic Bacteria¹ in Formation Water of the Reservoirs Submitted to Microbiological Treatment (Lazar et al, 1982b)

Reservoir	Number of Bacteria/ml							
	Before Treatment				After Treatment			
	1975	1976	1977	1978	1977	1978	1979	1980
Baicoi	4 × 10 ⁴				1.6 × 10 ⁹	3.5 × 10 ⁶	3.5 × 10 ³	3.8 × 10 ³
Vata				1.0 × 10 ²			3.6 × 10 ³	2.7 × 10 ³
Suta Seaca		1.2 × 10 ³			6.4 × 10 ²	4.3 × 10 ²	1.7 × 10 ⁸	2.9 × 10 ³
Bragadiru		4.0 × 10 ³			2.4 × 10 ⁴	1.7 × 10 ⁵	1.5 × 10 ⁴	3.2 × 10 ³
Tintea		1.2 × 10 ³			8.0 × 10 ⁴	9.2 × 10 ⁴	1.7 × 10 ³	7.8 × 10 ²
Beciu			9.6 × 10 ²			2 × 10 ⁵	1.2 × 10 ³	2.2 × 10 ³
Moreni				1.1 × 10 ⁴			5.6 × 10 ⁵	2.5 × 10 ²

¹In addition to those mentioned at Table 1: *Corynebacterium*, *Nocardia*, *Alcaligenes*, *Acinetobacter*.

TABLE 4—The Evolution of Anaerobic Heterotrophic Bacteria (Type *Clostridium*) in Formation Water of the Reservoirs Submitted to Microbiological Treatment (Lazar et al, 1982b)

Reservoir	The Intensity of Bacterial Growth							
	Before Treatment				After Treatment			
	1975	1976	1977	1978	1977	1978	1979	1980
Baicoi	+				+++	++	++++	++++
Vata				+			++++	++++
Suta Seaca		++			+++	+++	+++	++++
Bragadiru		++			++++	++++	++++	+++
Tintea		+			++	+++	+++	+
Beciu			++			+++	++++	++++
Moreni				++			++++	+

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case of the reservoirs where the evident increases of oil production were reported, some decrease of oil viscosity, of water pH, as well as increases of acidity and some alterations in the salts content of formation water. As it was mentioned in a recent paper of Lazar et al,³⁶ such alterations were for a short time, even at the reservoirs where positive results were reported.

It is possible that at least some of the bacteria from the inoculum prepared and injected into formation water so far, utilize less or not at all the heavy fractions of the crude oil. Such bacteria have preferences for the light fractions of the crude oil (Dumitru et al, 1982). These results are not totally in accord with those obtained in Hungary^{3,17,18} and Poland.^{20,21} In framework of the investigations now in progress, special attention is given to aspects concerning the methods for obtaining "adapted" bacterial populations with ability to decrease the crude oil viscosity.

Production under the microbiological treatment: Details on the fluids flow extracted before and after the microbiological treatment of the wells are presented in two recent papers.^{31,36} As explained in these papers, from 7 reservoirs subjected to microbiological treatment, in the period 1975–1979, only for 2 reservoirs positive results were reported by the economic units which administered the respective oil fields.

From the other reservoirs, although in some wells the increased oil flow was registered, the general balance of the oil production did not give clear indications of increased production as the minus oil production of some wells was covered by the plus oil production of the others.

At the two reservoirs with positive results, namely those from Baicoi and Vata oil fields (Table 6), the increases of oil production, which in some wells reached to 200 percent for periods of 1 to 5 years, were registered. Thus, at the Vata oil field, the well which before microbiological treatment had a production of 0.2 ton of oil/day, after the treatment the oil production was 0.6 ton oil/day for 1 year, indicating a 200 percent increase.

At another well of the same reservoir from Vata, whose production before microbiological treatment was 8.8 tons of oil/day, after treatment an oil production of 10.2 tons of oil/day for one year was registered, indicating 16 percent increase. At another oil field, namely Baicoi, 1 from the 3 reaction wells of the reservoir subjected to microbiological treatment had, before

bacterial injection, an oil production of 0.9–1.0 ton/day, for 5 years. After the treatment its production was 1.4–1.9 tons/day, indicating an increased oil production of 50–90 percent. At present, for some of the reaction wells, the increase of oil production is still rising, while in other reaction wells the oil production declined to the initial level or even less.

The unsuccessful results from some reservoirs could be due to the fact that these did not meet all the conditions required by microbiological method. For instance, in one of the reservoirs (Beciu) the rock permeability was less than 200 mD; in the case of another reservoir (Moreni) it was established later that some deficiencies, under the aspect of strata continuity between injected well and reaction wells, exist. At a third reservoir (Bragadiru), it was established that there were sand movements. At a fourth reservoir, from Suta Seaca oil field, it is possible that the high temperature (52–56°C) and high salt concentrations (170–190 g NaCl/l) partially influenced negatively on the microbiological treatment, although at this reservoir, in 1 of the 5 production wells, an important increase of oil production was registered (Table 6).

The low percentage of success, that is 2 from 7 reservoirs, is proving once more how important the reservoir ecology is for the success of microbiological treatment. If the physiochemical, petrophysical and biological parameters of the reservoirs do not allow the reservoir to behave as a large bioreactor or fermenter, the growth and activity of bacteria as well as the production of the bacterial metabolites involved in oil release, are not possible.

CONCLUSIONS

1. The investigations concerning the use of bacteria for enhanced oil recovery, carried out in Romania during the last 10 years were in three stages. In the first two stages, the research was concentrated on the bacterial flora of the formation water from oil reservoirs and on the methods for obtaining and producing "adapted" bacterial populations; in the third stage, the attention was on the field trials.

2. The "adapted" bacterial populations that were injected into 7 reservoirs, having different physiochemical and petrophysical characteristics, had dominant mesophilic heterotrophic bacteria which in the presence of oil, formation water, and temperature of reservoirs, fermented molasses with intensive gas pro-

TABLE 5—The Evolution of Some Special Groups of Bacteria in the Formation Water of the Reservoirs Submitted to Microbiological Treatment (Lazar et al, 1982b)

Reservoir	Intensity of Bacterial Growth																									
	Before Treatment												After Treatment													
	1975			1976			1977			1978			1977			1978			1979			1980				
	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c		
Baicoi	-	+	-										++	++++	++	++	+++	++	+++	++++	+	+++	+	+++	+	+++
Vata													++	+	++					+	++++	++	++++	++	++	++
Suta Seaca	-	-	-							+	-	+	+	+	++	+	-	++	+++	++	++	++	++	++	++	++
Bragadiru		+	++	+						++	++++	++++	+++	++	++++	+++	+++	++++	+	+++	+++	+	++++	++++	++++	++++
Tintea		+	++	+						++	++++	+++	+++	++	++	+++	+++	++	++	++	++	++	++	-	-	-
Beciu																+	++	+++	++	++++	++	-	++++	+++	+++	+++
Moreni																				++++	++++	-	+	++	++	++

^a Sulfur oxidizing bacteria (*Thiobacillus*)

^b Sulfate reducing bacteria (*Desulfovibrio*)

^c Iron oxidizing bacteria (*Sphaerotillus*, *Leptothrix*, *Galionella*)

duction as well as other bioproducts as: biopolymers, biosurfactants, organic and mineral acids, etc. Such bacterial populations are releasing 19.5 to 48.8 percent oil contained in a collector and have also the capacity to partly degrade the crude oil.

In the field trials, the bacterial populations caused in 2 from 7 reservoirs subjected to microbiological treatment, an increase of oil flow up to 200 percent from 1 to 5 years. The unsuccessful results from the other 5 reservoirs proved the importance of the physiochemical, petrophysical and biological parameters.

3. Until the practical use of "superbacteria" obtained by genetical engineering techniques are available, the use of "adapted" bacterial populations remains a working possibility which could be improved by adaptation process and stimulation of bacterial activity in the formation. At the same time, more knowledge about the ecology of the reservoirs subjected to microbiological treatment as well as the transformations taking place *in situ* at the rock-oil-water-gases level is necessary.

REFERENCES

1. Beerstecher, E. (1954). *Petroleum Microbiology*, Elsevier, Amsterdam, 375.
2. Coty, V. F. (1975). *The Role of Microorganisms in the Recovery of Oil*. National Science Foundation, Easton, Maryland, 9-14 XI, 1975, 77.
3. Dienes, M., Jaranyi, I. (1973). Az olajkihozatal növelese anaerob bakteriumok retegbe telepítésevel a demjeni mesőben, *Kőolaj és Földgáz*, 6 (106), 205-208.
4. Dostalek, M., Spurny, M. (1957b). Release of oil through the action of microorganisms. The influence of physical and physical-chemical conditions in oil bearing rock, *Cesk. Mikrobiol.*, 2, 300-317.
5. Dostalek, M., Spurny, M. (1957a). Release of oil through the action of microorganisms. I. Preliminary experiment on a petroleum deposit, *Cesk. Mikrobiol.*, 2, 300-306.
6. Dumitru, L. (1980). Utilizarea titeiului de catre populatiile bacteriene prezente în unele zacaminte de

titei, *Lucrarile celui de al II-lea Simpozion de Microbiologie ind.*, Iasi, 25-26 mai, 1979, 445-450.

7. Dumitru, L., Faghi, M., Delcea, C. (1982). Influenta microorganismelor asupra modificarii proprietatilor fizico-chimice a titeiului brut, în unele zacaminte din tara noastra (stabilirea potentialului de biodegradare), *Lucrarile celui de al III-lea Simpozion de Microbiologie ind.*, Buc., 12-13 iunie, 1981.

8. Ekzertsev, V. A. (1958). Izuchenie protessesa rezusenii nefi mikroorganizmami v anaerobniikh usloviakh, *Mikrobiologiya*, 27, 626-633.

9. Final Report of the Conference on Microbiological Processes Useful in Enhanced Oil Recovery, August 29-September 1, 1979, San Diego, California.

10. Grigoriu, A., Lazar, I. (1977). Cercetari asupra bacteriilor sporulate din genurile *Clostridium* si *Bacillus*, raspindite în apele zacamintelor de titei, *Lucrarile I-lui Simpozion de Microbiologie ind.*, Iasi, 18-19, dec., 1976, 270-276.

11. Grigoriu, A., Lazar, I. (1982). Degradarea bacteriana a solutiilor de polimeri folosite pentru dislocuirea titeiului din zacaminte, *Lucrarile celui de al III-lea Simpozion de Microbiologie ind.*, Buc., 12-13 iunie, 1981, 675-680.

12. Grigoriu, A., Lazar, I., Mihoc, A., Zamfirescu, I., Dumitru, L. (1980). Cercetari privind actiunea diferitelor biocizi asupra bacteriilor prezente în sistemele de apa de injectie din industria de titei, *Lucrarile celui de al II-lea Simpozion de Microbiologie ind.*, Iasi, 25-26 mai, 1979, 509-514.

13. Herlea, V. (1980). Utilizarea titeiului brut de catre levuri izolate din apa de strat a unor zacaminte de titei, *Lucrarile celui de al II-lea Simpozion de Microbiologie ind.*, Iasi, 25-26 mai, 1979, 455-459.

14. Hitzman, D. O. (1959). Recovery of oil from oil sands, *U.S. Pat.*, 2, 907, 389.

15. Hitzman, D. O. (1962). Microbiological secondary recovery, *U.S.*, 3, 032, 472.

16. Hitzman, D. O. (1967). Oil recovery process using aqueous microbiological drive fluids, *U.S.*, 3,340,930.

TABLE 6—Oil Production of the Reservoirs Submitted to Microbiological Treatment (Lazar et al, 1982b)

Reservoir	Wells ^a	Oil Production/day (Tons)		Period	Increased Oil Production (Tons)
		Before Treatment	After Treatment		
Baicoi	142	1.0	0.8	1977-1980	2093
	143	1.0	1.9		
	907	0.6	1.0		
Vata	1016	1.6	2.0	1979-1980	1024
	1393	2.3	1.9		
	1863	0.2	0.6		
	1864	3.8	3.0		
	1869	8.8	10.2		
Suta Seaca	89	1.0	0.8	1978-1980	—
	92	1.5	1.9		
	93	0.5	0.5		
	903	1.0	0.6		
	904	0.5	0.6		

^a Reaction wells theoretically appreciated to be influenced by microbiological treatment.

17. Jaranyi, I. (1968). Beszamolo a nagylengyel tersegeben elvezgett köolajmikrobiologiai kísérletkről, M. All. Földtani Intezet Evi jelentese A, 1968, Evröl, 423-426.
18. Jaranyi, I., Kiss, L., Szalanczy, G. (1967). A mikrobiologia módszer alkalmazhatasaga különbözö permeabilitasu homokköves es meszköves köolajtarokbau, M. All. Földtani Intezet Evi jelentese A, 1968, Evröl, 345-349.
19. Johnson, C. A. (1979). Microbial oil release technique for enhanced recovery, in Final Report of the Conference on Microbiological Processes Useful in Enhanced Oil Recovery, San Diego, California.
20. Karaskiewicz, J. (1974). Zastosowanie metod mikrobiologeznyck w intensyfikacji eksploatacji Karpackick zloz ropy naftowej; wydawnictwo "Slask" Katowice, 66.
21. Karaskiewicz, J. (1977). Biologicheskoe aktivivovanie neftianikh zaleshei v polse, Ivestia Akad. Nauk. SSSR, Ser. Biol., 5, 790.
22. Kuznetsov, S. I., Ivanov, M. V., Lyalikova, N. N. (1963). Introduction to Geological Microbiology, McGraw-Hill, New York, N.Y., 252.
23. de Lamballerie, G. (1981). La recuperation améliorée du pétrole, La Recherche, 12, 119, 148-157.
24. Lazar, I. (1976). Folosirea bacteriilor în procesele de eliberare si migrare a titeiului din zacaminte, Mine, Petrol, Gaze, Bucuresti, Romania, 27, 10, 475-480.
25. Lazar, I. (1978a). Dispozitiv pentru prepararea de inocul bacterian folosit la injectarea sondelor în vederea stimulării eliberării si migrării titeiului remanent din zacaminte, Certificat de inovator nr. 44 eliberat de MEI la 6, III, 1978.
26. Lazar, I. (1978b). Dispozitiv folosit pentru stabilirea capacitatii bacteriilor de a influenta eliberarea si migrarea titeiului continut într-un colector, Certificat de inovator nr. 45 eliberat de MEI, la 6, II, 1978.
27. Lazar, I. (1978c). Sistem pentru obtinerea de populatii bacteriene adaptate conditiilor din zacamintele de titei si pentru înmultirea unor astfel de populatii în vederea injectării zacamintelor, Certificat de inovator nr. 46 eliberat de MEI, la 6, III, 1978.
28. Lazar, I. (1978d). Microbiological Methods in Secondary Oil Recovery, European Symposium on Enhanced Oil Recovery, Heriot-Watt University, Edinburgh, 5-7, VII, 1978.
29. Lazar, I. (1979). Cercetari privind folosirea slamului de la fabricile de zahar, în procesele de adaptare si înmultire a bacteriilor destinate injectării zacamintelor de titei, St. si Cerc. Biol. Seria Biol. Veget., 31, 2, 123-128.
30. Lazar, I. (1980). Utilizarea titeiului de catre populatiile bacteriene prezente în inoculul folosit pentru injectarea sondelor în vederea stimulării eliberării titeiului, Lucrarile celui de al II-lea Simpozion de Microbiologie ind., Iasi, 25-26 mai, 1979, 435-438.
31. Lazar, I. (1982). Some characteristics of the bacterial inoculum used for oil release from reservoirs. In MEOR Monograph edited by Prof. E. J. Zajic.
32. Lazar, I., Dumitru, L., Balinschi-Zamfirescu, I., Grigoriu, A., Mihoc, A. (1977a). Capacitatea bacteriilor de a elibera titeiul dintr-un colector, Lucrarile I—lui Simpozion de Microbiologie ind., Iasi, 28-29 XII, 1976, 297-303.
33. Lazar, I., Grigoriu, A., Mihoc, A. (1982c). Aspecte ecologice ale raspindirii bacteriilor într-un sistem de apa de injectie de la un zacamint de titei din jud. Dimbovita, Lucrarile celui de al III-lea Simpozion de Microbiologie ind., Buc., 12-13 iunie, 1981, 536-543.
34. Lazar, I., Grigoriu, A., Mihoc, A., Zamfirescu, I., Dumitru, L. (1980). Cercetari privind rolul bacteriilor prezente în sistemele de apa de injectie din industria petroliera în corozionul metalelor, Lucrarile celui de al II-lea Simpozion de Microbiologie ind., Iasi, 25-26 mai, 1979, 473-477.
35. Lazar, I., Grigoriu, A., Mihoc, A., Zamfirescu, I., Dumitru, L., Trifu, L. (1982a). Experimente de santier privind folosirea bacteriilor în stimularea eliberării titeiului din zacaminte. I. Obtinerea inoculului bacterian folosit la injectarea sondelor Lucrarile celui de al III-lea Simpozion de Microbiologie ind., Buc., 12-13 iunie, 1981, 513-518.
36. Lazar, I., Mihoc, A., Grigoriu, A., Zamfirescu, I., Dumitru, L., Trifu, L. (1982b). Experimente de santier privind folosirea bacteriilor în stimularea eliberării titeiului din zacaminte. II. Tehnologia injectării sondelor si efectele tratamentului microbiologic, Lucrarile celui de al III-lea Simpozion de Microbiologie ind., Buc., 12-13 iunie, 1981, 519-527.
37. Lazar, I., Zamfirescu-Balinschi, I. (1977). Instalatii si modalitati de obtinere la scara industrială de bacterii adaptate conditiilor din zacamintele de titei, Lucrarile I—lui Simpozion de Microbiologie ind., Iasi, 18-19 dec., 1976, 284-289.
38. Lazar, I., Zamfirescu, I. (1979). Dispozitiv pentru cultivarea selectiva a bacteriilor destinate utilizării lor în mobilizarea titeiului din zacaminte, Brevet de inventie nr. 73891 acordat de OSIM la 28, XII, 1979.
39. Lazar, I., Zamfirescu, I., Dumitru, L. (1978a). Instalatie pentru selectarea bacteriilor active în mobilizarea titeiului dintr-un colector, Certificat de inovator nr. 47 eliberat de MEI, la 6, III, 1978.
40. Lazar, I., Zamfirescu, I., Dumitru, L., Grigoriu, A., Mihoc, A. (1976). Research on the bacterial flora occurring in the subsurface and injection waters of some oil fields in the districts of Prahova and Dimbovita, Rev. Roum. Biol. Ser. Bot., 21, 1, 53-58.
41. Lazar, I., Zamfirescu, I., Dumitru, L., Grigoriu, A., Mihoc, A. (1977b). Obtinerea de bacterii adaptate conditiilor din zacamintele de titei, Lucrarile I—lui Simpozion de Microbiologie ind., Iasi, 18-19 dec., 1976, 290-296.
42. Lazar, I., Zamfirescu, I., Dumitru, L., Grigoriu, A., Mihoc, A. (1978b). Metoda bazata pe folosirea bacteriilor pentru stimularea eliberării si migrării titeiului remanent din zacaminte, Certificat de inovator nr. 77 eliberat de MEI, la 28, X, 1978.
43. Lazar, I., Zamfirescu, I., Dumitru, L., Mihoc, A., Grigoriu, A. (1979a). Metoda si dispozitiv pentru determinarea capacitatii bacteriilor de recuperare a titeiului

din zacaminte, Brevet de inventie nr. 73892 acordat de OSIM la 28, XII, 1979.

44. Lazar, I., Zamfirescu, I., Dumitru, L., Mihoc, A., Grigoriu, A., Popea, Fl. (1979b). Cercetari asupra microorganismelor care populeaza apa de strat a zacamintelor de titei din jud. Prahova, Volumul de Comunicari si Referate al Muzeului de Stiintele Naturii, Ploiesti, 1979, 183-189.

45. Mihoc, A., Lazar, I. (1982). Cercetari asupra microorganismelor dintr-un sistem de apa de injectie de la un zacamint de titei din jud. Arges; rolul acestora in fenomenul de corozie, Lucrarile celui de al III-lea Simpozion de Microbiologie ind., Buc., 12-13 iunie, 1981, 544-550.

46. Mihoc, A., Popea, Fl., Lazar, I. (1977). Cercetari asupra bacteriilor chimioautotrofe aerobe si anaerobe din apele de strat ale zacamintelor de titei, Lucrarile I-lui Simpozion de Microbiologie ind., Iasi, 18-19 dec., 1976, 277-283.

47. Senyukov, V. M., Yulbarisov, E. M., Talbukina, N. N., Shishveva, E. P. (1970). Mikrobiologicheskii metod obrabotki neftianoi zalezii s visokoi mineralizatsii plastovikh vod, Mikrobiologiya, 39, 705-710.

48. Updegraff, D. M. (1957). Recovery of petroleum oil, U.S. Pat. 2,807,570.

49. Zamfirescu, I., Dumitru, L., Lazar, I., Grigoriu, A., Mihoc, A. (1980). Studiul microorganismelor prezente in sistemele de apa de injectie din industria extractiva de titei, Lucrarile celui de al II-lea Simpozion de Microbiologie ind., Iasi, 25-26 mai, 1979, 466-472.

50. Zamfirescu, I., Grigoriu, A. (1980a). Cercetari asupra capacitatii unor tulpini bacteriene de a utiliza titeiul, Lucrarile celui de al II-lea Simpozion de Microbiologie ind., Iasi, 25-26 mai, 1979, 439-444.

51. Zamfirescu-Balinschi, I., Grigoriu, A., Lazar, I. (1980b). Rolul microorganismelor in degradarea anaeroba a titeiului din zacamintele cantonate in gresii, Lucrarile celui de al II-lea Simpozion de Microbiologie ind., Iasi, 25-26 mai, 1979, 460-465.

52. Zamfirescu, I., Lazar, I., Dumitru, L., Mihoc, A., Grigoriu, A., Popea, Fl. (1977). Consideratii ecologice asupra microflorei din zacaminte de titei cu particularitati diferite de adancime, temperatura si grad de mineralizare al apei, Lucrarile I-lui Simpozion de Microbiologie ind., Iasi, 18-19 dec., 1976, 252-269.

Microbially Enhanced Oil Recovery from the Upper Cretaceous Nacatoch Formation, Union County, Arkansas

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ABSTRACT

A field test to evaluate the gaseous bacterial fermentation of sugar as a means of enhancing oil recovery is described. The test was conducted on the Lisbon Unit, Union County, Arkansas, using a two-spot pattern with 400 feet between wells. Laboratory experiments, which preceded the field test, showed that bacterial gases produced within the reservoir rock prior to waterflooding led to better oil recoveries than could be obtained by either waterflooding or gas flooding alone.

A two percent solution of beet molasses in fresh water was injected into the Nacatoch formation for a period of six months. Eighteen 220 gallon inoculations of *Clostridium acetobutylicum* were injected at scheduled intervals. Fresh water began to appear at the production wells seventy days after initial injection began. Fermentation products appeared 80-90 days after the first inoculation. Fermentation reactions are discussed which explain the products recovered.

An increase in oil production rate occurred soon after breakthrough of fermentation products. Increased production, its significance and the possible mechanisms responsible are discussed.

INTRODUCTION

At the time this work was done, enhanced oil recovery processes were employed primarily to reestablish reservoir pressures depleted during primary production. Repressuring a petroleum reservoir was usually accomplished by the injection of gas, water or other fluids, but as laboratory experiments had shown, repressuring could be achieved by means of bacterial fermentations within the reservoir. However, in order for a bacterial process to yield a recovery advantage over other methods it was reasoned that it should result in a mechanism of oil release not obtained during the course of the usual recovery procedures. Such a mechanism could be provided by the *in situ* production of gases which, by resisting displacement, serve to alter the flow of injected water, thereby improving sweep efficiency.

If gas-producing bacteria can be flooded into a reservoir, fed and encouraged to grow there, pressure build-up due to gas formation would be initiated in many small loci within the formation. The forces responsible for pressure build-up are thus exerted internally, rendering unnecessary the huge expenditures of energy required for surface gas injections. Of greater importance, however, is the probability that pressure bleed-down after fermentation would result in gas enucleation and movement in and from areas not reached by injected gases. Therefore, certain quantities of oil not reached or affected by normal flooding procedures could be forced into existing streamlines by fermentation pressures.

The anaerobe *Clostridium acetobutylicum* was chosen to test this theory principally because of its ability to produce a free gas phase (hydrogen) under reservoir conditions. Fortunately, it also grew vigorously in 2 percent beet molasses-water media and was not affected by sodium chloride concentrations up to 3 percent. Higher salt concentrations, however, inhibited both growth and gas production.

Fermentations using the test bacteria were carried out in the laboratory using core samples and sand packs under a variety of conditions designed to simulate reservoir environments. Total gas production, measured at one atmosphere and 100°F, ranged from 8-30 volumes per volume of medium fermented. The carbon dioxide/hydrogen ratio ranged from 50/50 to 55/45 (compared to a 60/40 theoretical ratio). Resulting gas pressures in closed non-liquid filled systems varied from 100 to 300 psig, while those in hydrostatic systems ranged from 600 to 1,300 psig. The addition of carbonate growth media increased total gas production. However, in closed systems it only slightly affected total pressure. No adverse effects of pressure on the growth of bacteria were ever detected.

FIELD TEST

The principal purpose of the field experiment was

to study the progress and problems of a very large underground bacterial fermentation. Laboratory experiments, though encouraging in their results, were conducted on an infinitesimally small scale when viewed from the standpoint of practical application. In the laboratory, bacterial fermentations can be carried out under conditions of pinpoint control with respect to maintenance of pure cultures and proper environmental conditions (pH, temperature, substrate concentration). Such control would seem to be extremely difficult, if not impossible, in a petroleum reservoir. The selection of a suitable environment was based primarily on a knowledge of what the bacteria would require for optimum growth and activity. Bacterial oil release was, of course, one of the chief aims in all experimentation. But the areas most suitable for field fermentation studies were not necessarily suitable for oil release measurements. After much careful study Magnolia's Lisbon Unit, Union County, Arkansas, was selected as the test location because of its near ideal conditions for the growth of *Clostridium acetobutylicum*. At the same time it was recognized that this field was quite undesirable for oil release studies.

The Lisbon Field was discovered in 1925. It covers an area of approximately 4400 acres and is divided into north and south sections. The north section, in which the test area was located, had been on water flood for five years prior to the test. The field produces from the Nacatoch sand, a loosely consolidated sand of high permeability and porosity, at an average depth of 2100 feet subsurface. Carbonate content of the sand ranges from 1 to 16 percent over the field and 8 percent in the test area. The test was conducted on a two-spot pattern, using Well 30 as the injection well and Well 31 (400 feet distant) as the producing well. Very late in the test, Well 32 (665 feet from Well 30) was opened to production and Well 31 closed in. These three wells are indicated by arrows on the north section isopachous map (Fig. 1). Field data indicated that the area around the test wells had been almost completely flooded out at the beginning of the test.

Wells 112 through 137 were new wells drilled for the north section water flood. All of these wells were cored. Cable tool cores were taken with oil, and rotary cores with a high water loss water-base mud. The weighted average core analysis data were:

Residual oil saturation, rotary cores,	
percent pore space	4.5
Residual oil saturation, cable tool cores,	
percent pore space	8.5
Interstitial water saturation, rotary	
cores, percent pore space	77.3
Interstitial water saturation, cable tool	
cores, percent pore space	72.3
Porosity, percent	30.5
Permeability to air, mD	408
Permeability range, mD	1-5770

Reservoir fluid properties at the beginning of the water flood were:

Estimated oil formation volume factor,	
reservoir bbl/stock tank bbl	1.00
Formation oil viscosity at 100°F, cp ...	4.48
Formation oil gravity at 60°F, °API ...	36
Fresh injection water viscosity	
at 100°F, cp	0.59
Formation water salinity, ppm NaCl ..	42,000

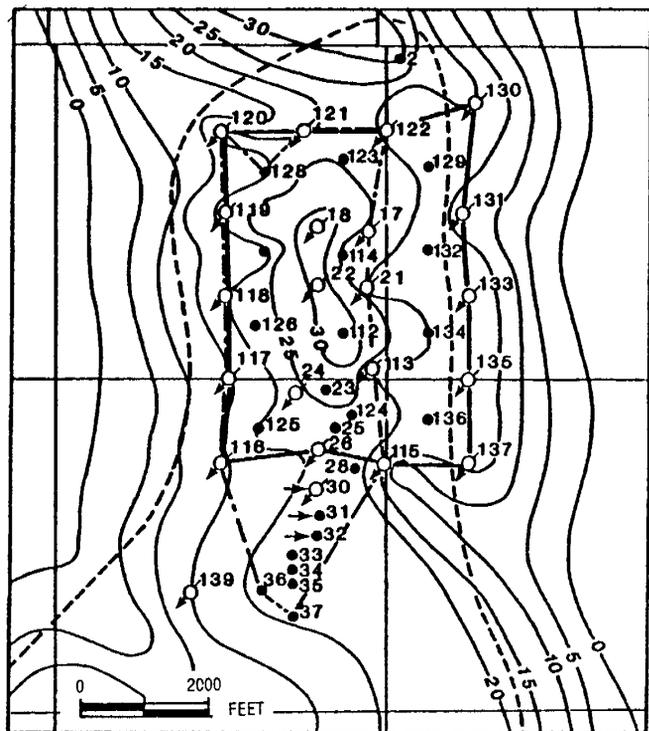
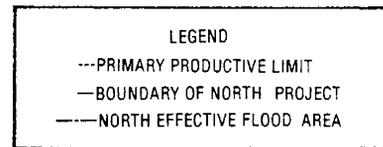


FIG. 1—Isopachous map, Lisbon unit.

The structure of the Lisbon Field is believed to be a terrace on a continuation of the El Dorado anticline. Therefore, the subsea depth to the top of the Nacatoch sand is fairly uniform throughout the field. This depth is about 1920 feet in the test area, and it is the average depth for the field. The net pay thickness ranged from 0-30 feet with a field average of 11.3 feet. The test area is located between the 20 and 25 foot contours on the isopachous map (Fig. 1). Bottom hole temperatures in this area range from 90°F to 105°F.

Normal secondary recovery operations already in progress continued throughout the test period. According to bottom-hole pressure data the water moved in a southward direction. Since Wells 30 and 31 are located near the south end of the north section, flood waters were constantly being pushed around and through the two-spot pattern. The effect, if any, that these operations may have had on the fermentation and its results were not determined. However, it is certain that the rate of flow between Wells 30 and 31 was determined by the field flood rather than injections into Well 30. Plots of total north section water injection, injection into the four wells immediately north of Well 30, and into Well 30 itself are shown in Fig. 2.

The field test was begun in May, 1954, with the injection of fresh water (less than 200 ppm sodium chloride) into Well 30. Previously, a mixture of produced and fresh waters (20,000-25,000 ppm sodium chloride) had been used for flooding. Two months later beet molasses was added to the injection water—first in a 60 bbl slug and then in amounts necessary to maintain a

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two percent concentration. This mixture was injected continuously for 5½ months. At first the injection mixture was allowed to flow into the well under gravity at a rate of approximately 100 bbl/day.

Later, because of a reduction in injectivity due to either gas or bacterial cells, it became necessary to install an injection pump in order to maintain rates of 100 to 500 bbl/day. Soon after the last bacterial inoculation, initial injectivity was restored.

Bacterial inoculations were begun simultaneously with the addition of molasses. Approximately 4000 gallons of heavy bacterial suspensions were flooded into the test well in 18 separate batches of 220 gallons each during the four month period (July–November). Bottom hole samples taken from the injection well in September gave ample evidence that an active clostridial fermentation was proceeding at the sand face. Samples of gas, water and oil were also taken at varied intervals from the test production well and all other producing wells in the vicinity of the test area throughout the experimental period. Analyses were performed on all samples for bacterial fermentation products, and on produced water samples for bacteria and salinity.

Fresh water breakthrough occurred in 70 days. During this period the average injection rate was 160 bbl/day in the test well. Fermentation products and sugar (unused molasses) appeared in produced water and gas 80 to 90 days after the initial inoculation. Average injection rate during this period was 70 bbl/day. The production of sugar, acids and carbon dioxide throughout the test is shown in Figs. 3, 4, and 5, respectively. After 286 days, fermentation products were also recovered from a producing well 665 feet from the test injection well and 265 feet from the test production well. All other producing wells were more than 1000 feet from the injection well and failed to show any evidence of bacterial fermentation after eleven months.

The principal fermentation products recovered in the produced gas and water from the test well were seven short chain fatty acids (formic through caproic, and caprylic) and carbon dioxide. Very small amounts of ethyl and butyl alcohol and acetone were also detected. The total bacterial acid production over the test period is calculated to be approximately 77,000 lb. This amount represents 59 percent of the sugar injected.

The carbon dioxide content of the gas produced from the test well varied between 39 and 82 percent (air free) during the experimental period. Wells not affected

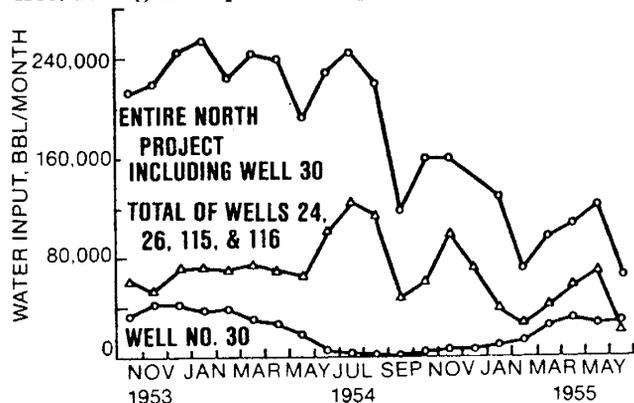


FIG. 2—Lisbon unit water injection.

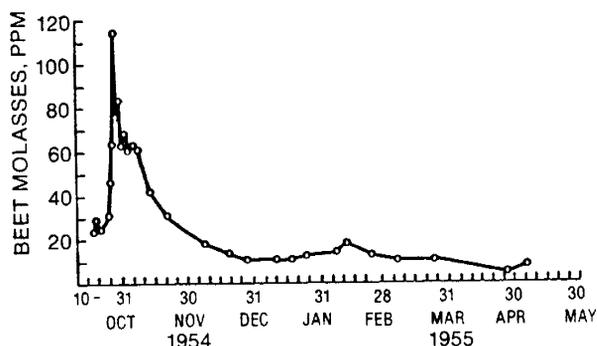


FIG. 3.—Beet molasses produced from Well No. 31.

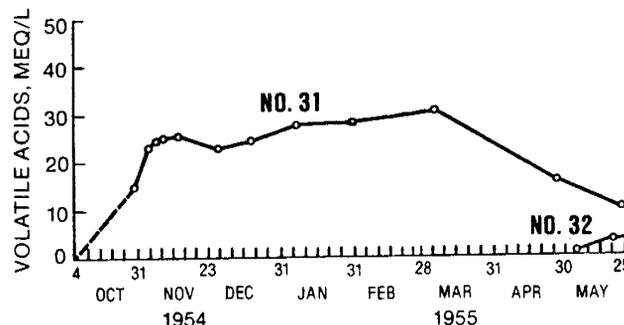


FIG. 4.—Volatile acids produced from Well Nos. 31 and 32.

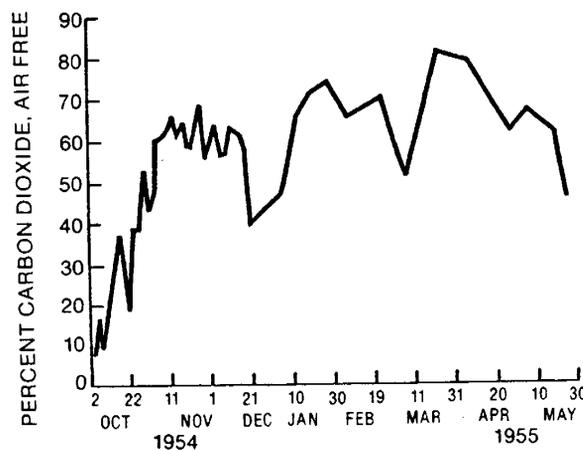


FIG. 5.—Carbon dioxide production, Well No. 31.

by the fermentation ranged from 0.3 to 8.8 percent. The total carbon dioxide produced in the fermentation was calculated to be approximately 200,000 cu ft (25,000 lb) which represents 19 percent of the sugar injected. The remainder of the gas from the test well was primarily methane. Little or no hydrogen was ever detected.

These results would not be expected from a pure *Clostridium acetobutylicum* fermentation. These organisms alone would produce three (formic, acetic, butyric) rather than seven acids, accounting for 35 to 40 percent of the sugar used. Also, 47 percent and 2 percent of the sugar would be converted to carbon dioxide and hydrogen, respectively. Presumably competing

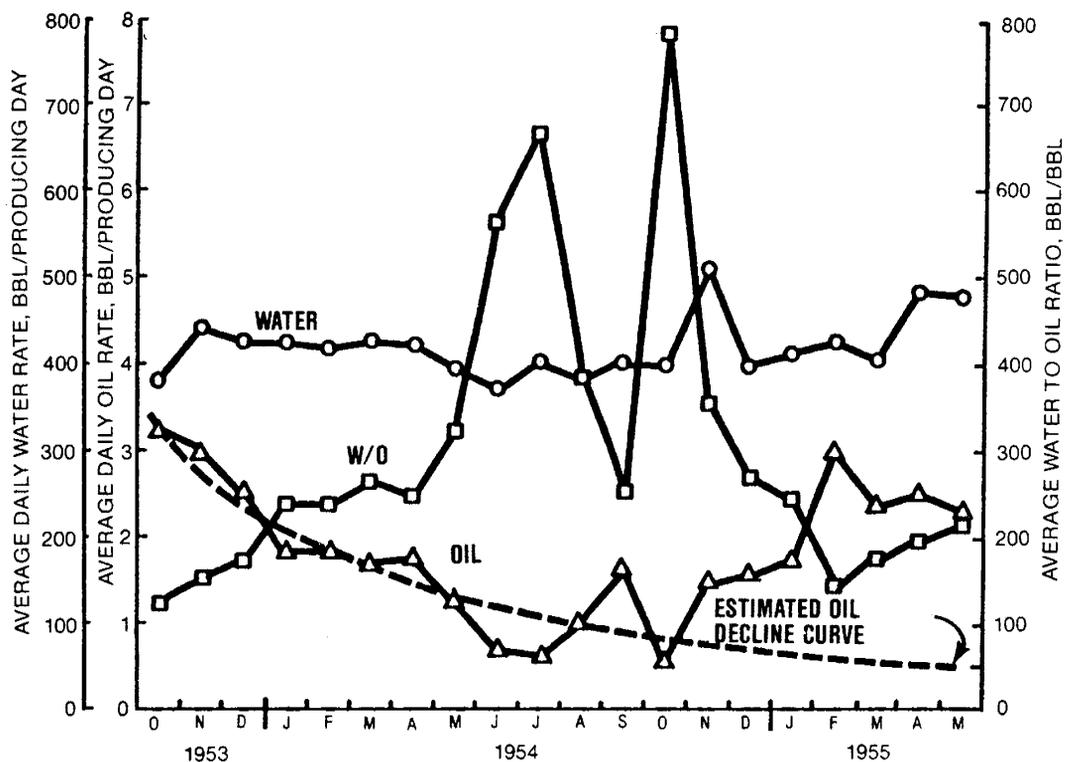


FIG. 6—Average daily production per producing day, Lisbon Well No. 31.

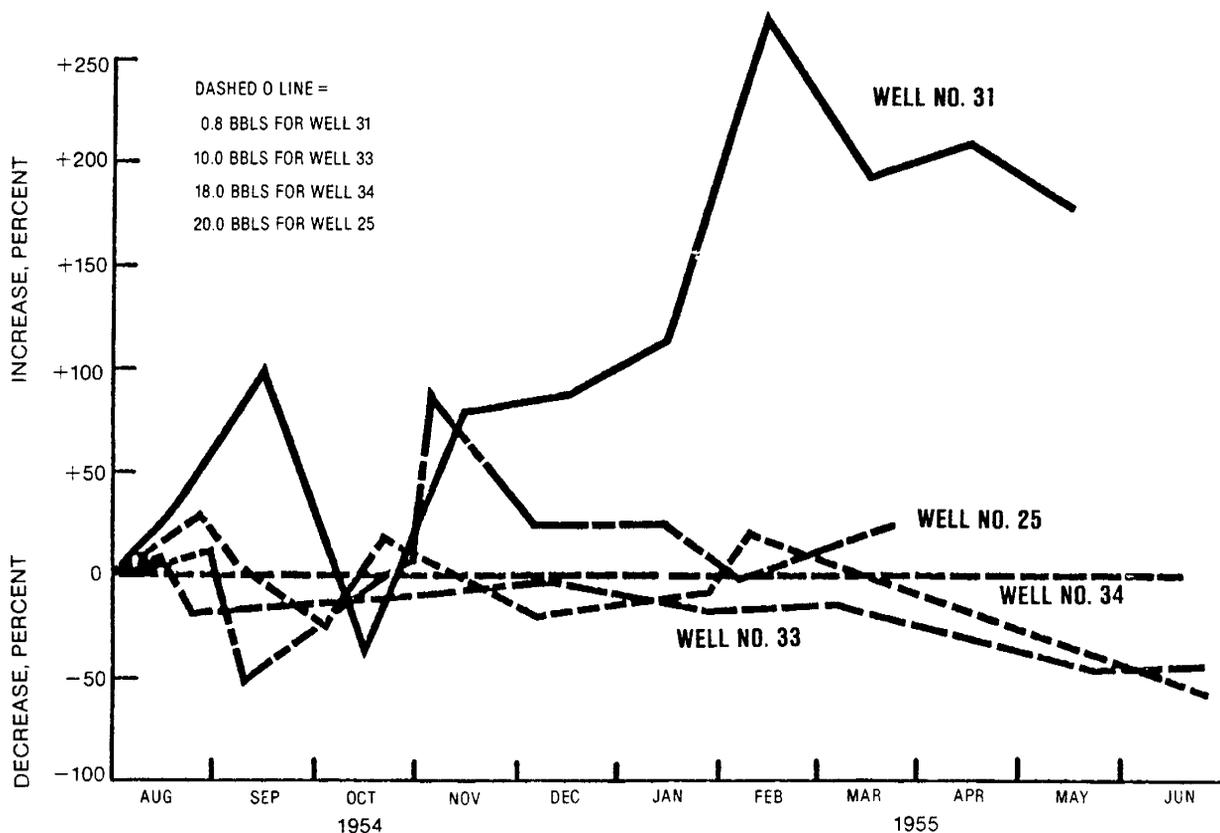


FIG. 7—Percent change in oil production from August 1, 1954.

MEOR Field Applications

fermentations (utilization of molasses) and secondary fermentations (utilization of primary fermentation products) occurred in the test area. Many bacteria, but not *Clostridium acetobutylicum*, were isolated from the produced water during the test. However, the numbers of the various types were too small and erratic to be correlated with the fermentation products recovered.

The actual occurrence of some of the postulated reactions has been demonstrated in the laboratory. Evidence for the conversion of the shorter chain acids (formic to butyric) into longer chains (valeric and caproic) has been obtained using a large sand pack in which field conditions were simulated. After four months, caproic acid appeared in the effluent. The bacterium responsible for this conversion was isolated and identified as *Clostridium kluyverii*. The bacterial conversion of carbon dioxide and hydrogen to methane was also demonstrated using field waters. Additional evidence for this latter fermentation was obtained by measuring the carbon isotope ratios (C^{14}/C^{12}) in the methane from the test well.

Twenty percent of this gas was found to be derived from a modern carbon source (presumably molasses), indicating that some of the carbon dioxide and hydrogen produced in the fermentation were converted to methane. Using the same analytical technique it was also shown that 20 percent of the produced carbon dioxide came from an ancient carbon source—presumably the result of acid attack on reservoir carbonate.

Samples of oil taken from the test well contained neither viable bacteria nor any of the fermentation products found in gas and water samples. These samples were also analyzed for various direct and indirect effects of bacterial action. Statistical evaluation of the data obtained showed no significant differences between samples taken from the test well and those from producing wells outside the test area.

The effect of the fermentation processes on oil recovery was studied by taking daily production data on Well 31 and by making periodic production tests on nearby wells. Figure 6 shows the production data for Well 31 from the time it was reopened on October 1, 1953, until its abandonment on May 27, 1955. A statistically significant increase in oil production occurred after October 1954. The water/oil ratio dropped correspondingly since water production was fairly constant.

Since Well 31 was watered out, the quantity of produced oil was not large, but the relative increase in oil production is significant. Based on the assumed normal decline curve shown as a dashed line in Fig. 6, the

average oil production rate for the period November 1954 to May 1955 should have been approximately 0.6 bbl/day while the actual average production was 2.1 bbl/day. The actual rate was 3.5 times the "normal" or an increase in oil production of approximately 250 percent.

As previously stated, the test area was affected by the regular field water flood operations. Therefore, the production rates of nearby wells were checked to see if the oil increase at Well 31 was unique or to be expected. The nearest regularly producing wells to 31 were 25 (northeast), 33 and 34 (southwest). Beginning with August 1, 1954, as a zero point the average daily production for each month from each of these four wells (25, 31, 33, 34) is plotted in Fig. 7 as percentage increase or decrease with respect to production on August 1.

It can be seen that none of these wells except 31 showed any significant increase in oil production. In fact, 33 and 34 declined on the average. Therefore, the increase in oil recovery rate at Well 31 differs from the norm and is assumed to have been favorably affected by the bacterial fermentation.

The mechanism responsible for increased oil production at the test well is not known. We can logically conclude that hydrogen was produced in the fermentation. It must have remained there, at least for some time, since no hydrogen was detected in produced fluids. Therefore, it is quite possible that after its production, free hydrogen resisted fluid displacement and acted as an effective blocking agent, forcing flood waters to seek new flow paths. This would, of course, cause previously unaffected oil-containing areas to yield oil.

Much carbon dioxide was produced both from fermentation and probably from the neutralization of fermentation acids by reservoir carbonate. This gas may also have exerted beneficial effects in the reservoir—such as lowering the pH of the water and viscosity of the oil. However, it is not likely that CO_2 contributed to a free gas phase. Reservoir pressures should have kept all of it in solution.

An analysis of recovery mechanisms must also include the unexpectedly high yield of organic acids resulting from the fermentation. The effect of these acids on reservoir carbonates must have been considerable. Many tons were required for neutralization. It is reasonable to suppose that dissolution of carbonate, with the concomitant production of CO_2 at the wetted surfaces, may have exerted appreciable effects on flood patterns and total recovery.

Microbial Enhancement of Oil Recovery in North Sea Reservoirs: A Requirement for Anaerobic Growth on Crude Oil

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All the field tests so far reported in which microorganisms have been injected directly into the reservoir strata have depended on nutrients, usually molasses, injected from the surface. It has never been clear how large a radius of effective action this has conferred on the injected microorganisms; however, it is probable that the nutrient itself, and hence microbial growth, would have been limited at most to a few tens of meters from the injection point. This may nevertheless not preclude the products of microbial action being carried further on a waterflood or that some bacterial cells might not similarly have been washed into the reservoirs, particularly if high permeability zones were present.⁴

The primary reason for a limited radius of action is not difficult to discern. Rich sources of nutrition, including molasses, promote relatively rapid bacterial growth. Under anaerobic conditions of fermentation, about 10 percent of a carbohydrate substrate is converted to cellular dry wt.⁶ Thus, 1 ton of molasses might yield 100 kg of microbial mass which, for small-sized organisms, would correspond to about 5×10^{17} cells.

The nature of bacterial growth, in which a mother cell enlarges and divides to give two daughter cells, means that a single bacterial cell in the course of some 59 generations would yield a progeny of 5×10^{17} cells and completely consume 1 ton of molasses. A very modest doubling time of, say, three hours, would result in total exhaustion of the molasses in about 7.5 days. Increasing the charge of molasses to 10 or even to 100 tons would delay exhaustion by only 10–20 hours. These values are, of course, very rough approximations but even if they contain two- or three-fold errors it is clear that the maximum period of *in situ* fermentation is likely to be very restricted indeed.

Valuable though microbial enhancement methods with surface feeding might be in many geographical locations, they would obviously pose severe problems for fields with wide well spacings, many of which are to

be found in offshore locations. In the North Sea, for example, the difficult weather conditions, coupled with the depth of water and the distance from the nearest shore base, have resulted in extremely high drilling costs. A small number of platforms are therefore sited in the most opportune portions in the fields and from them a minimum number of deviant wells are drilled to provide optimum drainage of the reservoir.

The oil-bearing strata themselves lie more than 2000 m below the sea bed. The combination of all these considerations has led to well spacings of the order of 1 km. Estimates vary of the fluid transit times required for water injected at one point to appear at a neighboring production well, but periods of 5–12 years are spoken of. It need hardly be remarked that under such conditions there is no prospect whatever of using *in situ* microbial enhanced oil recovery procedures based upon the injection of organic nutrients as growth substrates.

In theory an alternative lies readily to hand: crude oil, the very material it is sought to recover, and which is distributed in large quantities throughout the reservoir, might itself be a potential substrate for microbial growth. The ability of many microorganisms to metabolize and grow on crude oil is well known, but normally requires the presence of molecular oxygen as an electron acceptor.

Because of its corrosive effect on steel pipework, oxygen is usually removed from injected floodwater, but even if it were not the quantities dissolved in seawater would be inadequate to maintain microbial growth on crude oil for the requisite periods of time. The only feasible microbial methodology for these deep, offshore reservoirs is one in which crude oil would act as a carbon and energy source under conditions of strict anaerobiosis in which the electron acceptor would be a substance other than molecular oxygen.⁵

The question of whether or not constituents of crude oil can be metabolized by bacteria under such anaerobic conditions has been approached by the use of

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anaerobic enrichment techniques. In this paper we shall be concerned with demonstrating only that such growth is possible and not with the identity of the microorganisms involved or with any details of their biochemical mechanisms.

Microbial populations were obtained from a variety of industrial and natural locations: from oil-water separator tanks at refineries, oil-soaked soil, the marine sediment beneath production platforms in the North Sea and from soils in the vicinity of natural surface hydrocarbon. The presumed mixed populations in such samples were maintained for many months at 28°C under nitrogen with a suitable mineral medium and Forties crude oil (kindly supplied by British Petroleum Ltd.) as the sole carbon source.

Activity in the mixed cultures was monitored at weekly intervals by measurement of the volume of gas produced. Periodic subcultures into fresh mineral medium plus additional crude oil were made. The mineral media were selective for bacteria using nitrate as a terminal electron acceptor in the respiratory chain and for methanogenic bacteria.

Nitrate-reducing cultures were incubated under helium or argon gas so that the concentration of nitrogen in the tubes after incubation might be determined, and

the experiments included suitable controls. At each sampling the concentrations of carbon dioxide and of nitrogen in the head space of the cultures were analyzed by gas chromatography. The pH, absorbance at 650 nm and the concentrations of protein and of nitrite were determined for the aqueous phase of each culture.

Under these conditions no significant pH changes were observed over a 14-18 day incubation period. However, nitrite concentration increased during the first 4 days and then declined over the period 5-10 days, possibly as a consequence of further reduction to nitrogen gas (Fig. 1). Carbon dioxide concentrations also rose during the first 10 days of incubation (Fig. 1) showing that anaerobic metabolism of crude oil was taking place since no other carbon source was present. Protein concentration increased markedly during the first 3 days of incubation, thereafter remaining constant until 18 days, while the absorbance of the culture measured at 650 nm increased for 6 days and then remained fairly constant (Fig. 2).

TABLE 1—Anaerobic Gas Production on Various Substrates

A natural mixed population of sediment bacteria was incubated anaerobically with various substrates at 28°C. Gas production was measured at 10-day intervals.

Carbon Source Added	Cumulative Gas Production During 168 Days (as Percent of Control)	Lag Period Before Gas Production Exceeded That of Control (Days)
Control	100	—
Cyclohexane carboxylic acid	102	67
Decane	60	—
Dekalin	156	49
Forties crude A	209	31
Forties crude B	177	49
Gas oil	5	—
Hexadecane	121	127
Naphthenic acid	133	67

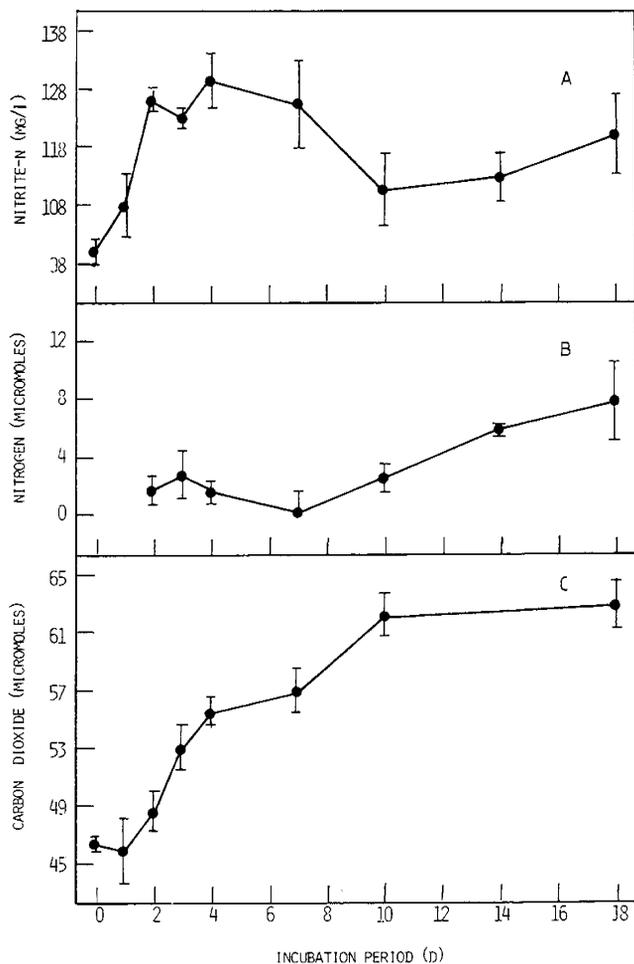


FIG. 1—Release of nitrite (A), nitrogen (B) and carbon dioxide (C) by mixed cultures of nitrate-reducing bacteria cultured anaerobically on Forties crude oil.

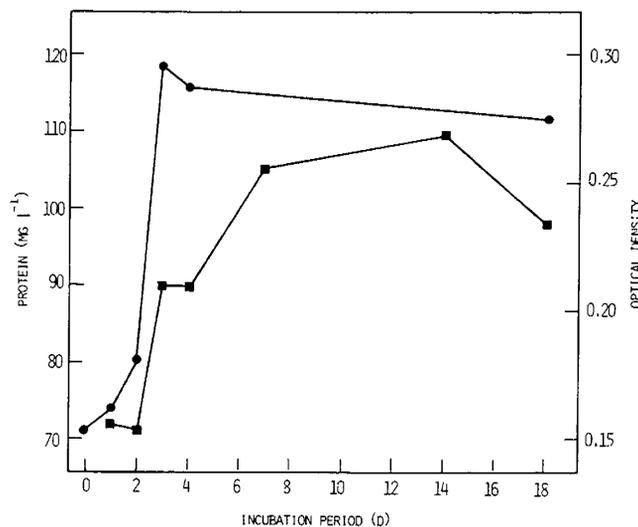


FIG. 2—The effect of incubation period on optical density (■) and protein concentration (●) in mixed cultures of nitrate-reducing bacteria grown anaerobically on crude oil.

Prolonged incubation of cultures in this way appeared, in the course of time, to favor the growth of a few bacterial species. Samples were removed from enrichment cultures which had been subcultured many times over a period of several months and were plated onto Bakker's medium¹ supplemented with 1 percent glucose and 2 percent agar. After incubation anaerobically for 4 weeks at 28°C, individual cultures were plated onto Bakker's agar supplemented with 1 percent Forties crude oil emulsified into the agar by ultrasonic agitation.

The plates were again incubated anaerobically for 4 weeks at 28°C and growth of bacterial colonies was observed on all the plates. Microscopic examination of the colonies showed an apparently homogenous population of bacteria in all cases, although microscopic observations of growth in liquid culture indicated the presence of at least two bacterial types on morphological criteria. Further investigations are in progress.

Enrichment cultures were set up strictly anaerobically under nitrogen gas on Ferry & Wolf medium,² selective for methanogenic bacteria, with Forties crude oil as the sole carbon source. Incubation was at 28°C and by the weekly addition of more crude oil and mineral medium continuous gas production has been maintained for many months (Fig. 3). In the 18 days after subculturing methane concentration showed a steady rise after an initial 2-day lag (Fig. 4).

Measurement of the gases produced during a 20-week incubation showed a methane:carbon dioxide ratio of $1.51(6) \pm 0.23(1.26-1.90)$. These data demonstrate the occurrence of methanogenesis in mixed microbial cultures in a suitable mineral medium with crude oil as a sole source of carbon. Methanogens are generally recognized to be among those bacteria showing the least tolerance to molecular oxygen.³ The observation of methane production thus demonstrates the effectiveness of our precautions to exclude oxygen.

The abilities of natural mixed populations of sediment bacteria to degrade anaerobically a range of hydrocarbons and related materials were investigated by incubating a slurry of sediment in the presence of the carbon source under test together with water from a waste lagoon at 28°C. No electron acceptors were

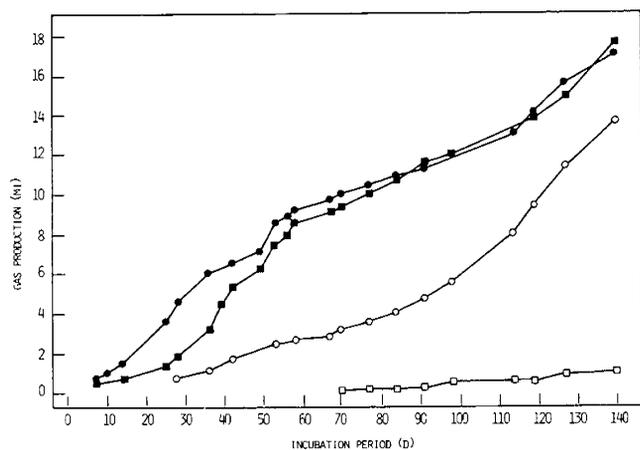


FIG. 3—Cumulative gas production from crude oil by anaerobic cultures enriched for methanogenic bacteria. Three parallel cultures (●○■); control without bacteria (□).

added; gas production was measured at roughly 10-day intervals over a 6-month period. Growth, as represented by gas production, was slow. Table 1 shows the production of gas as a percentage of that shown by the control, which received no carbon source. All the potential substrates were able to support growth except for decane and gas oil, both of which showed inhibition of gas production.

Table 1 reports the period in days before gas production with added substrates unequivocally exceeded that of the control. Once established, gas production in excess of the control was maintained at a constant rate throughout the incubation period. It should be noted that gas production in the control tubes was also linear for the whole 6-month experiment.

The susceptibility of ostensibly anaerobic growth on Forties crude oil to the presence of oxygen was determined. Three parallel cultures were incubated for 62 days in mineral medium selective for nitrate-reducing bacteria under our standard conditions of anaerobiosis in the presence of Forties crude oil. Linear rates of gas production were observed essentially from the commencement of the incubation period.

One vessel received no further treatment and acted as a control. The second received an injection of gaseous nitrogen, while the third was injected with air to give a concentration of oxygen in the gas space of about 13 percent (v/v). Figure 5 shows that the presence of oxygen rapidly terminated further gas production although the injection of nitrogen gas was without effect. Experiments are currently in progress to determine more precisely the concentration of oxygen necessary to achieve inhibition.

The experimental results reported in this communication show that anaerobic microbial growth on hydrocarbons and related materials is possible. It is of particular interest that, among the potential substrates tested, Forties crude oil promoted the fastest growth

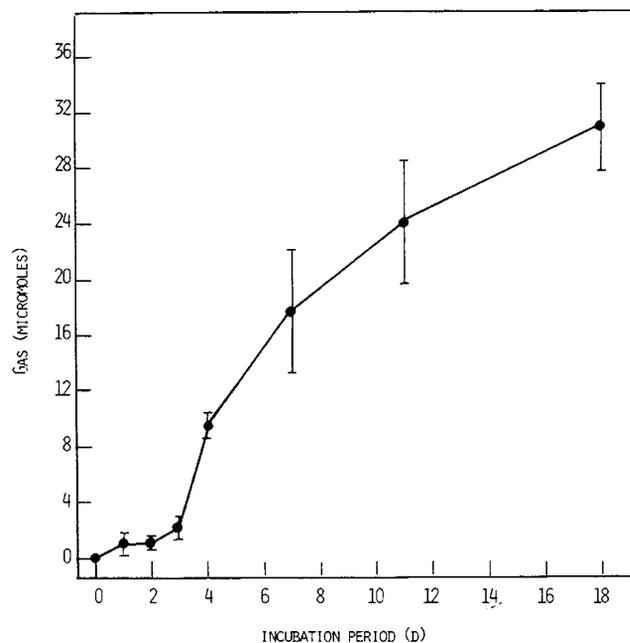


FIG. 4—Release of methane from crude oil by an anaerobic culture enriched for methanogenic bacteria.

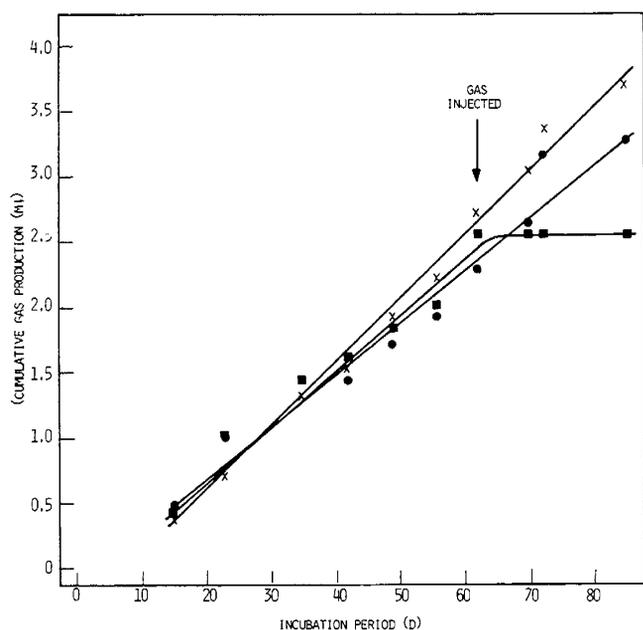


FIG. 5—Effect of molecular oxygen on anaerobic gas production from Forties crude oil by cultures enriched for nitrate-reducing bacteria. Control (x); nitrogen injected (●); oxygen injected (■).

rate. This observation suggests the presence in such crude oil of a growth substrate(s) more favorable than any of the pure substances which were tested. At this time we are not able to identify the chemical nature of the substrate(s).

It has been our immediate purpose in conducting these experiments to determine whether or not anaerobic growth on crude oil is a biological reality which can be incorporated into a scheme for developing a technology for microbial enhanced oil recovery. It is

our conclusion that such growth is indeed feasible. It must nevertheless be noted that growth rates were very low.

Whether faster growth would be necessary or desirable for an operating system cannot be decided with certainty until other aspects of such a system have been determined: these would include defining the nature and quantities of the microbial products required for oil mobilization and the relationship between microbial growth rate and the synthesis of such products. It does seem likely, however, that more rapid growth rates will be necessary and it remains to be seen whether or not they can be achieved.

The experimental work reported in this paper was carried out under contract to the United Kingdom Department of Energy.

REFERENCES

1. G. Bakker (1977). Ph.D. Thesis, University of Delft.
2. J. G. Ferry and R. S. Wolf (1976). *Arch. Microbiol.* 107, 33.
3. G. Gottschalk (1981). In "The Prokaryotes" (ed. by M. P. Starr, H. Stolp, H. G. Trüper, A. Balows and H. G. Schlegel), Vol. II, p. 1421. Berlin, Heidelberg, New York: Springer.
4. I. Jaranyi (1968). *M. All. Földtani Intezet Evi Jelentese Az 1968, Evröl* 423.
5. V. Moses and D. G. Springham (1982). "Bacteria and the Enhancement of Oil Recovery." London: Applied Science Publishers.
6. R. Y. Stanier, E. A. Adelberg and J. L. Ingraham (1977). "General Microbiology" (4th ed.), p. 285 (Table 9.2). London: Macmillan.

Some Constraints on the Use of Bacteria in Enhanced Oil Recovery

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All but a small proportion of U.K. oil production presently comes from the offshore fields of the North Sea. Production and exploration here are dominated by the depth of the water, and by the unfavorable weather which makes supply, and even access, a major problem. Because of the expense of maintaining platforms, fields are exploited by divergent drilling, many wells being serviced from a single platform with typical well-spacings of several hundreds of meters.

Water injected at one well is likely to take seven years or more to penetrate to a production well so any enhanced oil recovery process needs to operate with an unusually high degree of stability with respect to time and distance. Chemical procedures for enhanced oil recovery would require an enormous investment because of the amounts of materials needed to treat the large swept volumes between wells coupled with the cost of maintaining and operating platforms for several years before any return in the form of increased oil output could be expected.

Even the transport of materials to the platforms would pose severe logistic problems during the large part of the year when the weather is particularly unfavorable.

The North Sea fields are clearly difficult targets for enhanced oil recovery (EOR) but their size and their dominant position in U.K. oil production dictate that, despite the difficulties, careful attention should be given to the possibility of EOR operations. Our group has been conducting a study, funded by the U.K. Department of Energy and the Science & Engineering Research Council, to investigate the feasibility of microbial enhanced oil recovery (MEOR) methods in the North Sea fields. It was clear at an early stage that problems of cost and supply ruled out the provision of a carbon source for bacterial growth via the waterflood.

An alternative approach which we envisaged promised considerable reductions in cost and in the prob-

lems of transporting materials to the rig and this is described in a later paper (Moses *et al.*, these Proceedings). With this in mind we were encouraged to consider how some of the other major problems might be overcome.

MEOR processes depend on the injection of a bacterial inoculum with the waterflood. Success depends on adequate penetration of bacteria into the reservoir rock. Since the injected bacteria constitute an inoculum it is not essential that they all penetrate: in principle a single bacterial cell is sufficient to start growth at one particular site. Nor is it essential in all cases for the bacteria to penetrate throughout the reservoir. Thus, for instance, bacterial surfactants, generated *in situ*, may be able to move ahead of the cells and mobilize oil in regions not reached by the cells, and operations dependent on selective plugging by bacterial cells or their gaseous products require, by definition, uneven distribution of cells.

Nevertheless, an MEOR operation on anything but a small scale, demands the ability to predict with some confidence how cells will move through the reservoir. In a particular case we need to know with confidence whether cells will move away from the injection face and, if so, the linear rates of movement which can be expected, so that the time taken to establish bacterial growth throughout a specified portion of the swept pattern can be calculated.

The converse of penetration by bacterial cells is plugging. It is well known that fine particles in aqueous suspension can reduce the permeability of a porous matrix and, under unfavorable circumstances, bacteria in injection waters could decrease injectivity. Any MEOR operation other than the most casual field trial must attempt to assess the dangers of plugging; a large scale operation on a valuable field could only proceed on the basis of a clear understanding of the risks involved and of the operating procedures necessary to reduce them to negligible proportions.

There are many reports in the literature on bacterial penetration and plugging based on both field expe-

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rience and on laboratory experiments (for a review, see Moses & Springham)¹⁰ and this topic is considered in detail by some of the later papers at this Conference. It is clear from theoretical considerations and from experimental evidence that many factors can influence the penetration or plugging of cells in a porous matrix, these include:

1. Properties of the rock matrix such as permeability, pore-throat size, porosity, chemical properties such as surface charge, presence of oil, salinity of formation water.

2. Properties of the cells such as size, shape, whether the cells grow singly or in clumps or chains, surface charge, production of capsules, slimes, chemical precipitates, acids or gases and, possibly, whether or not cells are motile.

3. Properties of the injection system such as water flow rate, salt content and the density of the cell suspension.

While it is undoubtedly true that bacterial cells do have the potential to plug porous rock, reports of problems caused by bacteria must be interpreted with some caution in relation to the potential dangers of MEOR. Plugging associated with bacterial cells may be due either to the cells themselves or to their metabolic products. Bacteria which reduce sulfate to sulfide or oxidize iron may produce inorganic precipitates which are far more troublesome than the bacterial cells themselves. The lesson for MEOR is that many problems may be avoided by the choice of an appropriate bacterial strain and it is perhaps significant that none of the field trials reported in the literature seems to have encountered any serious problem from plugging.

To predict the behavior of bacteria in reservoir rock, a quantitative model is required which takes into account all the important variables. An approach to such a model is described in the paper by Teh Fu Yen later in this Conference.

Our approach to the study of penetration and plugging has made use of bacterial cells labelled with ³²P. Bacterial cells are grown in defined media containing limiting amounts of inorganic orthophosphate and to which high specific activity ³²P-orthophosphate is added. After growth, cells are washed to remove excess radioactivity and suspended at a known concentration in pre-filtered buffer. For most of the preliminary experiments we have used columns of glass microbeads with diameters less than 60 μ, packed to give various permeability values. Cores of Clasach sandstone, an outcrop rock of very uniform structure have been used in occasional experiments.

Suspensions of radioactive cells are injected into the column or core by a precision syringe pump at a rate sufficient to give a linear movement of the liquid of about 1 ft per day. The fluid emerging is passed through the counting chamber of a continuous flow scintillation counter; counts from emerging bacterial cells are summed over each 10-min period by a scaler and the results recorded on a teleprinter.

The counter has three channels which can be used in parallel to monitor three experiments simultaneously. A solid state pressure transducer, connected across the column, monitors pressure changes to assess the extent of any plugging. As an alternative,

emerging cells can be monitored by viable counts, a procedure which is more laborious than the isotope method but which, unlike the latter, is suitable for use with growing cell suspensions. The isotope method is designed to permit the rapid collection of data as three experiments can be run at once and the continuous presence of an operator is not required.

Some problems have been experienced due to the effects of the isotope on the living cells. For instance, ³²P has an inhibitory effect on growth if the specific activity is high^{9,11} and cells incorporating ³²P into their DNA lose viability when the radiophosphorous decays. The specific activity of the ³²P-phosphate used must therefore be chosen to compromise between a value high enough to provide adequate sensitivity of cell-detection and a value low enough to permit growth. At present we are using specific activities of about 18.5 MBq/μ mole. This permits us to detect as few as 10³ cells per ml.

Another problem arose when we passed labelled cell suspensions of *Escherichia coli* through a column. Cells lysed at some point during their passage through the column with the result that radioactive debris of some sort became adsorbed onto the surface of the flow cell producing a very high background count which made accurate measurement of the concentration of cells emerging from the column impossible. This problem was not encountered with *Pseudomonas putida* and *Bacillus megaterium* which were used for subsequent experiments but we have had problems with soluble radioactive compounds which leak from the cells.

This phenomenon is easy to detect by filtering cell suspensions on membrane filters and measuring the radioactivity in the soluble and particulate fractions respectively but loses the advantage of the automatic measurements. At present we are trying to solve this problem by pretreatment of cells to prevent loss of label.

The North Sea fields, besides their other difficulties are characterized by high values of temperature, pressure and salinity. For instance, Forties field has a temperature of around 90°C and an initial pressure of 22 MPa (Hawes, et al).⁴ These are not the most extreme conditions to be expected, thus Ninian field has a temperature of 102°C and initial pressure of 48 MPa and for Magnus field the figures are 120°C and 40 MPa respectively.

Most bacteria will tolerate pressures up to 20 MPa but above this an increasing proportion of strains are inhibited by pressure even when all other conditions are optimal (for reviews see Zobell and Kim,¹² Marquis,⁷ Marquis & Matsumura,⁸ and Marquis, these Proceedings).

Temperature appears to be a more serious limiting factor. Until recently 85°C appeared to be about the upper limit at which reproducible growth of bacteria under laboratory conditions could be clearly established. If this were indeed an absolute limit it would place considerable restrictions on the exploitation of deep reservoirs by MEOR techniques. There was, however, some reason to believe that bacterial growth could occur above this temperature under the right circumstances.

The growth of bacteria in hot springs in Yellowstone National Park, U.S.A., has been known for some years and Thomas Brock and his collaborators have

made a long and detailed study of microbial life at the highest temperatures. They were unable to culture organisms above 85°C but, in an extensive survey in which microscope slides were immersed in hot springs in Yellowstone and in Iceland and New Zealand they found microbial growth on the slides in the vast majority of cases, including those in which pool temperatures were as high as 101°C.^{1,2,3}

In interpreting this work it is essential to establish that the bacteria detected are really growing and are not merely non-growing contaminants from outside. In this instance the experiments were performed by a well-respected group with considerable experience in working with thermophiles. They took considerable care to establish that cells on the slides did not arise by contamination from outside and there appears to be no reason to doubt their conclusion that bacterial growth is indeed possible up to about 100°C.

Since many earlier attempts to isolate organisms capable of growth above 85°C have been unsuccessful, and lacking easy access to suitable hot springs, we decided to try to increase the temperature limit for existing cultures of extreme thermophiles by selection of mutants. We reasoned that thermal tolerance, like all or most other properties of living organisms, would be under genetic control, and that in a population of bacterial cells there would be some degree of variation in tolerance from cell to cell due to spontaneous mutation. A thermotolerant mutant could, in principle, be isolated by growing the culture at a sufficiently high temperature.

Figure 1 shows the typical relationship between temperature and growth rate for bacterial cells in the absence of nutrient limitation. For a given culture growth rate will increase with temperature to a maximum value at the optimum growth temperature (T_{opt}). Above this temperature the growth rate will decline rapidly with increasing temperature. T_{max} is the maximum temperature at which growth is possible. The exact values of T_{opt} and T_{max} will vary over a very wide range among different species and strains of bacteria, and, for a given strain, will vary to a smaller extent under the influence of other factors such as pressure, salinity and the types of substrate used for growth.

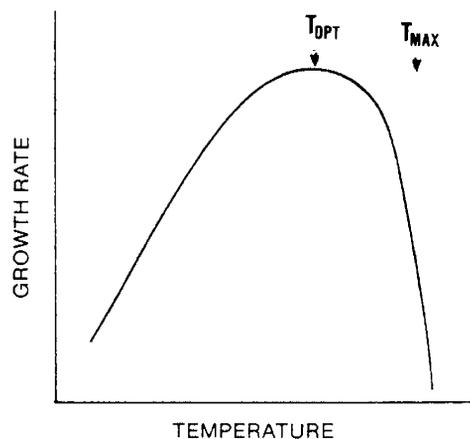


FIG. 1—Generalized relationship between temperature and bacterial growth rate.

If a culture is grown in the region between T_{opt} and T_{max} under conditions where nutrients are nonlimiting, a mutant with a slightly improved temperature tolerance would be expected to grow slightly faster than other cells in the population. If a system of continuous culture were used, the tolerant mutant would eventually supplant the other cells in the culture vessel. *A priori* we assumed that only slight improvements in thermal tolerance would be obtained at a single step: all cellular components need to be able to tolerate the highest growth temperature, above this a particular component would become rate-limiting and to increase thermal tolerance this component would need to be changed by mutation. At this point another component would presumably limit thermal tolerance and this in turn would have to be changed by mutation to achieve a further improvement and so on. We thus assumed that a series of mutations would have to be selected, each resulting in a small increase in thermal tolerance.

The most commonly used system for laboratory-scale continuous culture, the chemostat, works by limiting the nutrient supply to the bacterial culture. As we wished to work with cultures where growth was limited by temperature rather than by nutrient supply, the chemostat was unsuitable and we had to use an alternative system known as the turbidostat. To our knowledge, no commercial instrument of this sort was available at the time, so we had to construct our own. Figure 2 is a block diagram of the instrument. It is based on a commercial chemostat with modifications to the temperature control and readout (not shown) to permit precise temperature control up to at least 90°C.

In the turbidostat fresh growth medium is added whenever the cell density reaches a predetermined value as measured by light absorbance of the culture in the external flow cell. The culture is thus diluted, but when sufficient growth has occurred to restore the original cell density more medium is added and so on. This system ensures that adequate medium is always present and permits the growth rate to be controlled by temperature. In our apparatus each medium addition is of a fixed volume and is recorded as a bar on a chart recorder.

The rate of addition of medium, which is proportional to bacterial growth rate, can thus be simply obtained by inspection of the chart. The system worked well in trials with *Escherichia coli*, and with *Thermus thermophilus* at temperatures well below T_{max} , but at about 80°C, a temperature very close to T_{max} , erratic operation occurred due to growth of cells on the surface of the flow cell. The apparatus has been redesigned to eliminate this problem but no further experiments have been performed with it. Instead we have been investigating reports which have appeared in the literature of organisms able to grow at or near 100°C in laboratory culture.

Thus Heinen and Lauwrrers⁵ and Lauwrrers, Heinen and Mulders⁶ reported that a strain of *Bacillus caldolyticus*, originally isolated from a hot spring in Yellowstone Park, could be grown in continuous culture at temperatures up to 100°C (105°C with pressurization), using media based on mineral salts and yeast extract. We attempted to repeat this experiment using the media described by Heinen, both in a conventional chemostat and in an exact replica of the continuous culture vessel used in his experiments. Despite visiting his laboratory

and using two different cultures of *B. caldolyticus* which he kindly supplied, we were unable to obtain growth in continuous culture at any temperature above 73°C. We cannot say for certain why we were unable to repeat the work of Heinen's group but we have now shifted our attention to another organism. This is a methanogen and is presently growing in our laboratory at 95°C.

We are now investigating the effects of temperatures and pressures in the range of interest on some thermophilic strains. This work is at an early stage but good growth has already been observed at 70°C and 30MPa pressure and we are seeking to extend the range. We are now optimistic about the chances of getting bacterial growth at pressures and temperatures representative of a significant proportion of the North Sea fields.

The exploitation of deep fields still presents many special problems. Assuming that bacteria able to grow in these extreme environments can be found it is unlikely that they will possess the other necessary attributes for MEOR such as, for instance, anaerobic growth on hydrocarbons, the ability to mobilize oil and to spread throughout the rock formation without causing plugging. Strains with a complete range of appropriate properties will thus have to be constructed using the techniques of genetic manipulation to transfer attributes between strains: most probably this will require genetic information to be transferred from other strains into a strain which is temperature, pressure and salt tolerant.

There are two other serious problems which have to be faced in MEOR operations. One is the growth of unwanted bacteria: there is much evidence of bacterial activity on oil fields where waterflooding has occurred and the injection of organic nutrients such as molasses is likely to stimulate growth of the indigenous population which may compete with bacteria injected deliberately. Nutrient penetration may also be a problem since nutrients added from the surface will tend to be removed by bacteria in the immediate vicinity of the injection well, thus restricting bacterial growth to a small region of the reservoir. It has not escaped our attention that these problems are likely to be much less serious in deep hot fields where crude oil is the only carbon source.

REFERENCES

1. Bott, T. L. and Brock, T. D. (1969). *Science*, *164*, 1441.
2. Brock, T. D., Brock, M. L., Bott, T. L. and Edwards, M. R. (1971). *J. Bacteriol.* *107*, 303.
3. Brock, T. D. and Darland, G. K. (1970). *Science* *169*, 1316.
4. Hawes, R. I., Sayers, J. J. and Matthews, J. D. (1980). *Proc. Europec. Conf.* *1*, 473.
5. Heinen, W. and Lauwrens, A. M. (1981). *Arch. Microbiol.* *129*, 127.
6. Lauwrens, A. M., Heinen, W. and Mulders, J. W. M. (1981). *Arch. Microbiol.* *130*, 159.
7. Marquis, R. E. (1976). *Adv. Microbial Physiol.* *14*, 159.
8. Marquis, R. E. and Matsumura, P. (1978). In: "Microbial Life in Extreme Environments," ed. D. J. Kushner, p. 105. Academic Press, New York.
9. McFall, E., Pardee, A. B. and Stent, G. S. (1958). *Biochim. Biophys. Acta* *27*, 282.
10. Moses, V. and Springham, D. G. (1982). "Bacteria and the Enhancement of Oil Recovery." Applied Science Publishers, London.
11. Schmidt, C. F. (1948). *J. Bacteriol.* *55*, 70.5.
12. ZoBell, C. E. and Kim, J. (1972). In: "The Effects of Pressure on Organisms," eds. M. A. Sleight and A. G. MacDonald, p. 125. Symposium No. XXVI. Society for Experimental Biology. Cambridge University Press, Cambridge.

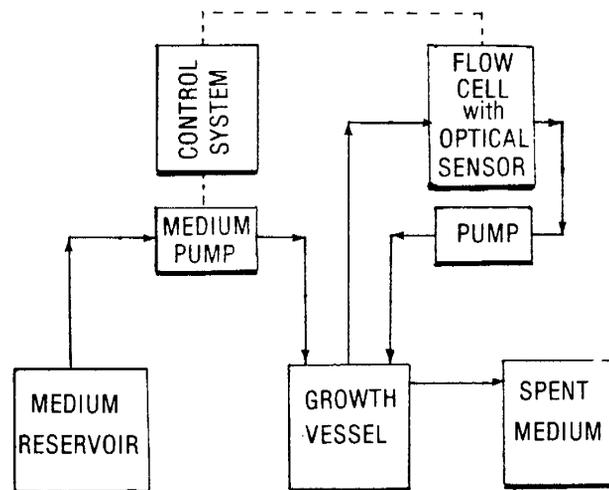


FIG. 2—Turbidostat: block diagram. Flows of medium with or without cells are shown by arrows. Dotted lines show control signals. Provisions for stirring, temperature control and aeration are not shown.

Petroleum Microbiology and the History of Its Role in Enhanced Oil Recovery

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INTRODUCTION

The world moves on an oil economy. Thus even a small oil shortage results in higher prices which are magnified in economic and social effects in both producer and consumer countries. This situation has resulted in a concentrated effort to find and produce more oil.

It is now recognized that only about 30 percent of the oil discovered in the world can be recovered using current technology. The realization that most of the oil is left in the reservoir after primary and secondary oil recovery techniques has prompted the widespread investigation of tertiary oil recovery methods. While there are many proposed tertiary processes such as chemical flooding, steam, micellar, CO₂, polymer, etc., no single one will be applicable to all oil reservoirs due to the wide variations which occur between reservoirs. In addition, these tertiary recovery processes while technologically successful, must also be economically successful. Unfortunately, in many cases, it appears that the costs of tertiary processes have escalated at the same rate as the price of oil due to their dependence on raw material or energy costs so their cost effectiveness becomes reduced.

One tertiary oil recovery process that is now receiving renewed interest worldwide is the use of microorganisms and their metabolic products to stimulate oil production. I have termed this use of microorganisms as a tertiary oil recovery method the MORE (Microbial Oil Recovery Enhancement) process. Basically the MORE technique involves the injection of selected microorganisms into the reservoir and the stimulation of their growth *in situ* in order that their presence improves oil recovery. This unique process differs from

other conventional tertiary oil recovery processes in that a self-duplicating unit is injected into the reservoirs and by its multiplication *in situ*, magnifies its effects out of proportion to its small initial volume. Some of the mechanisms proposed by which these microbial agents could stimulate oil release are shown in Table 1. (See Table 1.)

That microorganisms could exert such massive changes in oil reservoirs has been demonstrated in the past, much to concern of the oil industry, by reports of entire oil and gas reservoirs turning sour with H₂S due to microbial action. If such uncontrolled microbial action has occurred, it is possible that a similar controlled microbial process can be employed that will lead to additional oil recovery. This report compiles and summarizes the available data from past field tests in which wells and reservoirs were purposely inoculated or microbial growth was encouraged to produce such a favorable oil release.

BACKGROUND

The introduction of microorganisms into reservoirs for the purpose of enhancing oil recovery was first suggested by Beckman in 1926. The pioneering studies by C. E. ZoBell under API Research Project 43A (1943-53) showed possible mechanisms by which microorganisms could cause oil release. Workers in the USSR, including S. I. Kuznetsov, I. L. Andreyevsky, L. D. Shturm, V. M. Senyukov, and others, were extremely active and completed extensive examinations of the microflora of oil field waters and developed a strong geomicrobiological base. Such work was complemented by M. Dostalek and M. Spurny in Czechoslovakia and by J. Karaskiewicz in Poland and I. Jarnyi, M. Dienes, and L. Kiss in Hungary who were involved in various aspects of the laboratory and field tests of the technique. In 1954, V. F. Coty, D. Updegraff and H. Yarborough conducted a field test for Mobil Oil in the United States while other field tests were made in Czechoslovakia (1954-58).

Various techniques, cultures, modifications, and studies were made during this period but most results were restricted by proprietary interests and were released only through the patent literature (D. Bond and D. Hitzman). In the 1960's additional field tests were conducted in Poland, Hungary, and USSR but interest in the United States had declined due to low oil prices. Such is not the case today.

TABLE 1—Proposed Mechanisms of Microbial Oil Release

1. *In situ* generation of CO₂ for pressure and solubilization
2. Microbial production of organic acids
3. Conversion of hydrocarbons to lower molecular weight
4. Production of surface active agents
5. Encourage consolidation of oil to droplets
6. Enzymatic modification of hydrocarbons
7. Viscosity improvement

More comprehensive discussions of the theory, laboratory tests, cultures, and the advantages and disadvantages of the MORE technique have been covered in previous publications and reviews. However the area that has received limited attention, except for generalized statements, is that of actual field results. The reasons for this omission are varied but include factors such as: the data are published in obscure or nonbiologically oriented journals, not readily available and usually requiring translation, test results span years and are reported only as fragments in different publications, proprietary interests delaying or preventing publication of results, etc. Since the actual field tests provide the most valuable data to determine the success of the MORE process, I have attempted to gather together the available published information on all field tests of the MORE process.

The information reported is restricted to field test data and does not cover research laboratory tests which may have been made preceding or in conjunction with the field tests. It must be recognized and emphasized that this compilation is not complete, and will contain some discrepancies and inaccuracies due to the revisions of the data in later publications, translation errors, etc. However, in spite of these limitations the data do provide a background for evaluating the field tests and are offered as a preliminary effort to document such field tests. It is requested that if additional data on these or other field tests are available, or corrections can be made, that such information be made available so this record of field tests can be made as accurate and complete as possible. It is anticipated that additional field test data will be presented at this meeting that will supplement this information.

DISCUSSION

The purpose of this paper is to provide a record of past field tests of the MORE process. Each field test has been recorded in the addendum to this paper and the reports are divided by country and within each country by the year of the test. Thus the tests are designated as Poland field test, P-I through P-XVIII, Hungary, H-I through H-X, Czechoslovakia, C-I through C-VII, USSR I and II, Netherland, D-I and D-II, Romania, R-I, and United States as USA-I through USA-IV. In some of such designated field tests there was more than one test well inoculated, and in others there is an overlap between tests with multiple inoculations, etc. However the data are organized in an attempt to segregate out "cause and effect" events for each field test. The data are reported in a standardized format so that comparisons can be made more easily. In each such

TABLE 2—Number of Wells in European Countries Treated with Microbial Cultures

	Number of Wells Treated with Cultures
Poland	17
Hungary	10
Czechoslovakia	6
U.S.S.R.	2
Romania	9
Netherland	2
U.S.A.	178

field test section the figures and tables have been numbered for that test (example, Fig. P-I-1, Table P-I-1, etc.) for ease of reference.

No attempt has been made to draw conclusions from the data which are presented in the same form as in the original report or have been reorganized from different reports to a more concise form. All the factual data offered by the investigators are reported but their interpretations were minimized except where it concerned events which influence the observed data. By an examination of the individual field tests followed by a study of the combined data, each investigator can compare and evaluate the data for his own interests.

However, an overview of the data is provided by the comparison and summarizing tables which follow. In the years since 1954 over 200 wells have been treated with microorganisms (see Table 2). While this number looks impressive, most of the wells in the U.S. were in the stripper well category with minimum control and almost no data available due to proprietary considerations. A more realistic number of tests with some data would be 40-50. Most of these were field tests run 15-20 years ago when techniques were being developed.

Of the wells treated the depth of the reservoir varied between 50 and 1550 meters. (See Table 3.)

As could be expected the earliest tests were mainly in the very shallow reservoirs but a trend to treating deeper producing formations occurred as confidence in the process was gained. In almost all cases the wells treated were very poor, marginal, or closed wells. Most wells fell in the category of 1 to 2 bbl/day. This probably influenced the results reported, either favorably or unfavorably, but in some cases the control wells and their response was more revealing of changes in reservoir conditions due to microbial growth than that observed in the inoculated well.

Considering the small number of wells treated the diverse range of parameters encountered in the reservoirs was quite large. (See Table 4.)

The comparison of parameters to microbial action

TABLE 3—Depths of Wells Treated with Microbial Cultures

Well Depth (m)	Number of Wells Inoculated
50-250	7
251-500	1
501-750	16
751-1000	4
1001-1250	5
1251-1500	2
Over 1500	1
Reported as between 250-1550	38

TABLE 4—Extremes Reported in Reservoir Parameters

Temperatures (°C)	22 to 97
Porosities (percent)	11 to 36
Permeability (md)	10 to 8100-highly fractured
Oil types	Heavy asphaltic-light paraffinic
Rock	Sandstone-limestone

and oil production show several interesting relationships. Permeability will play a key role in the MORE process while the effects of pressure and temperature are less clear. It appears that the temperature tolerance for microbial growth may be higher than would be expected from laboratory tests. Besides the one test at 97°C, it has been reported that reservoirs in this same temperature range have gone sour due to microbial action. It is possible that thermophilic cultures will play a more important role in deeper reservoirs than thought or suspected at present.

The cultures employed in the tests varied depending on the investigator and country (see Table 5).

The predominant species in early tests were mixtures of aerobic and anaerobic organisms (except the Mobil test using *Cl. acetobutylicum*). As testing continued it became evident that the anaerobic forms were preferred and the use of aerobes, except as contaminants, declined. Initially sulfate reducing bacteria were favored but due to their reputation involving corrosion and H₂S generation, their importance decreased and the inocula evolved into simple mixtures designated as stable mixed anaerobic cultures. The use of polymer (gum) producing types was restricted to special cases where the need was for an improved water viscosity.

A trend in the size and amount of inoculum and molasses also seemed to develop from a very small quantity used in early tests to the larger amounts used later (see Table 6).

This observation must be tempered by the fact that as larger and deeper wells were treated, and techniques improved, the larger quantities would be needed. An important point and one which is critical is the observation that these relatively small amounts of inoculum were able to reproduce to the extent disclosed. Considering the volumes of fluids injected, produced, and remaining in the reservoir, the self-duplication of the inoculum or the stimulation of the native microflora seems impressive. In most cases crude molasses was employed as the nutrient due to its low cost, availability, and nutritive value. The use of supplemental mineral additions seems to have declined with field practice. Whether this was due to adequate microbial growth without its addition, its cost, or lack of effectiveness, etc., is not known. Certainly the addition of molasses alone, with the inoculum, or its addition later seemed adequate to show significant microbial growth stimulation.

TABLE 5—Cultures Used as Inoculum

Poland—Mixed aerobic and anaerobic
Hungary—Thermophilic mixed cultures
—Sewage sludge
Czechoslovakia—Sulfate reducing and hydrocarbon utilizing types
—Sulfate reducing bacteria
U.S.S.R.—Mixed aerobic and anaerobic
Netherland— <i>Betacoccus dextranicus</i>
—Slime forming bacteria
Romania—Adapted mixed enrichment cultures
U.S.A.— <i>Clostridium acetobutylicum</i>
—Mixed cultures

TABLE 6—Amounts Reported Injected

Inoculum Size (l)	No. of Wells Treated	Molasses (kg)	No. of Wells Treated
10-50	4	10-100	4
51-100	6	101-500	4
101-200	1	501-1000	
201-300		1001-1500	3
301-400	2	1501-2000	10
401-500	9	2001-10,000 ...	1
501-600	2	Over 10,000 ...	4
601-700			
701-800	1		
Over 800	4		

A summarized comparison of the techniques used to inoculate and treat a well shows that different countries had different methods. The microbial treatment advanced from the simplest technique of only injecting a small inoculum into the well such as the Czechoslovakia early trials (see Table 7) to a staged operation involving cleaning the well, adding a buffer formation water slug, followed by the inoculum and nutrients and pushed by water and an oil slug (see Tables 8 through 12). In some cases the well was shut in to allow cell growth while in others the well was produced or water-flooded immediately. Thus in some cases the microbial injection acted as a modified well cleanout treatment while in other tests the purpose was to favor inoculation of the entire reservoir so the production response would occur in the control wells. Variations of all these approaches were tried including "huff and puff," viscosity improvement, etc. In addition, the effect of fracturing with microorganisms was tested and compared to fracturing in the absence of microorganisms. Within each country the method was improved with experience and it is evident that the inoculum and treatment will evolve into even more favorable practices in the future.

The data from the individual field tests in each country have been summarized (Poland, Table 13; Czechoslovakia, Table 14; Hungary, Table 15; USSR, Romania, Dutch and USA, Table 16) for comparative purposes. These tables show the progress of techniques, treatments, objectives, and responses. To make the data more amenable to review they have been condensed to an overall tabulation in Table 17.

It is unfortunate that all the information is not available that would allow a more intensive evaluation of the results of microbial action on an oil reservoir. However by a more detailed examination of individual

TABLE 7—Method of Microbial Treatment of Wells (Czechoslovakia)

	Period	
	Early	Later
Culture: Sulfate reducing + hydrocarbon bacteria		Sulfate reducing
Injection sequence:		
1. Inject culture		1. Clean well
		2. Inject culture
		3. Inject nutrient
		4. Increase pressure

TABLE 8—Method of Microbial Treatment of Wells (Poland)

	Period	
	Early	Later
Injection sequence:		
1. Formation water (l)	3050	50000
+ Molasses (kg)	250	2000
+ Culture (l)	80	500
+ Oil (kg)	—	150
2. Shut-in well	3-4 months	
3. Well frac sometimes done following treatment.		

Culture preparation—Mixed inoculum grown in 10 l bottles with formation water + 4 percent molasses + oil + sand (32°C).

sections of the tables it is possible to formulate some interesting generalized statements.

Some of these are:

1. Microbial activity occurs in some reservoirs under some conditions and a positive oil response occurs.
2. *In situ* microbial growth can be stimulated by the addition of nutrients.
3. Such microbial growth influences the properties of the fluids in the reservoir and the changes are both physico-chemical and petrophysical.
4. The flow patterns in the reservoir can be traced by following microbial growth patterns.
5. Most of the mechanisms proposed for the MORE process (such as CO₂, acid, surfactant, etc., and changes in oil viscosity, gas production, etc.) have been shown to play a role in oil mobilization.
6. Some reservoirs have conditions such as low permeability, salinity, pH, etc., that could limit microbial growth.

7. The temperature and pressure conditions of some reservoirs may not be as restrictive as previously considered..

8. Fracturing with microorganisms seems to be a successful technique in some cases.

9. Even a small inoculum, or the stimulation of the native reservoir population, appears to exert an effect out of proportion to what would be expected from size alone.

10. Anaerobic organisms seem to be preferred and more effective than aerobic forms.

11. The technique employed to introduce the inoculum into the reservoir and their rate of penetration and action is dependent on the formation characteristics and must be carefully considered to have a successful project.

12. Better records and monitoring must be made to truly evaluate the success or failure of a test.

While no mention has been made of the economics of the MORE process the reported data indicate that the oil production was obtained at a cost which would be competitive to other tertiary oil recovery processes. However this remains to be proven under vigorous field test conditions.

The data emphasize a key factor which must be considered in any application of the MORE technique. The success of a project will depend on close cooperation between the microbiologists and the reservoir and production engineers. In the future, genetic recombination studies may also play a role by the development of optimized cultures.

While much remains to be done to evaluate the potential role of microorganisms in tertiary oil recovery, the available data suggest the MORE process may be a cost effective oil recovery technique in some reservoirs.

TABLE 9—Method of Microbial Treatment of Wells (Hungary)

	Period		
	Early	Mid	Later
	1962-65	1968	1973
Injection sequence:			
1. Formation water (m ³)	20	20	—
2. Formation water (m ³)	100	100	?
+Molasses (T)	4	2	2
+KNO ₃ (kg)	120	70	50
+Na phosphate	50	10-20	5
+Sucrose (kg)	100	50	none
3. Culture (l)	100	*Injected 1/2 of solution	?
		100	
		*Injected 1/2 of solution	
4. Formation water (m ³)	50	50	?
5. Close well	5-6 months		
	“then huff and puff.”	Remained closed except to add more nutrients.	Halted supplemental nutrients after 2-3 yr to eliminate microbial products.

TABLE 10—Method of Microbial Treatment of Wells (U.S.S.R.)

Geobioreagent: Consists of mixed aerobic and anaerobic types. Together with hydrolysis substances from peat and silts.
Culture preparation: Culture grown adjacent to wells in pits containing formation water and oil.
Injection sequence: 1. Inject geobioreagent; 2. Inject fresh water; 3. Waterflood

TABLE 11—Method of Microbial Treatment of Wells (Romania)

Divide reservoirs into 3 types—treat differently

Reservoir Type	Treatment Based on
1. Producing oil	Hungarian process
2. Producing only water	Hungarian process
3. Waterflooded	Hitzman process (U.S. Pat. 3,340,930)

TABLE 12—Method of Microbial Treatment of Wells (U.S.A.)

Mobil process

Culture: *Clostridium acetobutylicum*

Injection sequence:

1. Culture in 2 percent molasses injected in 18 separate injections (4000 gal water) over 5.5 months.
2. Waterflood at 100–500 bbl/day.

Pure Oil fracturing process

Culture: *Desulfovibrio*

Frac sequence:

1. Inject 5000 gal of lactate medium with gel agent and culture.
2. Apply pressure.
3. Close well 3 months.

TABLE 14—Summary of Available Data on Microbial Treatment on Oil Fields in Czechoslovakia

Test	I	II	III	IV	V	VI	VII
Date	1954	1955	1955	1957–58	?	?	?
Reservoir	Deposit Z	Deposit Z	Deposit H	Deposit H	Hodonin	Petrova Ves	Petrova Ves
Well treated	Z1	Z1	H1	78	78	404	481
Control wells	Z2,4,5,7	Z2–8	H2–8	10 wells	10 wells	15 wells	9 wells
Depth (m)	50	50	150	150	150	700	700
Porosity, percent	24–32	24–32	36	36	—	—	—
Permeability (mD)	3000–8000	3300–8100	3300–8100	3300–8100	3300–8100	Tens of mD	Fractured
Well treatment							
Inoculum (l)	40	40	40	60	60	None	?
Type	SRB+HC	SRB+HC	SRB+HC	SRB+HC	SRB	—	?
Molasses (kg)	None	30	30	50	100	200	Amt unknown
Reported results							
Bacteria count	Increased	Increased	Increased	Increased	Increased	Increased	Increased
Oil prod.	Unchanged	Small increase	Small increase	Increased	Increased	Increased	Increased
Water			(4 percent)	6.85 percent			
Oil viscosity			Unchanged	Decreased			
Observations	Little growth	Oil increase as	Oil increase as	Oil increase as	Bacteria follow	Native bacteria	Faults stop
	no change	bacteria	bacteria	bacteria	flow pattern	stimulated in	migration
		increased	increased	increased		permeable zones	

SRB = sulfate-reducing

HC = hydrocarbon oxidizers

MEOR Field Applications

TABLE 13—Summary of Available Data on Microbial Treatment on Oil Fields in Poland

Test	I	II	III	IV	V	VI	VII	VIII	IX
Reservoir.....Potok		Kroskienko	Potek	Jakub	Swierchowa	Weglowka I	Weglowka I	Swierchowa	Weglowka I
Inoculation date.....May 1961		June 1961	April 1962	May 1963	May 1964	July 1965	Sept. 1965	Dec. 1965	March 1966
Well inoculated.....P4		K18	L140	R13	SW14	W169	W254	Z-12	W256
Control wells.....	T4	K21,K31	L138,L143	J17,J20,J21, J23,R14,R18	SW8,SW9, SW13	None	None	Z-7	None
Well depth (m).....558.7		578.8	715.13	403.9	519	510	533.5	529.5	514.9
Porosity, percent.....13				25.1					
Well treated with									
Inoculum (l).....400		480	400	750	600	500	500	600	500
Molasses (kg).....1100		1100	1100	2300+	200	2000	2000	200	2000
Water (l).....48000		43000	48000	57000	50000	40000	40000	50000	40000
Oil (kg).....150		150	150						
Shut in time (days).....138		175	138	Till 1968	566	208	122	217	150
Production before test...P4	T4								
Oil (kg/month).....4460	4650	60 kg/yr		1200	1500	7500	600	1200	6000
Water (kg/month).....16610	16590			600	3000	9300	279000	3000	37000
Gas (m ³ /month).....3783				None	None	730	None	None	11000
Production after test									
Tons of extra oil after 1 year.....	16.5	3	Increased		22.12	32.18	16.94	4.77	33.14
Extra water produced.....					250 kg/day	Increased	Same	20% increase	Increase
Extra gas (m ³ /month).....	1000				Some	Increased		Some	Increase
Wellhead pressure (atm).....		To 8		To 2.5	To 7			To 17	
Total extra oil due to microbial action.....	636 tons in 9.5 yr	29.5 tons in 5 yr	Large increase after well shot	260 tons in 8 yr	230 tons in 7 yr	212.7 tons in 5 yr	87.7 tons in 6 yr	73.55 tons in 7 yr	221.84 tons in 4 yr
Percent oil increase reported.....	360			150-200	112	28	340	184	
Bacterial count (no/ml).....				Results in 4 control wells					
Before test.....1 × 10 ³	1 × 10 ³	1 × 10 ³		1300-3000					
During test.....7.5 × 10 ⁴	6 × 10 ⁴	5 × 10 ⁸	Increased	8000-600000	Increased	Increased	Increased	Increased	Increased
pH.....8.8-6.7	8.7-8	8.7-6.8	Decreased	8.7-7.4-7.9	8.4-7.4	8.4-7.3	8.4-7.3	8.8-7.0	
Viscosity (cSt).....1.05-1.4			1.29-1.29	10.67-4.4	16-10	6-7-4		9-11-10	7-6.6
Key observation.....Oil, CO ₂ prod. increase signif.		Increased production	Fracturing after inoc. very successful	Stimulated oil prod. added nutrient for more response	Good increase initially and sustained	Good initial response sustained	Late peak of oil but sustained production	Quick response to bacteria action	Well frac. in 1970 large oil increase

MICROBIAL ENHANCEMENT OF OIL RECOVERY

TABLE 13 (continued)—Summary of Available Data on Microbial Treatment on Oil Fields in Poland

Test	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII
Reservoir	?	Bobrka	Bobrka	Rowne	Weglowka II	Weglowka II	Weglowka II	Weglowka II	Weglowka II
Inoculation date	1967?	Dec. 1967	May 1968	July 1968	April 1969	April 1969	April 1969	April 1969	April 1969
Well inoculated	W8	B118	B117	A35	W176	W266	W271	W288	W273
Control wells	None	None	None	None	None	None	None	None	—
Well depth (m)	597	740.3	802	578	1100 11	1144.2 11	1124.4 11	1125 11	1100
Well treated with									
Inoculum (l)	200	?	?	500	500	500	500	500	None
Molasses (kg)	240+	?	?	1600	2000	2000	2000	2000	None
Water (l)	5600	?	?	45000	40000	40000	40000	40000	None
Oil (kg)									None
Shut in time (days)	120	145	85	100	90	90	90	90	90
Production before test									
Oil (kg/month)	6200	3600	3200	3100	5500	22700	9400	7100	8000
Water (kg/month)	900	2000	2400	4600	37500	25900	14400	2800	3200
Gas (m ³ /month)				200	15450	10500	11000	6000	3800
Production after test									
Tons of extra oil after 1 yr ... Slimy oil produced		31.95	33.85		55.9	6.2 tons/ 1.5 yr	No increase	No report	No increase in 2 yr
Extra water production				Increased					
Extra gas (m ³ /month)			Increased						
Wellhead pressure (atm)		To 7		To 2.5					
Total extra oil due to microbial action		152.83 tons in 4 yr	93.75 tons in 3 yr	85.56 tons in 3 yr		139 tons in 1.8 yr includes hydraulic frac 1970			90 tons oil loss
Percent oil increase reported		143	46	68	32				
Bacterial count (no/ml)									
Before test	2×10^5								
During test	3×10^6	Increased	Increased	Increased					
pH	8.5-7.8	8.4-7	8.5-7.5	8.5-7.5					
Viscosity (cSt)	2.02	11-13-9.9	Increased 1	4.5-3.8					
Key observation	Unknown slimy oil	Good bacteria response	Slow response quick decline	Good bacteria stimulation	Perforation success with bacteria	Frac. success with bacteria low	Perm. too low	No report	No response in well

MEOR Field Applications

TABLE 15—Summary of Available Data on Microbial Treatment on Oil Fields in Hungary

Test	I	II	III	IV	V	VI	VII	VIII	IX	X
Date		1961						1969-70	1970	1970
Reservoir		Lendvaufalu				Nagy Lengyel		Demjen East	Demjen East	Demjen West
Rock type	Sandstone	Sandstone	Sandstone	Sandstone			Limestone	Frac. sandstone		-
Oil type		Naphthenic		Light paraffinic	Highly paraffinic		Naphthenic			
Well inoculated						Well closed 2 yr before inoculation	Unproductive for 7 yr	DK114	DK192	De61
Control well		100 m distant			300 m distant	288 to 1700 m distant		Many		-
Depth (m)	200	700	1400	994	1392	2457	700	300-1000	300-1000	200
Temperature (°C)	25-35	50	50	70	72	97	50	25-30		-
Permeability (mD)		600-700	10-70	150-300	Low			67-104		-
Well treatment	Unknown		Unknown	Unknown	Unknown		Unknown			
Inoculum (l)		50				100		100		-
Type		Thermophilic				Sewage		Sewage sludge		-
Molasses (kg)		2000				40,000		20,000		-
Water (m ³)		60				120		170		-
Other additions	Nutrients added	KNO ₃ , PO ₄				KNO ₃ , sugar		KNO ₃ , PO ₄ , sucrose		-
Shut in time (days)		150				90		Closed except to add nutrients	Closed	Closed
Reported results										
Bacteria	Increased	Increased				Increased		Increased	Increased	Increased
pH	Reduced	9-6-7		7-6	Unchanged	9-6	7-6	Decreased	Reduced	Reduced
Viscosity	Reduced	42-18-26		3-3	Unchanged		600-300	Reduced		
Production										
Oil	18-26 pct increased	Increased	Unchanged	10 pct increased	Unchanged	60 pct increased	60 pct increased	126 pct increased	117 pct increased	
Water		Unchanged					Decreased	Decreased	Decreased	
							oil/water ratio			
Gas pressure										
CO ₂		To 33 atm To 40 m ³ /day		Increased	Unchanged	To 228 atm	To 60 pct			
Observations		Effect lasted 8 mo., CO ₂ stimulated	No effects, perm. too low		No effects, low perm.	High temp. growth and oil recovery	Effect lasted 2 wk to 18 mo in different wells	Added nutrients increased effects	Effects decreased as nutrients used	No evaluation, combustion process tested in well

MICROBIAL ENHANCEMENT OF OIL RECOVERY

TABLE 16—Summary of Available Data on Microbial Treatment on Oil Fields in USSR, Romania, Dutch and USA

Test	USSR I	USSR II	Romania	Dutch I	Dutch II	USA I	USA II	USA III	USA IV
Date	1966		1975-78	1956?		1954	1957?	1977-81	1977-82
Reservoir	Arlansk	Sernovodsk				Natotoch		Mid-Continent	Mid-Continent
Type	Sandstone		Sandstone				Sandstone	Varied	Varied
Well treated	1455					400' distant		150 treated	24 treated
Control wells	Many								
Depth (m)	1200	1000	500-1500			608	912		90-1400
Reservoir temp. (°C)	22-24		27-56			34		Variable	Variable
Porosity (percent)	20-23					30			
Oil type	Heavy asph.							Variable	Proprietary
Permeability (mD)	1D		80 to 1000		Variable	1-5700		Proprietary	
Well treatment			Hungarian or Hitzman process						
Inoculum	190 m ³	?		Inoculated molasses sol.	3000 l.	18 injections over 5.5 mo.	5000 gal in lactate and gel fluid		
Type	Geobioreagent	Geobioreagent		Betacoccus dextranicus	Slime forms bacteria	Clostridium acetobutylicum clastus	Desulfovibrio hydrocarbon-clastus	Bacillus Clostridium	Mixed
Molasses	?	2000	2-4 pct	1000 l. of 10 pct sol.	100 m ³ of 50 pct sol.	4000 gal of 2 pct sol.		Proprietary	Proprietary
Water	650 m ³ /day					100-500 bbl/day			
Supplemental additions	Fresh water			Uninoc. molasses			Sand frac		
Shut in time	None	6 mo			5 days			90 days	
Results of test									
pH	5-6.5-6.5-8.3		Changed						
Viscosity (cSt)	Decreased	40.3-49.3		Increased water visc.					
Bacteria	Increased								
Production									
Oil	Increased	37 T-40 T/mo	Increased 30-100 pct of production			1-3 bbl	15-25 bbl	Increase	Of 24 wells 4 double prod. for 6 mo. 12 increased 50 pct for 3 mo. 1 increased 6 fold. 30 control wells stimulated 75 pct of wells had 10-200 psi increase
Water, percent	Reduced	7.5-4-6 decrease			Oil/water ratio 1:50-1:20	Unchanged			
Gas factor	8-18-18-66	11-15-16				CH ₄ +CO ₂			
Pressure	Increased	To 1.5 atm						Increase	
CO ₂	To 3 pct	Increased				To 80 pct		Increase	
Operator				N.V. deBataafsche Petroleum Co.		Mobil	Pure Oil	A. C. Johnson	Petrogen
Observation	Bacterial growth gave 18 pct oil increase	Oil viscosity increased prod. increase		Increased pore vol. of oil from 40-70 pct.	Increased oil/water viscosity	Too much molasses		Stripper wells treated	24 wells had overall increase of 42 pct

TABLE 17—Summary of Microbial Tests in Oil Fields Throughout the World

Test	Year Started	Test Duration (Yr)	No. of Control Wells	Inoculum		Molasses (kg)	Shut In Time (Days)	Reservoir		
				Amt (l)	Type			Temp. (°C)	Depth (m)	Porosity (Pct)
P-I	1961	9.5	1	400	Mixed	1100	138		558	13
P-II	1961	5	2	480	Mixed	1100	175		579	
P-III	1962	3	2	400	Mixed	1100	138		715	
P-IV	1963	8	6	750	Mixed	2300	5 yr		404	25.1
P-V	1964	7	4	600	Mixed	200	566		519	
P-VI	1965	5	0	500	Mixed	2000	208		510	
P-VII	1965	6	0	500	Mixed	2000	122		533	
P-VIII	1965	7	1	600	Mixed	200	217		529	
P-IX	1966	4	0	500	Mixed	2000	150		515	
P-X	?	?	0	200	Mixed	240-	120		597	
P-XI	1967	4	0	?	Mixed	?	145		740	
P-XII	1968	3	0	?	Mixed	?	85		802	
P-XIII	1968	3	0	500	Mixed	1600	100		578	
P-XIV	1969		0	500	Mixed	2000	90		1100	11
P-XV	1969	1.8	0	500	Mixed	2000	90		1144	11
P-XVI	1969		0	500	Mixed	2000	90		1124	11
P-XVII	1969		0	500	Mixed	2000	90		1125	11
P-XVIII	1969	2	0	None	Mixed	None	90		1100	
H-I	?	1.6	?	Unknown	Unknown	*		25-35	200	
H-II	1961	.8	Some	50	Thermophilic	2000*	150		50	700
H-III	?			Unknown					50	1400
H-IV	?			Unknown					70	994
H-V	?		Some	Unknown					72	1392
H-VI	?			100	Sewage	40000*	90		97	2457
H-VII	?			Unknown					50	700
H-VIII	1969	1.5	Some	100	Sewage	20000*	Closed	25-35	300-1000	20-28
H-IX	1970			100	Sewage	20000*	Closed	25-35	300-1000	20-28
H-X	1970			100	Sewage	20000*		25-35	200	20-28
C-I	1954		4	40	SRB+HC	None			50?	24-32
C-II	1955		6	40	SRB+HC	30			50	24-32
C-III	1955		7	40	SRB+HC	30			150	36
C-IV	1957-8		10	60	SRB+HC	50			150	36
C-V	?		10	60	SRB	100			150	
C-VI	?		15	None		200			700	
C-VII	?		9	Yes?		Yes			700	
USSR-I	1966		Many	190 m ³	Geobioreagent		None	22-24	1200	20-23
USSR-II	?			?		2000	180		1000	
D-I	1956			1000	Betacoccus	10 pct sol.				
D-II	?			3000	Slime bacteria	100 m ³ -50 pct sol.	5			
Rom-I	1975-8	9 wells inoc.—2 methods			Mixed	2-4 pct sol.		27-56	500-1550	
USA-I	1954		1	4000 gal.	Cl acetobutylicum	2 pct sol.		34	608	30
USA-II	1957				Desulfovibrio	Lactate medium*	90		912	
USA-III	1977-81	150 wells inoc.		Variable	Bacillus & Clost.	Yes*	10-15	Variable	60-500	Variable
USA-IV	1977-82	24 wells inoc.			Mixed			Variable	90-1400	Variable

*—supplemental nutrients added + = small positive effect observed
 **—test failure or incomplete ++ = some positive effect observed
 +++ = successful test

Increase or effect noted
 +slight
 ++more
 +++large
 —unchanged

SRB = sulfate reducing bacteria
 HC = hydrocarbon utilizing bacteria

POLISH FIELD TEST I

Year of test: 1961. Location: Potok Reservoir

Geological data—The test area lies in the region of the Potok anticline which is part of the central Carpathian depression filled mainly with Krasno strata. Older strata from the Eocene and upper Cretaceous periods emerge from these layers and form several larger anticlinal structures. In cross section the Potok anticline is represented by a steeply folded form with dislocations running crosswise and lengthwise (Fig.

P-I-1). The wells selected are located in a region of tectonic elements bordered by two transverse faults running north and south. Wells selected have only one oil producing zone and produce oil with water. The II Cieżkowicki sandstone is productive (30 m thick) whose structure varies from fine to coarse granular and shows conglomerate arrangement. The area is heavily flooded.

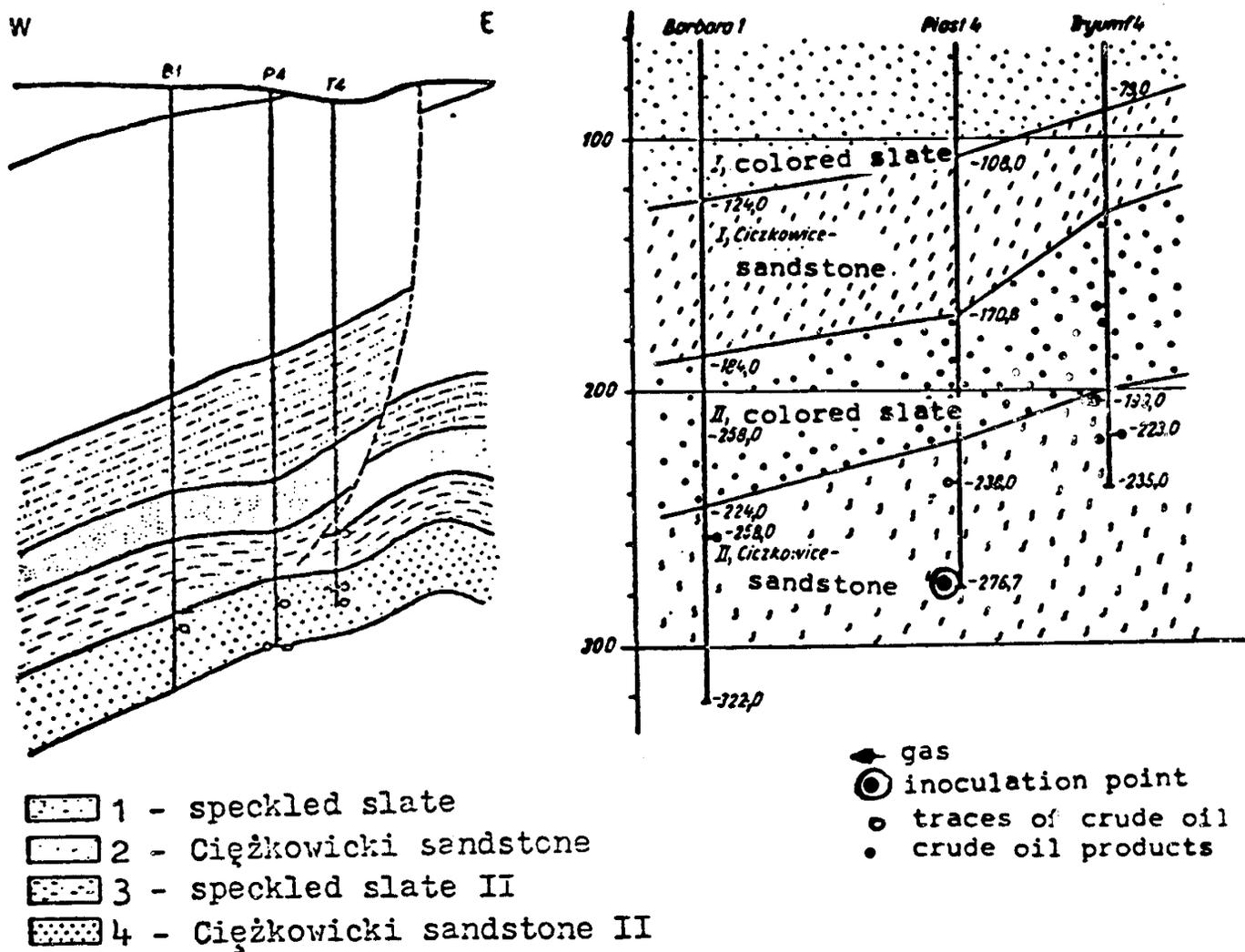


FIG. P-I-1—Geological profile in the area of the Potok crude oil deposit.

Depth: 558.7 m (production from Ciezkowice sandstone). *Porosity:* 13 percent. *Viscosity:* 1.05–1.4°E. (sp. grav. 0.823 to 0.840 g/cm³). *Permeability:* 1–120 mD. *Interfacial tension:* 26.5–28.3 dynes/cm.

Prior production history—The Piast 4 (P4) well was used for inoculation with the production wells, especially the Tryumf 4 (T4) well, serving as control wells and were within a radius of 100 m (see Fig. P-I-2). At the time of the test the area was not subject to other recovery methods.

Well P4: Had been producing since 1895 and had a 4 in. casing to 558.7 m. The well was operated by gas drive since 1933. In May 1961 the well produced 4460 kg oil without paraffin, 16,610 kg water, and 3780 m³ of gas. One month before inoculation it produced 2750 kg oil.

Well T4: Located 50 m from P4. In April 1961 (1 mo before test) the mean daily average oil production was 155 kg oil and 553 kg water.

Well L-160: Was a gas injection well (gas injected in this area for 30 yr) which had influenced Well P4 since 1933.

Microbial well treatment—Well P4 was inoculated May 25, 1961 with 400 l of bacterial suspension (2×10^6 cells/ml) together with 1100 kg molasses and 48,000 l of formation water at 35 atm. The above mixture was injected in 5 portions (each of 80 l culture, 300 kg water, 100 kg molasses) followed by the remaining molasses and water. Finally 150 kg of native oil was added and the well shut in for 138 days.

Culture employed: Mixed culture isolated from soil and water samples.

Generalized inoculum-preparation and well-treatment procedures—Poland

Cultures employed: Cultures were isolated from soil and water samples in vicinity of oil and also from waters from sugar factories. Identified organisms were: *Arthobacter*, *Clostridium*, *Mycobacterium*, *Peptococcus*, and *Pseudomonas* species. Pure cultures were isolated but mixed cultures were used for field tests.

Inoculum medium and procedure: Various media were employed for laboratory studies but for wells the inoculum was prepared in 10 l bottles containing 10 cm of sand plus culture inoculum + oil (.1 percent) plus formation water and 4 percent molasses. The bottles were sealed and incubated at 32°C. When growth occurred the bottles were emptied, refilled with 4 percent molasses, 0.1 percent ammonium phosphate (pH adjusted to 5.4 with 10 percent KOH) and the process repeated to cell density of 3×10^6 cells/ml. The inoculum was put in cylinders, 1 cm oil added and transported to the field.

Well selection: In selecting wells the geological structure of the deposit was analyzed in detail for an understanding of the tectonics, kind of reservoir rock, its petrophysical properties, the chemical composition of oil and water, the temperature and pressure in various horizons to obtain a correct lithostratigraphic picture. Wells were selected that were drilled and cased correctly and ranged in depth from 500–1500 m to insure a temperature optimum.

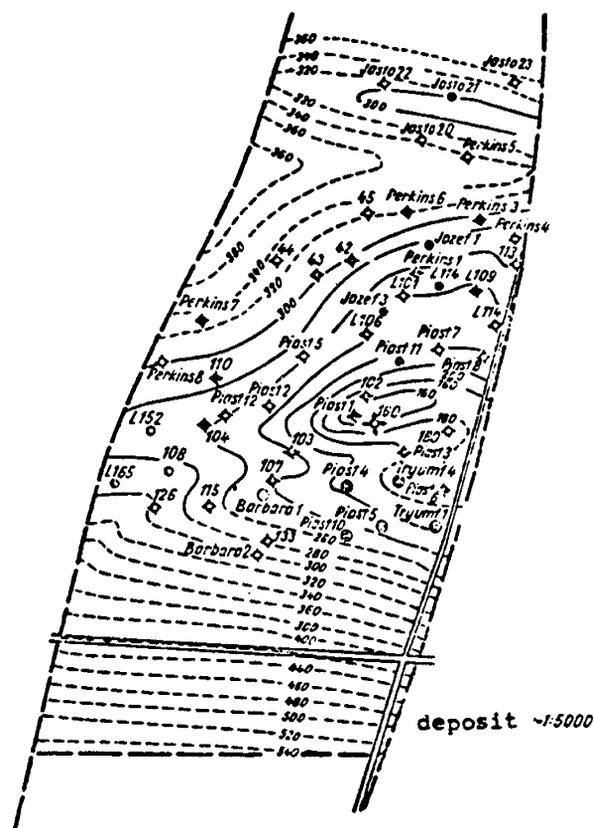


FIG. P-I-2—Structural map of the roof of the seam of II Ciezkowice sandstone in the area of the Potok deposit of crude oil.

Injection procedure: The culture and nutrients were injected by a simple fire engine pump using a siphon device (pressure never exceeded 100 atm). In the early tests a typical inoculum consisted of 3050 l of formation water, 250 kg molasses and 80 l of culture. In later tests the injection fluids generally consisted of 50,000 l of formation water, 2000 kg of molasses and 500 l of culture inoculum containing 6×10^6 cell/ml per well followed by 150 l of the native oil. The well was then shut in for 3–4 mo and then monitored. In some cases hydraulic fracturing was used following the inoculum. In this case a typical process used 14 m³ of formation water, 18 m³ of carrier formation water, 5 tons of quartz sand (0.3–0.8 mm diameter) and 9 m³ of wadding formation water. The minimum flow rate was 3 m³/min.

Sampling procedure: The well connection was washed with 50 percent ethanol. The initial sample was discarded and the microbiological samples collected in 250 ml opaque bottles with corks. The physico-chemical samples were taken in 1 l clear flasks. The gas samples were taken in 500 ml aspirators filled with saturated solution of NaCl. Microbial counts were made by microscopic observations.

Response—The test results have been divided into effects observed on control Well T4 and inoculated Well P4.

Control Well T4—75 days after inoculation of Well P4, Well T4 responded with a 360 percent increase which averaged 260 percent for 6 mo (Table P-I-1) (statistical increase of 16.5 tons of oil in 1961) and increased to 87 tons additional oil in 1962.

TABLE P-I-1—Oil Production in 1962 from Well T4
(kg/month)

Month	Expected Decline Curve Oil Production	Actual Oil Recovery	Extra Oil Produced	Percent Increase
Jan. 1962....	2480	8610	6130	250
February....	2240	9220	5980	268
March.....	2480	11430	8950	360
April.....	2400	10960	8650	360
May.....	2320	10870	8550	358
June.....	2250	8490	6240	278
July.....	2260	7940	5580	257
August.....	2170	7420	5250	242
September...	2100	6120	4020	190
October.....	2170	5620	3450	160
November...	2100	5840	3840	182
December ...	2170	6420	4250	196

Plots of the production from Well T4 from 1937-1962 (Fig. P-I-3) show the overall production history of this well while Fig. P-I-4 shows in more detail the production immediately preceding and during the test.



FIG. P-I-3—Influence of the microbiological inoculation process and the crude oil production curve for 1937-1962.

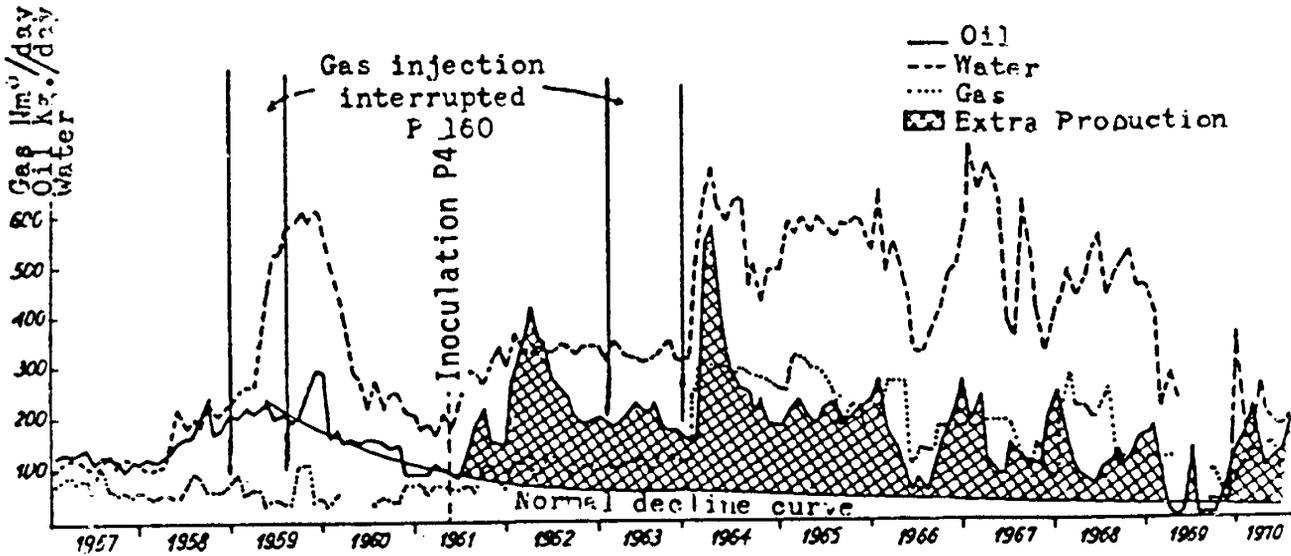


FIG. P-I-4—The effect of bacterial inoculation of Well P4 on production from Well T4.

In 1963 gas injection in Well P160 was interrupted (injection about 40,000 m³/mo) yet Well T4 still maintained a 52-ton/yr oil increase. In 1964 the gas injection in P160 was increased to 55-56,000 m³/mo which resulted in an increased oil yield during the first quarter of 1964 but oil production dropped in the second quarter. However, overall the increased yield in 1964 was 91 tons of oil. The production increased the following amounts for the years 1962-1970.

In 9.5 yr there was a total increased production of 470 tons of oil from Well T4. There was a minimum change in oil viscosity (0.5°E).

Well response injection Well P4: After being shut in for 138 days, Well P4 was put on production. Although there were periodic shutdowns the response in this well is shown (Fig. P-I-5).

Year	Increased Oil Production in Well T4 (Tons/Year)
1962	87
1963	52
1964	91
1965	65
1966	41
1967	46
1968	39
1969	12
1970	29.5

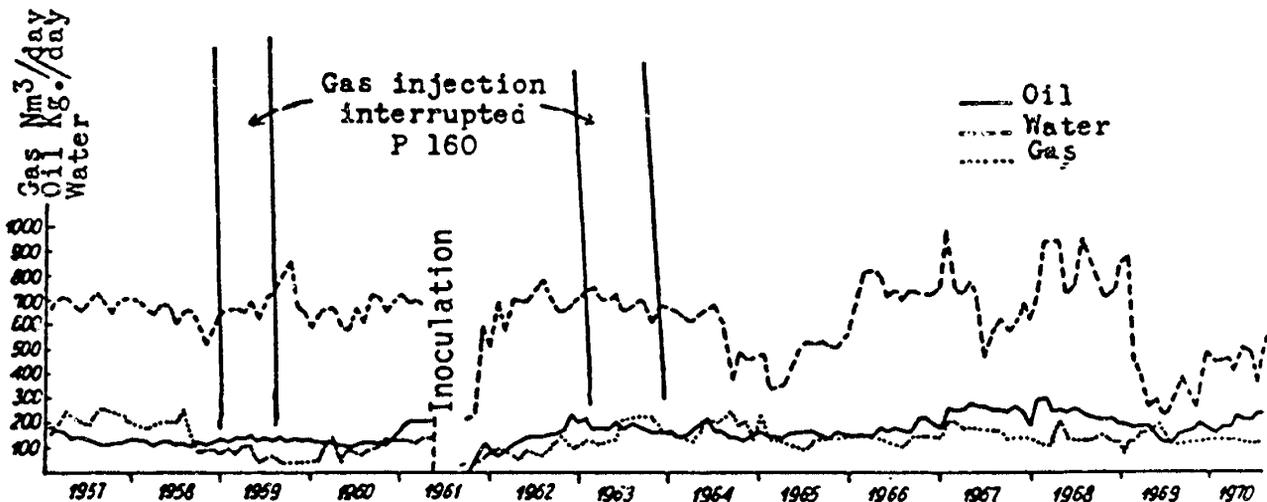


FIG. P-I-5—The effect of inoculation on production from Well P4.

TABLE P-I-2—Bacterial Counts and pH Changes in Wells P4 and T4

	Well P4		Well T4	
	Bacteria Numbers	pH	Bacteria Numbers	pH
Before inoculation	1.2×10^3	8.8	1×10^3	8.7
Days after inoculation				
15....			1.5×10^3	8.8
23....			2.0×10^3	8.6
43....			5.0×10^3	8.7
64....			7.0×10^4	8.7
75....			6.5×10^4	8.4
139....	7.5×10^4	6.7	6.3×10^4	8.0
360....	7.0×10^4	8.0	6.0×10^4	8.2

Bacterial counts and measured pH of Wells P4 and T4 were made periodically and showed an increasing count of bacterial numbers and a decrease in the pH (Table P-I-2).

Effects of inoculum on gas volumes and compositions: Gas production in Wells P4 and T4 are given in Table P-I-3 but are considered as estimates. The composition of gas from Well P4 (Table P-I-4) shows the observed increase in CO₂ and ethane.

Remarks—By 1970 the net oil production increase from Wells T4 and P4 was 636 tons of oil. The two wells sustained a 40 percent increased oil production for 4 yr (reported as a gain of 1-1.4 bbl/day at each well). There was an observed increase in bacterial numbers and a decrease in the pH.

TABLE P-I-3—Monthly Gas Yield from Wells P4 and T4

	P-4		T-4	
	1961	1962	1961	1962
	m ³		m ³	
I	4 460	3 710	2 330	3 610
II	3 810	3 130	1 400	3 080
III	4 770	3 300	2 170	4 200
IV	4 530	2 400	2 010	2 220
V	3 780	2 440	1 860	3 720
VI	—	2 900	1 170	3 820
VII	—	2 600	2 080	3 600
VIII.....	—	4 350	1 860	3 240
IX	—	3 750	3 222	2 700
X	—	4 720	2 200	3 260
XI	2 740	4 830	2 210	3 420
XII.....	2 240	4 900	2 020	3 650

TABLE P-I-4—The Composition of Gas from Well P4

Gas Com- position	12/14/61	5/5/62	6/30/62	7/27/62	9/21/62	11/7/62
Air	2.6	2.1	0.3	2.05	1.9	2.1
Methane	81.0	79.9	75.56	79.80	79.0	79.5
Ethane	0.9	1.6	3.2	1.8	1.9	2.4
Propane	2.22	0.3	3.2	1.0	1.5	1.8
i-Butane	2.15	2.2	1.9	1.9	2.0	1.2
n-Butane	2.5	1.6	—	1.4	1.2	1.0
CO ₂	8.63	12.3	15.44	12.55	12.5	11.9

POLISH FIELD TEST II

Year of test: 1961. Location: Kroszienko Field

Geological data—The Kroszienko field is situated on the Potok ridge entering into the central formation of the Carpathian depression, filled mostly with Krasno layers. The area is divided into 3 fields. The test well K18 is in the southern portion of the central area with production from the Third Cieczkowice sandstone. This production zone is uniform but contains numerous variegated slate (black) and clays sometimes causing the isolation of the Second layer of sandstone. The oil zones occur essentially in 3 levels, situated in the top, center, and lower part of the Second Cieczkowice sandstone layer. The layer measures 60-80 m in width at a variable depth of 300-450 m.

The test area (consisting of 6 producing wells) is separated from the rest of the field by a series of faults. The inoculated Well K18 is positioned in the saddle section of the block at the highest point with respect to the surrounding 5 wells. Depth: 578.8 m.

Prior production history—Well K18 was drilled in 1902. In 1951 the depth was extended to 578.8 m. The production history of the well showed that in 1951 production was 8147 tons of oil. Between 1955-56 air (26,348 m³) was injected with no results and the well was shut down in 1975. In 1958 production was 180 kg, in 1959 production was 170 kg and in 1960 only 60 kg oil was produced. Production during 1958-60 was intermittent. There had been no gas production for many years.

Microbial well treatment—Well K18 was treated on June 28, 1961 with 480 l of inoculum (containing 2×10^5 cell/ml), 1100 kg molasses, 43 m³ of formation water, 150 kg oil by the same procedure used on Well P4 (see Field Test I). K18 was shut in for 175 days. The control wells were K21 and K31.

Culture employed: Mixed culture (same as in Well P4).

Response—After 110 days the wellhead pressure increased to 8 atm and a gas analysis showed a composition of methane 89.6 percent, ethane 6.1 percent, propane 2.8 percent, butane 0.5 percent and i-butane percent (converted to air free gas content). After days the well was put on production and within 10 days produced 2121 kg oil. Control wells K21 and

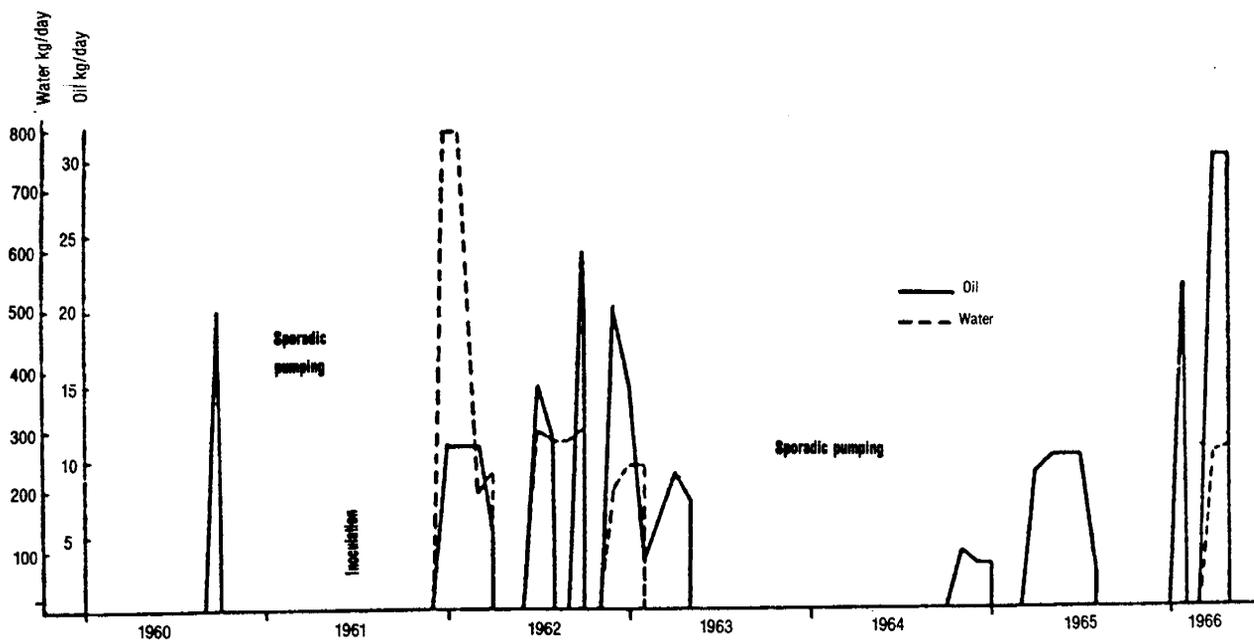


FIG. P-II-1—The effect of bacteria on the yield of oil in Well K18.

showed even more production. The additional oil production to the end of 1966 was:

Well K18	8.2 tons
K21	5.6 tons
K31	15.5 tons
Total	29.3 tons

Microbial counts increased from 1×10^3 to 5×10^8 in the reservoir waters and the pH decreased from 8.7 to 6.8. The effect of microbial treatment on oil production in Well K18 can be seen in Fig. P-II-1 and on production from Wells K21 and K31 in Fig. P-II-2.

Remarks—The inoculation of bacteria in Well K18 increased oil production in the control wells.

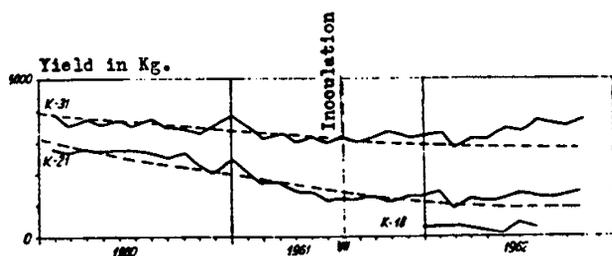


FIG. P-II-2—The effect of injected bacteria into Well K18 on the oil yield in Wells K21 and K31.

POLISH FIELD TEST III
 Year of test: 1961. Location: Potok Reservoir—
 Well L140

Geological data—Same as Test I. *Depth:* 715.13 m. *Viscosity:* 1.29.

Prior production history—Well L140 produced since 1914. In 1922 it was deepened to 715.13 m with production of 3600 kg oil/mo. The well was shot in 1958 with no success. Gas injection in an adjacent well did not increase production significantly.

Microbial well treatment—Well L140 was inoculated on Apr. 26, 1962 using the same procedure as in Well P4 Test I. The well was shut in 138 days. The control wells were L138, L130, L134.

Response—There was no immediate increase in oil production despite an observed increase in bacterial numbers, and a pH change. There was no major change in the viscosity of oil (Table P-III-1).

TABLE P-III-1—Changes in Oil Viscosity Observed After Inoculation of Well L140

Well Number	Measured Oil Viscosity at 20°C on		
	April 26, 1962	Sept. 1, 1962	Nov. 8, 1962
L130.....	1.21	1.07	1.23
L134.....	1.17	1.43	1.43
L138.....	1.15	1.15	1.19
L140.....	1.29	1.36	1.23
L143.....	1.25	1.19	1.17

However, in 1963 there was an observed increase in oil production (Fig. P-III-1).

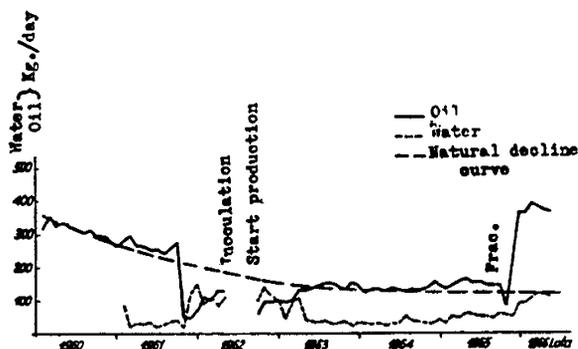


FIG. P-III-1—Result of microbial action and fracturing on production from Well L140.

The large increase in oil production in 1965 followed a fracturing of Well L140 on Oct. 25, 1965. A previous shooting of this well in 1958 resulted in no increase in production so it was suggested the fracturing increase was influenced by 3 yr of microbial action.

Remarks—There was a delayed response in oil production but fracturing the well 3 yr following inoculation was very successful.

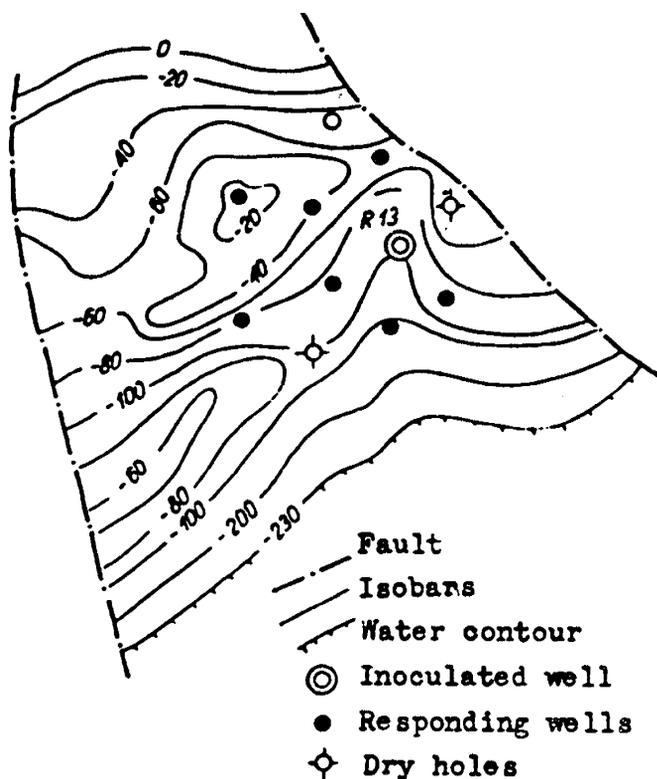


FIG. P-IV-1—Structural map of top of Cieszkowice sandstone.

POLISH FIELD TEST IV
Year of test: 1963. Location: Jakub Field

Geological data—The Cieszkowice sandstone of the Lipinek ridge has a large facial variability. Production is from the First Cieszkowice sandstone which has a large variation in sand grain size ranging from 0.04 to 0.3 mm with the major portion having a diameter smaller than 0.2 mm. It is expected that large differences in pore sizes and permeability exist in the field. In the Jakub field oil production is from the First Cieszkowice sandstone layer which is split into 2 layers. The inoculated Well R13 is drilled to the upper layer and is situated on the southern flank of the Lipinek anticline. The water contour is to the south of Well R14 and is not active. The control wells R12, R14, R17, R18, J21 and J23 are 50 m from the inoculated Well R13 and are all structurally lower (Fig. P-IV-1).

Depth: 403.9 m. *Porosity:* Calculated at 25.1 percent but variable. *Viscosity:* Paraffinic oil. *Permeability:* Variable.

Prior production history—Well R13 has been producing since 1942. In April 1963 production was 1200 kg oil and 600 kg light-brine.

Microbial well treatment—Well R13 was inoculated on May 7, 1963 with 750 l of inoculum, 2300 kg molasses and 5700 l of formation water and the well closed. Response on the control wells was such that the well was left shut in until March 1968 when the well was examined by pulling the pipe and 8 m of fill was found. On Mar. 10, 1968 the pipe was returned (with the pump cylinder) to a depth 10 m from the bottom of the well and 100 kg of molasses in 3000 l of formation water heated to 60°C was injected. The well was again shut in.

Culture employed: Mixed culture as in Well P4.

Response—Control Well J21 responded 22 days after inoculation with increased production of 150-200 percent. Wells R17 and R18 responded next, followed by R14, J20, J21 and J23. The increased production in the control wells equaled the losses by R13 closure (production in 1963 was 1140 kg oil). R18 responded only in 1964 and 1965 while R12, R14, R17, J20, J21 and J23 maintained increased production to the end of 1971. Since the response was so favorable during the observed test period (1963-1972), Well R13 was not reopened but was reinjected with 100 kg molasses in 3000 l of formation water on Mar. 30, 1968 and shut in. Production from the control wells attributed to the bacterial treatment was estimated at 260.2 tons by the end of 1971 (see Table P-IV-1).

TABLE P-IV-1—Production from Control Wells Influenced by 1963 Inoculation of Well R13

Year	R12		R14		R17		J20		J21		J23	
	Norm.	Surpl.										
1964..	7.69	2.31	11.15	7.11	1.40	5.60	2.55	0.65	9.06	9.94	2.67	3.53
1965..	10.01	0.51	10.17	8.08	2.00	4.72	2.87	0.71	9.63	9.97	6.83	6.62
1966..	9.68	1.08	8.97	8.16	2.67	4.11	2.62	0.98	8.70	4.13	6.11	3.57
1967..	8.29	2.23	8.74	9.26	2.67	1.19	2.50	1.10	8.86	5.75	5.93	1.44
1968..	5.27	8.40	7.59	10.31	1.11	0.33	2.22	1.38	7.91	7.35	6.87	6.88
1969..	8.89	9.91	7.31	10.69	0.21	0.98	2.44	1.16	7.05	10.95	4.36	11.64
1970..	8.26	7.30	6.27	8.25	2.00	0.85	1.86	1.74	6.89	11.33	4.90	7.77
1971..	7.25	10.24	6.04	8.56	2.48	0.83	1.83	1.85	7.38	9.41	4.08	6.03

The effect on Wells J21 and J23 has been shown in Figs. P-IV-2 and P-IV-3.

Following inoculation the viscosity and specific gravity of the oil was measured (Table P-IV-2). The oil viscosity decreased from 10.67 cSt and sp. grav. of 0.8516 to 4.37 cSt and 0.8320 g/cm³.

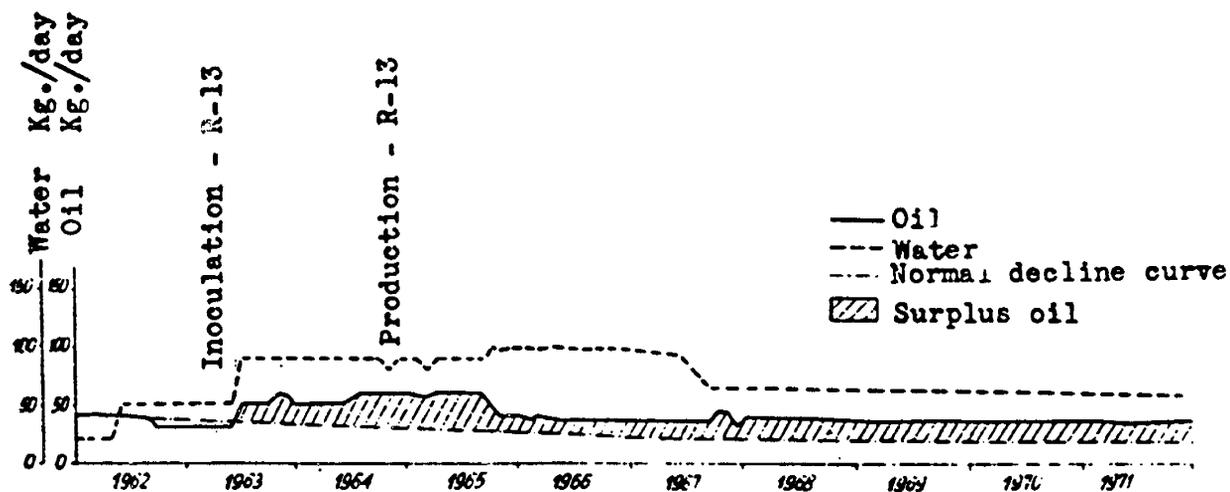


FIG. P-IV-2—The effect of bacterial inoculation of Well R13 on oil production in Well J21.

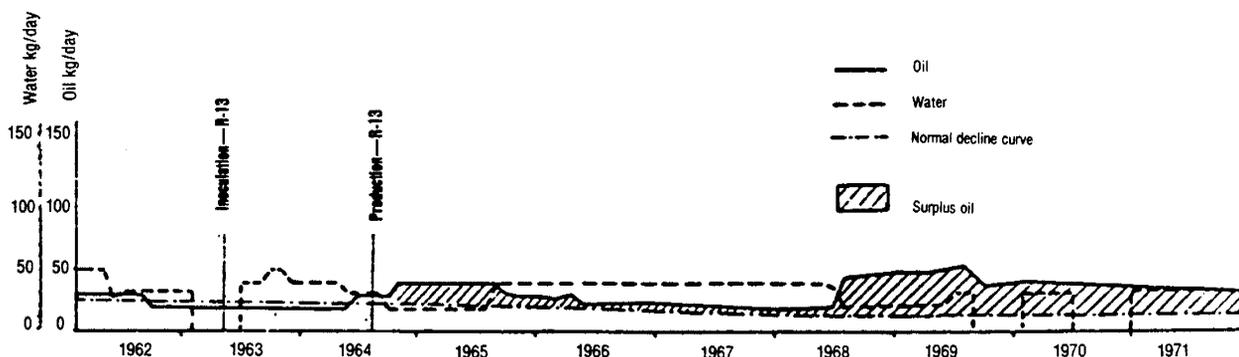


FIG. P-IV-3—The effect of bacterial inoculation of Well R13 on oil production in Well J23.

TABLE P-IV-2—Viscosity and Specific Gravity of Oil from Well R13 and Control Wells

Well Number	Date Sampled	Viscosity, cSt at 20°C	Specific Gravity, g/cm ³ at 20°C
R13.....	Sept. 26, 1963	7.41	0.8623
	Oct. 26, 1963	6.48 @ 36°C	0.8516
	Nov. 23, 1964	10.67	0.8504
	Nov. 29, 1964	9.66 @ 30°C	0.8626
R12.....	Oct. 26, 1963	7.41	0.8510
	June 18, 1964	6.48	0.8439
	Oct. 29, 1964	11.13	0.8617
R14.....	Sept. 24, 1963	6.82	0.8421
	Oct. 26, 1963	6.33	0.8405
	Sept. 13, 1964	9.66	0.8568
	June 18, 1964	8.20	0.8506
	Oct. 29, 1964	10.84	0.8610
R17.....	Oct. 26, 1963	6.12	0.8423
	June 18, 1964	9.11	0.8579
J20.....	Apr. 10, 1964	6.46	0.8433
	Oct. 29, 1964	6.31	0.8440
J21.....	Sept. 24, 1963	4.40	0.8328
	Oct. 26, 1963	4.47	0.8349
	Sept. 9, 1964	4.37	0.8320
	June 18, 1964	7.39	0.8556
	Oct. 26, 1964	4.49	0.8368
J23.....	June 22, 1963	7.70	0.8550
	Sept. 24, 1963	4.85	0.8360
	Oct. 26, 1963	5.27	0.8420
	Apr. 11, 1964	4.95	0.8374
	June 13, 1964	4.79	0.8368
	Oct. 29, 1964	9.72	0.8615

The viscosity and specific gravity changes are most apparent in Well J23. Such changes were maintained for 9 mo after which return to normal values occurred.

A 10 step distillation of oil from Well J23 before and during the test period of microbial action showed an increase in the lighter fractions and a decrease in the heavier fractions (Fig. P-IV-4). The same observations were made to a lesser extent on the oil from the other wells as shown for Well R12 (Fig. P-IV-5).

The reservoir waters were examined for microbial counts and pH changes and showed increased microbial numbers and decreased pH values (Table P-IV-3).

Well R13 before inoculation had no gas cap and the water was not gassy. After 3 mo of being shut in the wellhead pressure increased to 2.5 atm which was maintained for several months. The gas was composed of methane 86.35 percent, ethane 2.01 percent, propane 0.23 percent, CO₂ 11.27 percent, nitrogen 0.11 percent (converted to air free gas). In the control wells there was a noticeable but small increase in gas production in the oil and formation water.

Remarks—Inoculation of the reservoir resulted in increased oil production. In the 5 yr after injection, increases in production were still being noted. Initially the treatment stimulated 4 wells and with time it stimulated 6 wells.

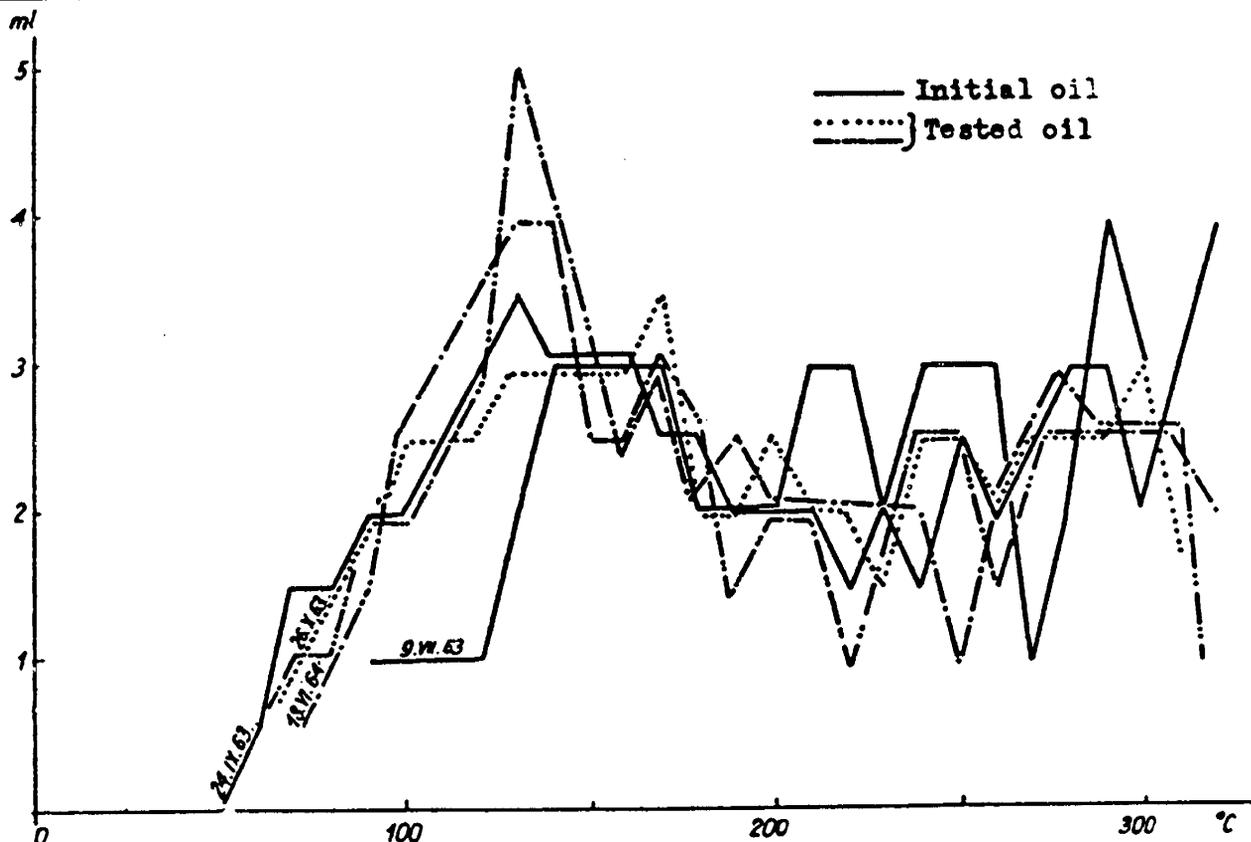


FIG. P-IV-4—The fractional composition of oil from Well J23 after injection of Well R13 (Engler distillation).

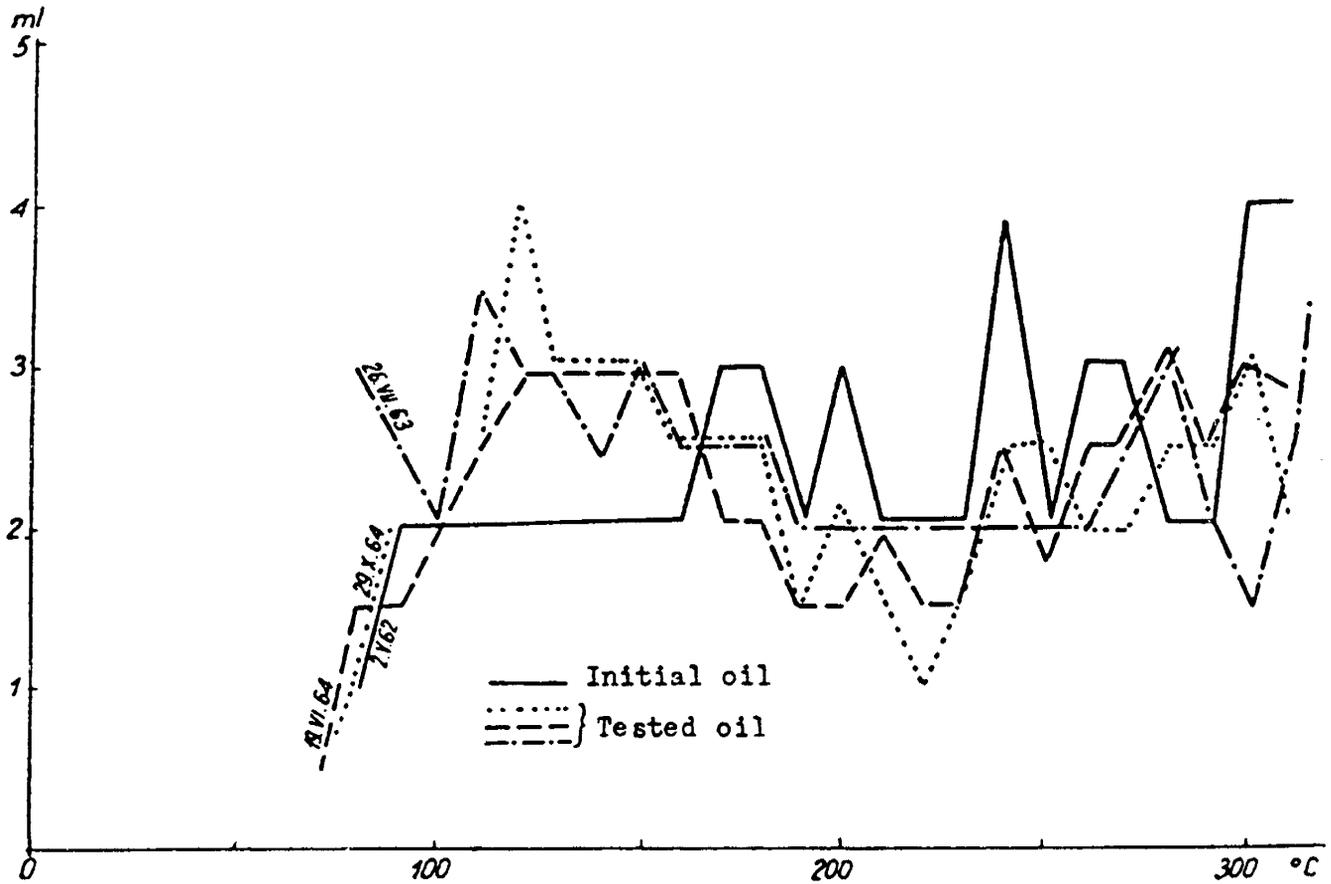


FIG. P-IV-5—The fractional composition of oil from Well R12 after injection of Well R13 (Engler distillation).

TABLE P-IV-3—Changes in Microbial Numbers (No/ml) and pH of Control Wells After Inoculation of Well R13

	R14		R17		J21		J23	
	Microbial Count	pH						
Before test	3,000	8.7	3,000	8.6	2,800	8.6	1,300	8.7
Days after inoc. R13								
15	8,500	7.9	7,800	8.0	8,000	8.2	40,000	8.4
22	8,700	7.6	8,000	7.8	8,500	7.9	65,000	8.0
43	70,000	7.4	600,000	7.7	8,000	7.7	60,000	7.9

POLISH FIELD TEST V

Year of test: 1964. Location: Swierchowa Field

Geological data—The test area is in the southern submerged extension of the Bobrki ridge whose center consists of layers of Eocene. On the edges of this formation are newer links of Krasno layers. The Eocene is separated into 4 layers of variegated fragments which are separated with 4 layers of Czarnorzeki sandstone. With respect to the tectonic formation a clear long split ridge is evident together with further long dislocations on the southern wing which are a result of tectonic action characteristic of the central Carpathian region. In addition to the lengthwise faults, the ridge in the area of Swierchowa and Zalezu are transected by a number of sideways faults. This double system of tectonic disturbances results in the oil being selectively placed in individual blocks. The oil bearing strip of tectonic blocks is found on the southern side. On two such adjoining blocks are situated Wells SW14 and Z12 (Fig. P-V-1). The Swierchowa field produces oil from the upper section of the Czarnorze sandstone in 4 separate blocks. Each of the mentioned wells is in a different tectonic block.

Depth: 519–530 m. *Viscosity:* Approximately 16 cSt.

Prior production history—Well SW14 was drilled in May 1951 to the Third Czarnorzeki sandstone (519.4 m) and produced 180 tons of oil. In 1953 production was 107 tons followed by a gradual decline until by 1963 oil production was only 20.5 tons. Monthly production in 1964 was 1500 kg oil and 3000 kg water.

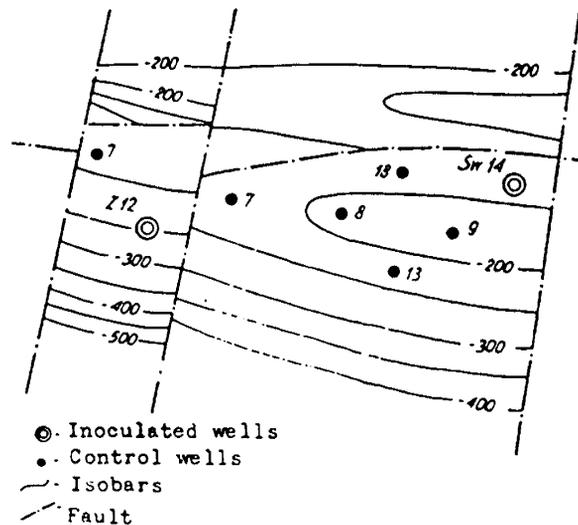


FIG. P-V-1—Structural map of the Czarnorzeki sandstone showing showing location of Well SW14.

Microbial well treatment—Well SW14 was inoculated on May 14, 1964 with 600 l of bacterial culture, 200 kg molasses, and 50,000 l of formation water and closed for 566 days. The control wells were SW8, SW9, and SW13.

Culture employed: Mixed culture.

Response—Well SW14 was opened and by the end of 1965 the extra oil produced was 22.12 tons (112 per cent increase) and water production increased from 100 to 350 kg/day (Fig. P-V-2). A large effect was noted on Well SW9 (Fig. P-V-3).

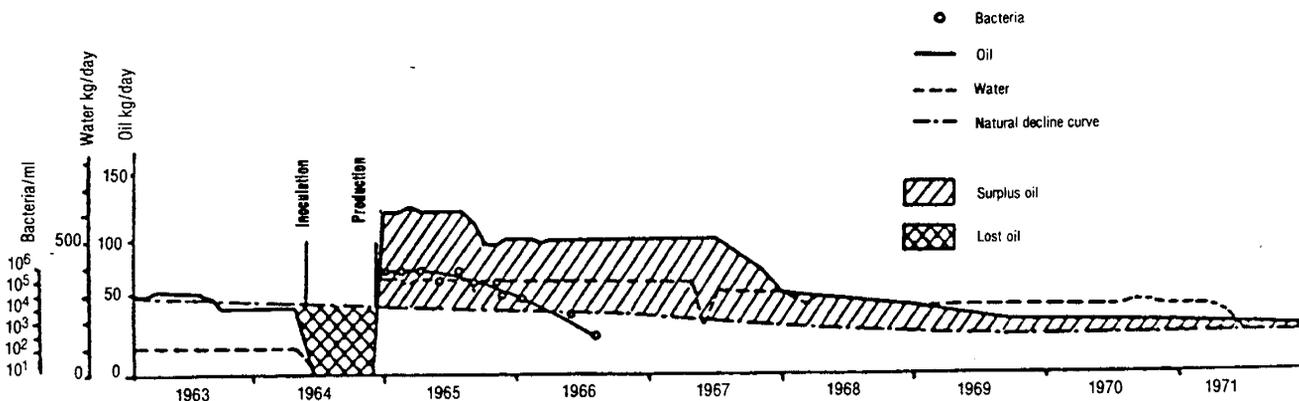


FIG. P-V-2—The effect of bacteria on production from inoculated Well SW14.

TABLE P-V-1—Production from Wells in Swierchowa Field After Inoculation of Well SW14—Tons of Oil

Year	SW14		SW8		SW9		SW18	
	Normal	Surplus	Normal	Surplus	Normal	Surplus	Normal	Surplus
1965	19.98	22.12	41.46	4.51	43.55	10.63	—	—
1966	16.34	15.78	32.66	5.58	40.75	4.47	7.44	0.56
1967	14.20	7.20	30.90	9.47	37.56	6.48	7.48	1.62
1968	10.13	6.53	27.73	12.64	33.74	10.30	8.0	3.0
1969	10.03	6.20	25.05	15.15	29.99	13.51	7.0	3.7
1970	10.99	3.53	24.91	15.02	31.01	12.89	7.24	3.65
1971	10.83	3.40	15.05	14.8	30.61	12.89	7.33	3.65

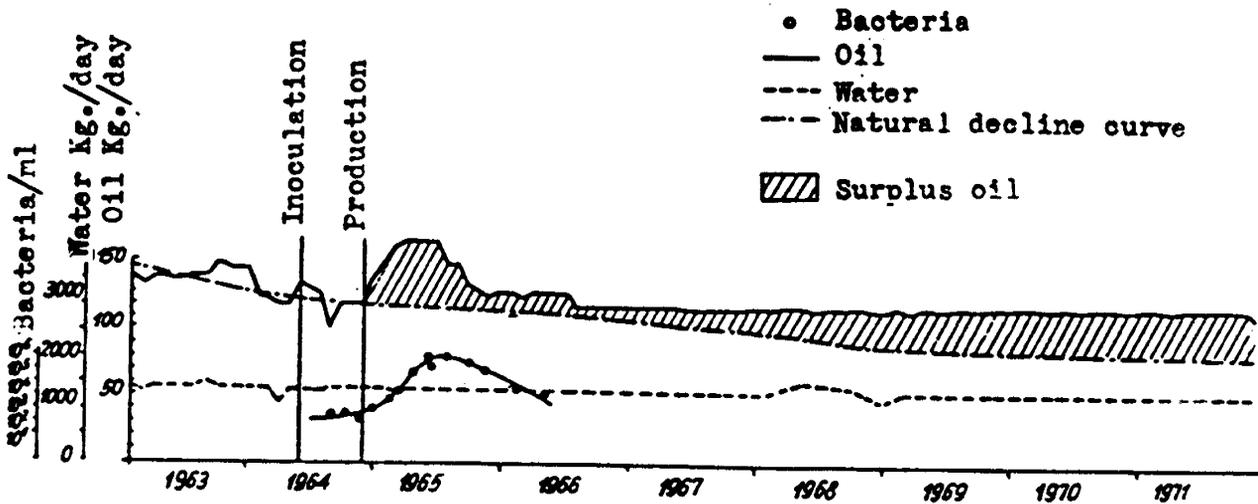


FIG. P-V-3—The effects on Well SW9 from inoculation of Well SW14.

A 7 yr total production increase of 230.3 tons of oil for all wells in this test area was attributed to microbial action (Table P-V-1). The increase was continuing in 1972 with additional stimulation of Wells SW9 and SW13.

The measurement of viscosity and specific gravity showed a notable decrease in Wells SW9 and SW13 (Table P-V-2) which was attributed to microbial action and increased oil production.

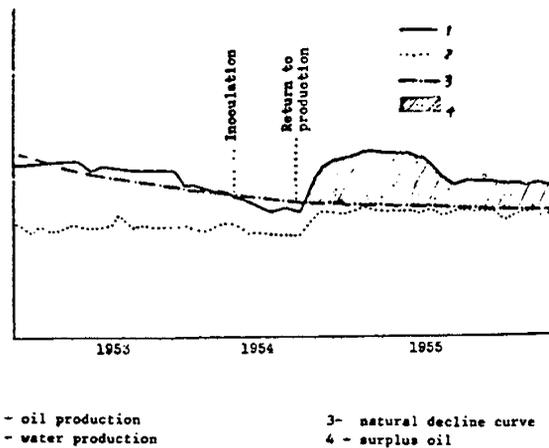


FIG. P-V-4—The effect of microbial treatment of Well SW14 on total production of Wells SW8, 9, 13, 14, and 18.

TABLE P-V-2—Changes in Oil Viscosity Observed in Well SW14 and Control Wells SW8, SW9, SW10, and SW13

Well Number	Date Sampled	Viscosity (cSt at 20°C)
SW14	Nov. 5, 1964	16.95
	Nov. 6, 1964	15.96
	Nov. 10, 1964	16.59
	May 24, 1965	15.00
	May 21, 1971	17.72
	Oct. 27, 1971	14.72
SW8	Dec. 16, 1964	13.93
	May 24, 1965	15.48
SW9	July 8, 1964	17.86
	Aug. 28, 1964	10.54
	Sept. 5, 1964	11.29
SW10	May 21, 1971	13.56
	July 8, 1964	13.94
	Aug. 28, 1964	12.28
	Nov. 5, 1964	13.21
SW13	May 24, 1965	14.38
	July 8, 1964	16.95
	Aug. 28, 1964	11.65
	Nov. 5, 1964	11.49
	May 27, 1971	15.42

In Well SW14 the pH declined in the light brine formation water. pH before inoculation was 8.4, 566 days after inoculation and at first production the pH had declined to 7.4, 5 mo later the pH was 7.9. The water volume increased for 6.5 yr (Fig. P-V-4) while the bacteria count increased for almost 2 yr. Before the test Well SW14 produced no gas while 3 mo later a pressure of 7 atm was noted. Its composition was methane 93.71 percent, ethane 3.44 percent, propane 0.59 percent, i-butane 1.59 percent, n-butane 0.47 percent (air free basis). There was a show of gas at the control wells but it was of no commercial value.

Remarks—The oil production increased significantly following microbial injection. The effect was noted for at least 7 yr following treatment.

POLISH FIELD TEST VI

Year of test: 1965. Location: Weglowka I Field Well W169

A fractionation of the oils showed an increase in the lighter fraction of the oil after microbial action (Fig. P-V-5).

Geological data—The test wells are found in the southern part of a block which was exploited for years as coal fields. The block was bisected by two lengthwise faults so a southern, central, and northern part can be

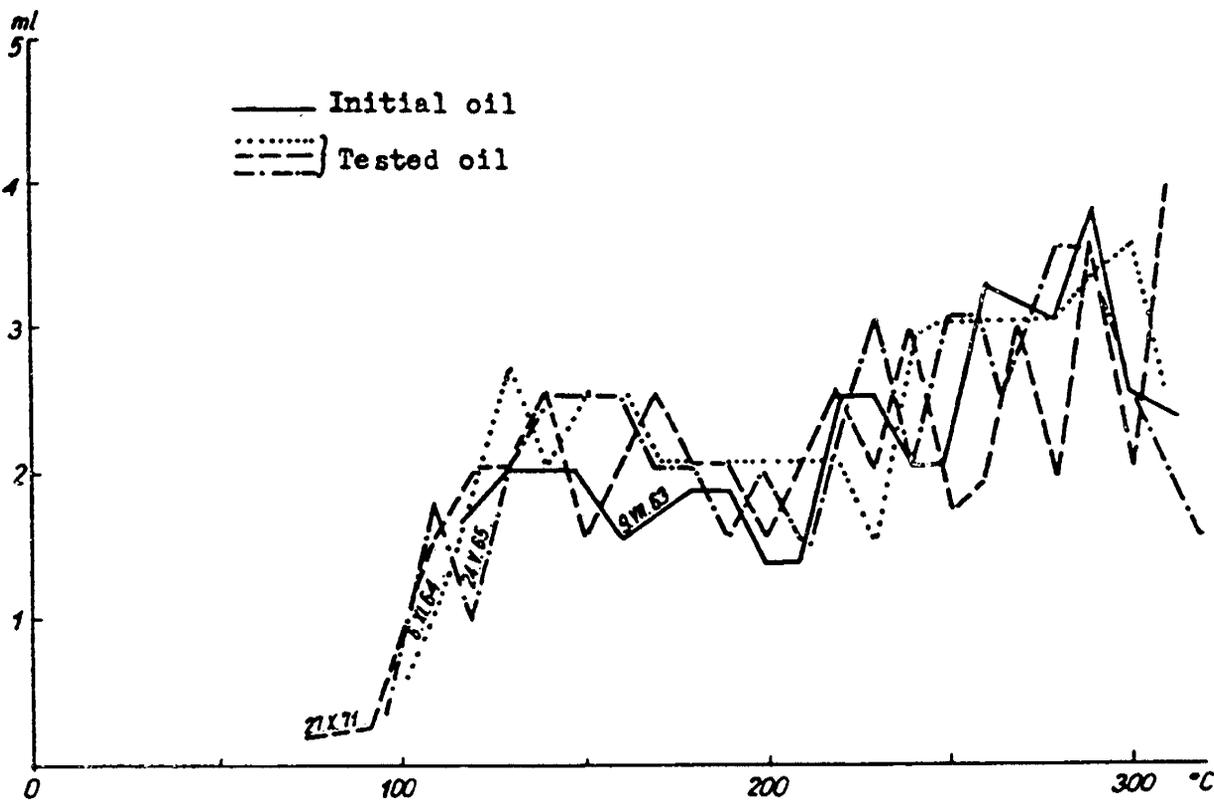


FIG. P-V-5—The fractional composition of oil from Well SW14.

TABLE P-VI-1—Oil Production from Well W169 Following Microbial Treatment

W169 Production	Tons Oil/Year	
	Normal	Surplus
1966	53.3	32.18
1967	78.33	26.87
1968	83.75	23.95
1969	81.28	28.22
1970	77.60	10.48

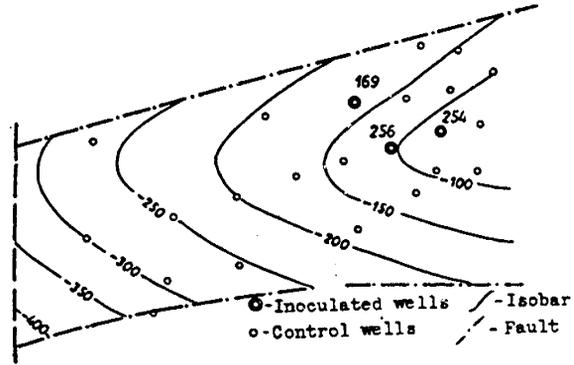


FIG. P-VI-1—Structural map of the Third horizon and location of test wells in Weglowka field.

separated. All three sections have oil-gas horizons with the central being most important. The oil bearing lower Cretaceous levels are found in the coal bearing sandstone (lower chalkpit). The test wells are in the central block which has two productive horizons, the First and Third (the Second is flooded). The surrounding water is found in the First horizon (~160 m). The depth of First layer is from 250 to 500 m with a thickness of 16 m, and a saturation coefficient of 0.5 (low because the block is drained by adjacent wells). The Third horizon layer is 360-670 m and the oil-water interface is 300 m with a thickness of 25 m and a saturation coefficient of 0.5 (Fig. P-VI-1).

Depth: Well W169, 510.3 m. **Porosity:** First horizon 11 percent, Third horizon 15 percent. **Viscosity:** 7 cSt.

Prior production history—Well W169 produced since 1957 from a depth of 510.3 m. Prior to inoculation produced 7500 kg oil, 9300 kg water and 730 m³ gas/mo.

Microbial well treatment—Well W169 was treated on July 19, 1965 with 500 l culture, 2000 kg molasses and 40,000 l of formation water. The well was shut in for 208 days.

Culture employed: Unknown.

Response—By the end of 1970 the well had produced 212.7 tons of excess oil which averaged as a 28 percent increase (Table P-VI-1).

After 1970 the well returned to the normal expected decline (Fig. P-VI-2).

The microbial count increased, then decreased. The pH decreased from 8.4 to 7.3 and was maintained at a level of pH 7.3 to 7.8 for 140 days after which it returned to normal. The water production increased greatly and remained thereafter at this high level.

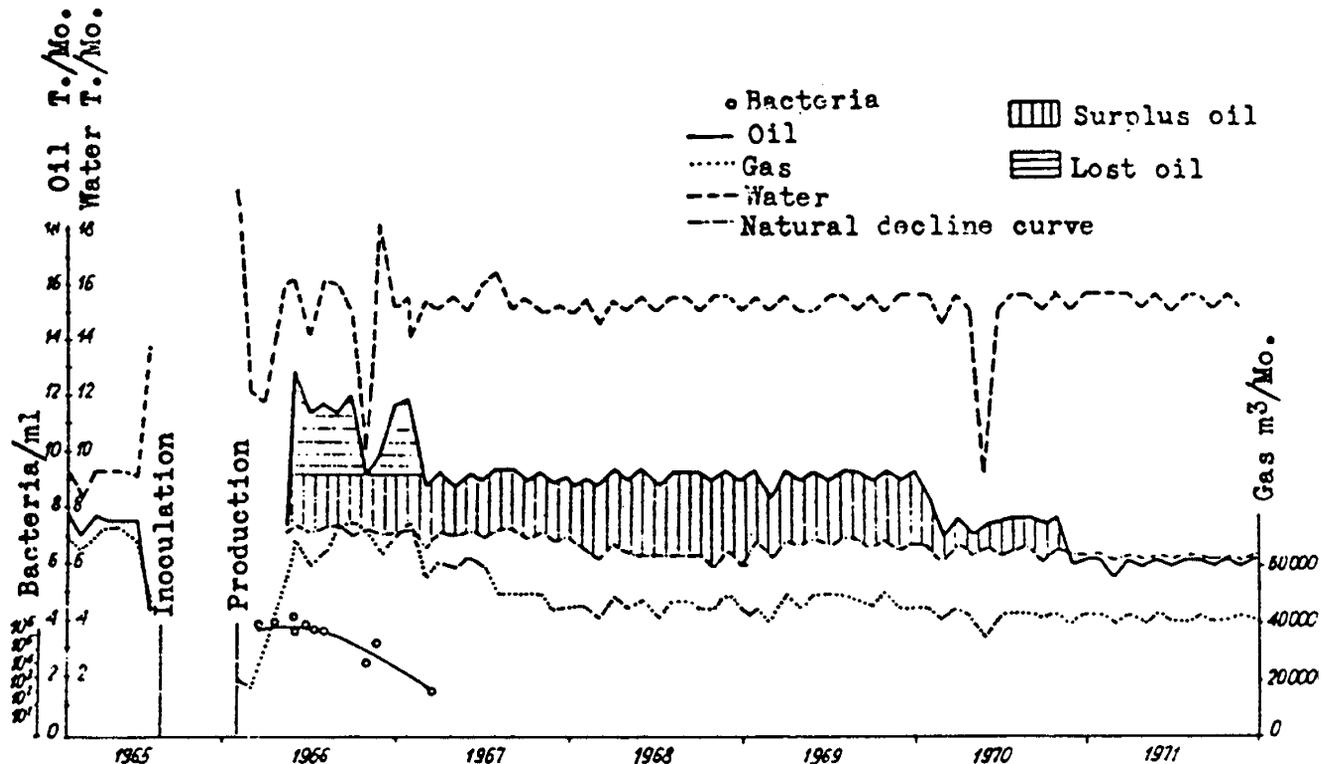


FIG. P-VI-2—The effect of bacterial inoculation of Well W169.

TABLE P-VI-2—Changes in Oil Viscosity Noted in Well W169

Date Well W169 Tested	Vis (cSt at 20°C)	Specific Gravity (g/cm ³ at 20°C)
July 8, 1965	6.65	0.8640
July 19, 1965 (inoculation)		
Oct. 9, 1965	6.07	0.8560
Apr. 26, 1969	4.25	0.8437
Dec. 12, 1970	7.06	0.8588
Apr. 12, 1970(?)	7.60	0.8590

The viscosity was measured for the 5 yr period and showed a drop, then a return to normal (Table P-VI-2). The decrease of 2 cSt was considered important to the oil release noted.

The gas production increased (especially in the first year) and in the third month following inoculation its composition was: O₂ 1.78 percent, N₂ 3.04 percent, CH₄ 83.83 percent, C₂ 3.54 percent, CO₂ 1.0 percent, C₃ 3.44 percent, i-C₄ 0.79 percent, n-C₄ 1.4 percent, i-C₅ 0.69 percent, n-C₅ 0.46 percent.

A distillation of the oil showed an increase in the lighter fractions (Fig. P-VI-3).

Remarks—Inoculation of the well showed a positive response in oil production. Water production increased. A change in viscosity was considered important in oil production increases.

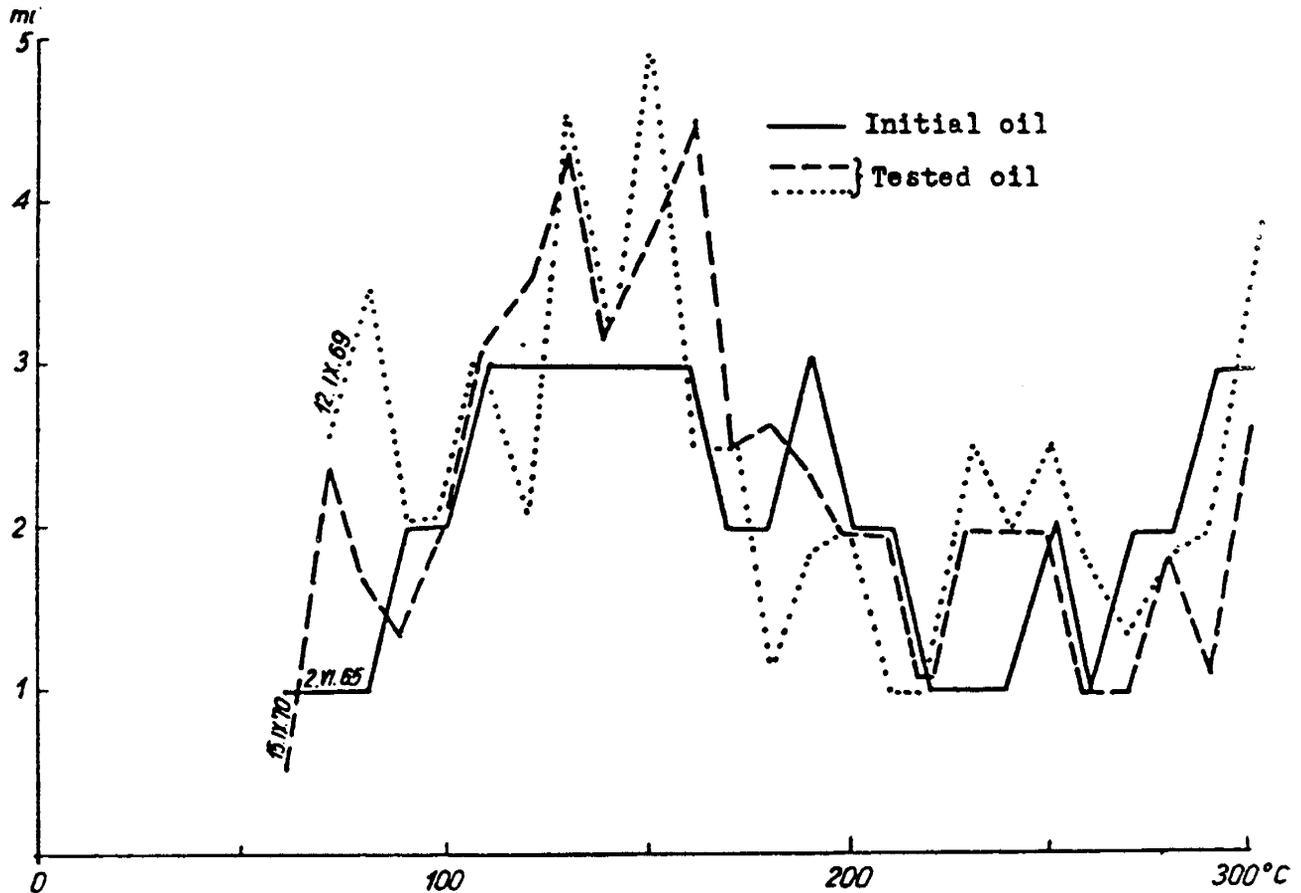


FIG. P-VI-3—The fractional composition of oil from Well W169 (Engler distillation).

POLISH FIELD TEST VII

Year of test: 1965. Location: Weglowka I Field Well W254

Geological data—See Weglowka I W169 test. Depth: 533.5 m.

Prior production history—Well W254 was drilled in March 1906 to depth of 533.5 m. In April 1965 the well produced 600 kg oil and 279 tons of water.

Microbial well treatment—Well W254 was inoculated on Sept. 22, 1965 using same technology as in W169 test. Well W254 was shut in for 122 days.

Culture employed: Same as in W169 test.

Response—In the 6 yr of observation the well produced 87.7 tons of excess oil (average increased yield of 340 percent) (Table P-VII-1).

TABLE P-VII-1—Effect of Microbial Inoculation on Well W254

W254 Production	Normal	Surplus
1966	3	16.94
1967	4.72	17.53
1968	4.77	13.03
1969	4.52	13.63
1970	4.43	12.77
1971	5.07	13.19

The bacteria count increased and the pH changes were similar to those observed in W169 (Fig. P-VII-1). The viscosity decrease was minimal.

Remarks—As in Well W169 an increased oil production followed microbial inoculation of the well.

POLISH FIELD TEST VIII

Year of test: 1965. Location: Swierchowa Field

Geological data—Same as Test V. *Depth*: 529.5 m. *Viscosity*: A paraffinic oil, 9–11 cSt.

Prior production history—Well Z12 drilled in 1950 to depth of 529.5 m and produced 267 tons of oil in 1951. In 1952 production was 9.4 tons and by 1963 only 10 percent of original production. Monthly production in 1963 was 1200 kg oil and 3000 kg water.

TABLE P-VIII-1—Production from Wells Z12 and Z7 After Microbial Treatment

Year	Tons of Oil			
	Z12		Z7	
	Normal	Surplus	Normal	Surplus
1965	16.12	4.77	—	—
1966	13.69	25.28	12.72	0.64
1967	11.69	6.66	12.22	2.46
1968	9.28	7.67	11.05	3.93
1969	8.81	6.69	10.31	2.09
1970	8.82	5.70	10.01	0.94
1971	8.75	5.56	9.82	1.16

Microbial well treatment—Well Z12 was inoculated on Dec. 16, 1965 with 600 l bacterial culture, 200 kg molasses, and 50,000 l of formation water and shut in for 217 days. The control well was Z7.

Culture employed: Mixed culture.

Response—Oil production increased in Wells Z12 and Z7 (Table P-VIII-1).

The total excess production from the wells in 7 yr was 73.55 tons and the increased rate was continuing. (1 yr after treatment 184 percent increase, 7 yr later 72.4 percent increase.)

The increase in production in Well Z12 is plotted in Fig. P-VIII-1.

There was a small increase in water production (20 percent) the first year which decreased to normal. The

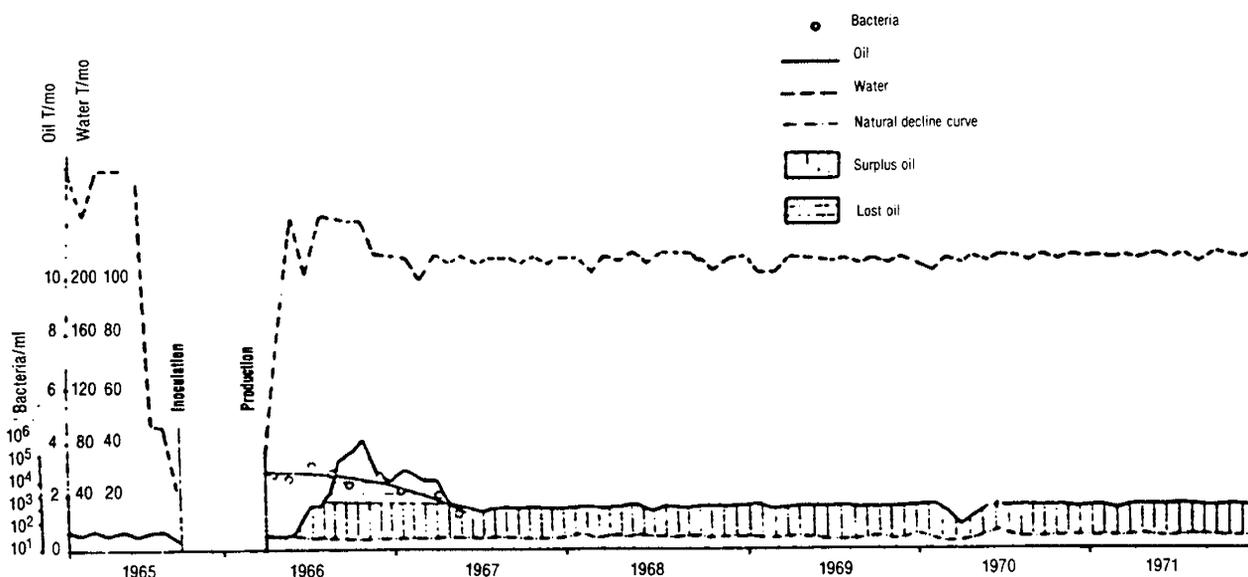


FIG. P-VII-1—The effect of bacterial inoculation of Well W254.

TABLE P-VIII-2—Changes in Oil Viscosity Observed in Wells Z12 and Z7

Date Sampled	Viscosity (cSt at 20°C)	
	Well Z12	Well Z7
Apr. 8, 1964	11.03	10.12
Oct. 29, 1964	9.26	10.09
Mar. 15, 1965	9.90	9.00
May 29, 1965	8.90	11.02
Inoculation Dec. 16, 1965		
May 12, 1971	10.50	10.48

microbial count increase paralleled the oil increase. The pH dropped from 8.8 before inoculation to 7.0 after being shut in for 217 days. It remained for 2 mo at 7.0 then returned to pH 8.8.

The viscosity of the oil in the test area was measured (Table P-VIII-2) and showed a decrease.

There was no gas before the test but built up to 17 atm during closure of the well. Its composition was methane 93.71 percent, ethane 3.44 percent, propane 0.59 percent, i-butane 1.5 percent, and n-butane 0.67 percent.

As observed in previous tests on Well SW14 a distillation showed an increase in the lighter fractions of the oil in Well Z12 (Fig. P-VIII-2).

Remarks—A positive response of oil production to microbial growth was noted.

POLISH FIELD TEST IX

Year of test: 1966. Location: Weglowka I Field
Well W256

Geological data—See Weglowka I field test VI (Well W169). Depth: 514.9 m. Porosity: 11 percent. Viscosity: 7 cSt.

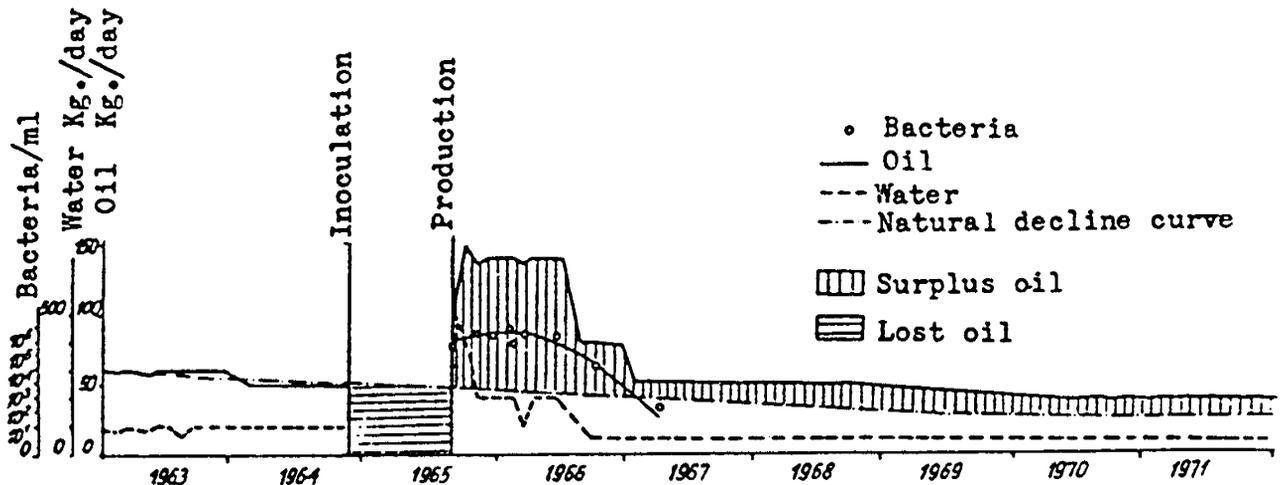


FIG. P-VIII-1—The effect of bacterial inoculation on Well Z12.

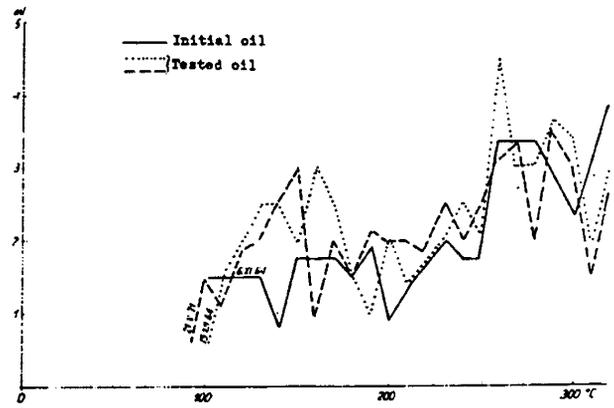


FIG. P-VIII-2—The fractional composition of oil from Well Z12 (Engler distillation).

Prior production history—Well W256 produced from 1956 from a depth of 514.9 m. In February 1966 Well W256 produced 6 tons of oil and 37 tons of water from the Weglowieckie sandstone (Lower Cretaceous).

Microbial well treatment—Well W256 was inoculated on Mar. 1, 1966 using the same technology as in W169 test. The well was shut in for 150 days. On Feb. 26, 1970 Well W256 was hydraulically fractured.

Culture employed: Same as W169 test.

Response—By the end of 1971 the well had produced 412.36 tons of excess oil. However, part of the production from 1970-71 could be due to the fracture treatment (Table P-IX-1 and Fig. P-IX-1).

TABLE P-IX-1—Effect of Treatments on Well W256

W256 Production	Normal	Surplus
1966	21.41	33.14
1967	50.50	60.73
1968	45.2	59.8
1969	41.33	68.17
1970	42.81	69.47
Well fractured		
1971	36.8	121.0

TABLE P-IX-2—Changes in Viscosity Observed in Well W256

Date Sampled	Viscosity (cSt at 20°C)	Specific Gravity (g/cm ³ at 20°C)
Oct. 9, 1965	7.28	0.8672
Mar. 1, 1966 (inoculation)		
Nov. 15, 1968	8.01	0.8653
Apr. 20, 1969	6.60	0.8589
Sept. 10, 1969	7.43	0.8600
Sept. 12, 1969	7.43	0.8607
Apr. 10, 1970	7.08	0.8601

Bacterial counts increased to a maximum 152 days after inoculation and paralleled increased oil production. The viscosity measurements (Table P-IX-2) showed:

A distillation of the oil showed an increase in the lighter fraction of the oil (Fig. P-IX-2).

Remarks—Good response from microbial action and an improved fracturing response when bacterial action is present.

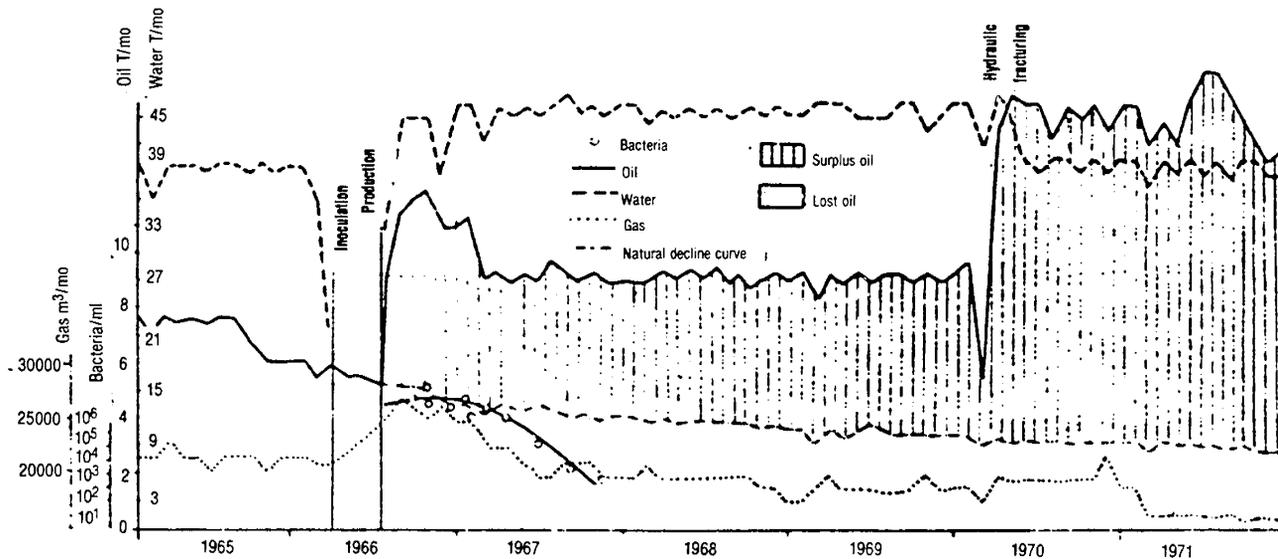


FIG. P-IX-1—The effect of bacterial inoculation and fracturing on production from Well W256.

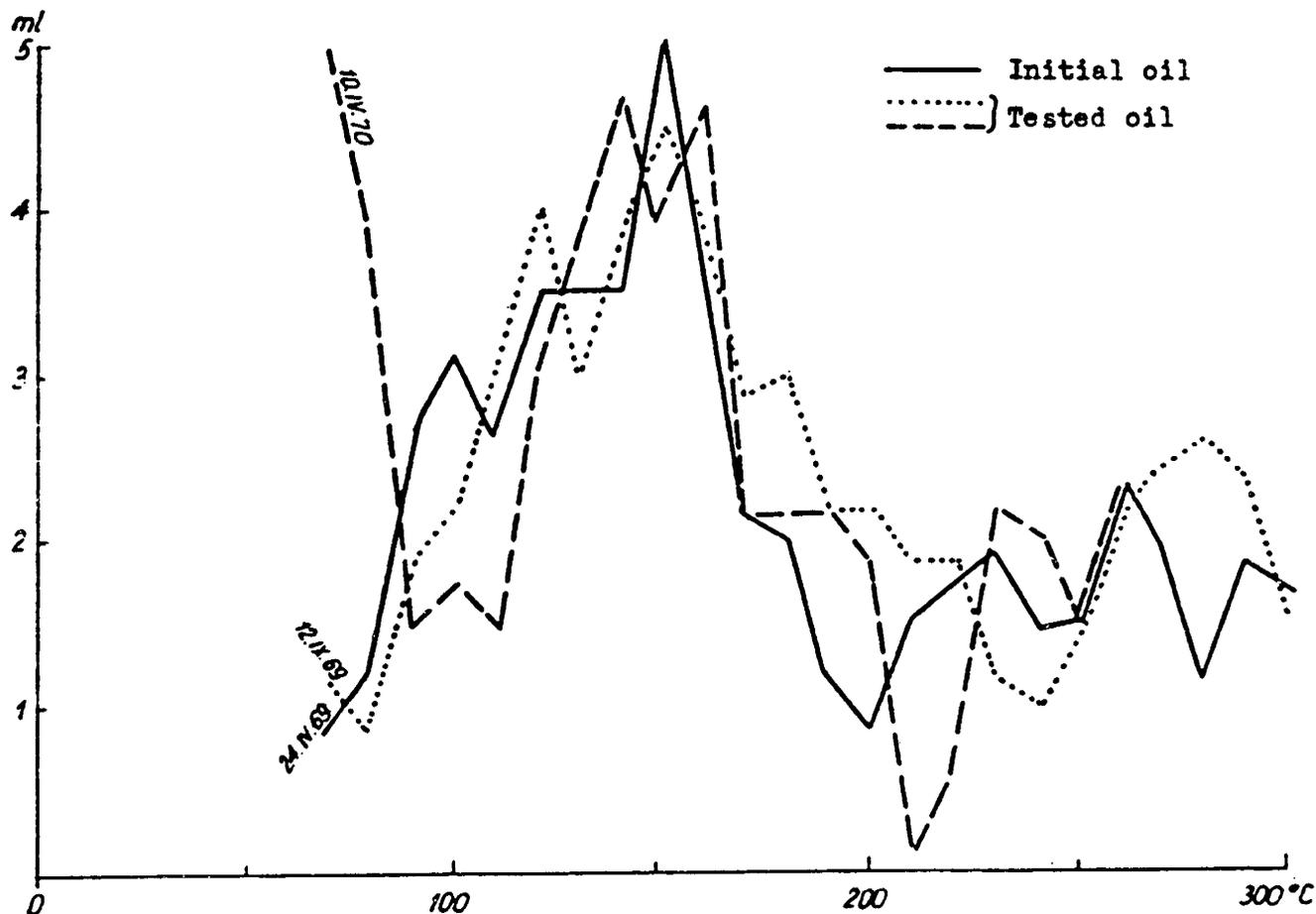


FIG. P-IX-2—The fractional composition of oil from Well W256 (Engler distillation).

POLISH FIELD TEST X

Year of test: 1967(?). Location: Unknown—Well W8

	pH	Dry Weight (g/L) After Drying at 180°C	Ionic Content of Cl, HCO ₃
Before test	8.5	9.722	
After opening well ...	7.8	10.176	Decrease
Months later	8.1	10.212	Increase

Geological data—Production of paraffinic crude from the Type I Cieszowice sandstone. Depth: 597 m. Viscosity: 2.02°E (specific gravity 0.876 g/cm³ at 20°C).

Prior production history—Well W8 was drilled in 1947 and had produced 6200 kg oil and 900 kg water/mo.

Microbial well treatment—Well W8 was inoculated with 200 l of bacteria, 240 kg molasses, 5600 kg of formation water. An extra amount of water and molasses was added and the well shut in for 4 mo.

Culture employed: Mixed culture isolated from sewage and industrial and petroleum sources.

Response—The water was characterized by a large quantity of very slimy crude. The chemical composition of the water changed:

The bacteria at the boundaries were maintained at a count of 2×10^5 and reached counts of 3×10^6 /ml.

A distillation of the oil showed the following changes in fraction content (Fig. P-X-1).

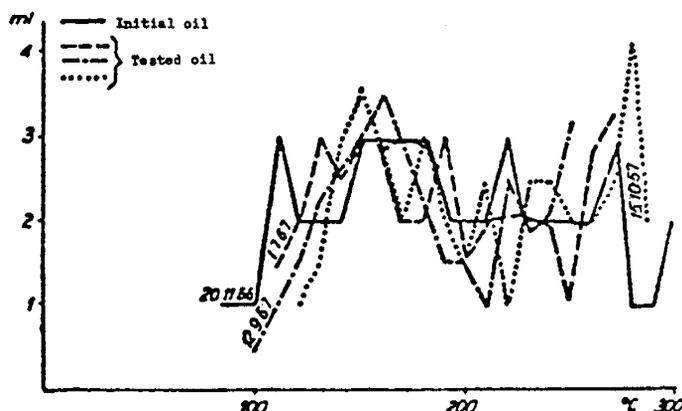


FIG. P-X-1—The fractional composition of oil from Well W8 (Engler distillation).

Remarks—The reason for this well producing a slimy oil is unknown. Since no production figures are given, the results of this effect are unknown.

POLISH FIELD TEST XI

Year of test: 1967. Location: Bobrka Field—Well B118

Geological data—The long narrow Bobrka field is bounded by faults with the southern end being rather steep and the north gradual. The center of the fold forms the surface of the First Type Eocene aggregated slate and the southern wing is made up of mineralite slate. The deep structure in the area of the Third Type Ciezkowice sandstone is present in small folds not more than 150 m wide. The best Third Ciezkowice sandstone area is in vicinity of B115 at -285 m. B115 is the top and descends on both sides with B117 at -282 m. The oil reservoir is the Third Ciezkowice at a depth of 650–680 m (30–40 m thick). Production is from the sand just below the Type Three slate (at -300 to -400 m). It is assumed there are two producing zones—the Third Ciezkowice sandstone and the nearby sand. These two zones are separated by large partitions composed of compressed sandstone of small grain size, impermeable, with irregular slate inserts. Wells B117 and B118 are in the southern area (Fig. P-XI-1).

Depth: 740.3 m. **Porosity:** Main porosity (variable). **Viscosity:** 11–13 cSt. **Permeability:** Core samples from an adjacent northern area showed a variable permeability.

Prior production history—Well B118 has produced from February 1956. In September 1967 from 740.3 m it produced 3600 kg oil and 2000 kg water monthly.

Microbial well treatment—The well was inoculated on Dec. 15, 1967 and shut in for 145 days (loss of 12 tons of oil).

Culture employed: Unknown.

Response—Upon resuming production in Well B118 the following production was obtained (Table P-XI-1).

The largest production increase occurred 4 mo after inoculation (300 percent increase) while production for the 4 yr increased by 143 percent.

TABLE P-XI-1—Oil Production (kg) from Well B118 Following Bacteria Inoculation

Month	Normal Prod. Expected	Actual Prod. Obtained	Surplus Production	Percent Increase ^a
Apr. 1968	1800	5350	3550	198
May	2640	5970	4330	125
June	2550	6400	3850	65
July.....	2600	10430	7850	300
Aug.	2540	5440	2900	120
Sept.	2450	4420	1970	80
Oct.	2500	5650	3150	125
Nov.	2400	5780	3150	140
Dec.	2450	4650	2200	90
Year	Tons of Oil			
1968.....	22.14		31.95	
1969.....	26.50		44.98	
1970.....	24.33		42.54	
1971.....	23.48		33.36	

The bacteria counts increased for 1.5 yr (Fig. P-XI-2). There was a minimal increase in water production. The pH decreased from 8.4 to 7 after 106 days of production.

The observed change in viscosity showed its greatest decrease 10 mo after inoculation after which it returned to normal (Table P-XI-2).

The wellhead pressure increased to 7 atm (gas cap) and showed the following composition: O₂ 4.80 percent, N₂ 13.35 percent, C₁ 71.11 percent, C₂ 3.55 percent, CO₂ 2.69 percent, C₃ 2.70 percent, i-C₄ 0.76 percent, n-C₄ 0.27 percent, i-C₅ 0.43 percent, and n-C₅ 0.34 percent. The gas yield was maintained for 1 yr.

Remarks—The injection of bacteria showed a significant improved oil recovery response.

TABLE P-XI-2—Effect of Bacterial Inoculation on Viscosity and Specific Gravity on Well B118

Date Sampled	Viscosity	Sp. Gravity
Feb. 5, 1968	12.2	0.8563
Apr. 12, 1968	9.91	0.8520
Sept. 12, 1968	9.88	0.8518
Oct. 30, 1968	14.25	0.8641
Feb. 20, 1969	8.92	0.8433
Apr. 4, 1969	10.92	0.8538
Dec. 2, 1969	11.65	0.8544
Sept. 18, 1970	13.19	0.8598

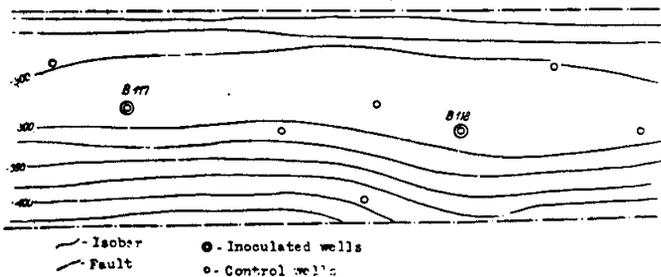


FIG. P-XI-1—Structure map of the top of the Third Ciezkowickiego sandstone and locations of Wells B117 and B118.

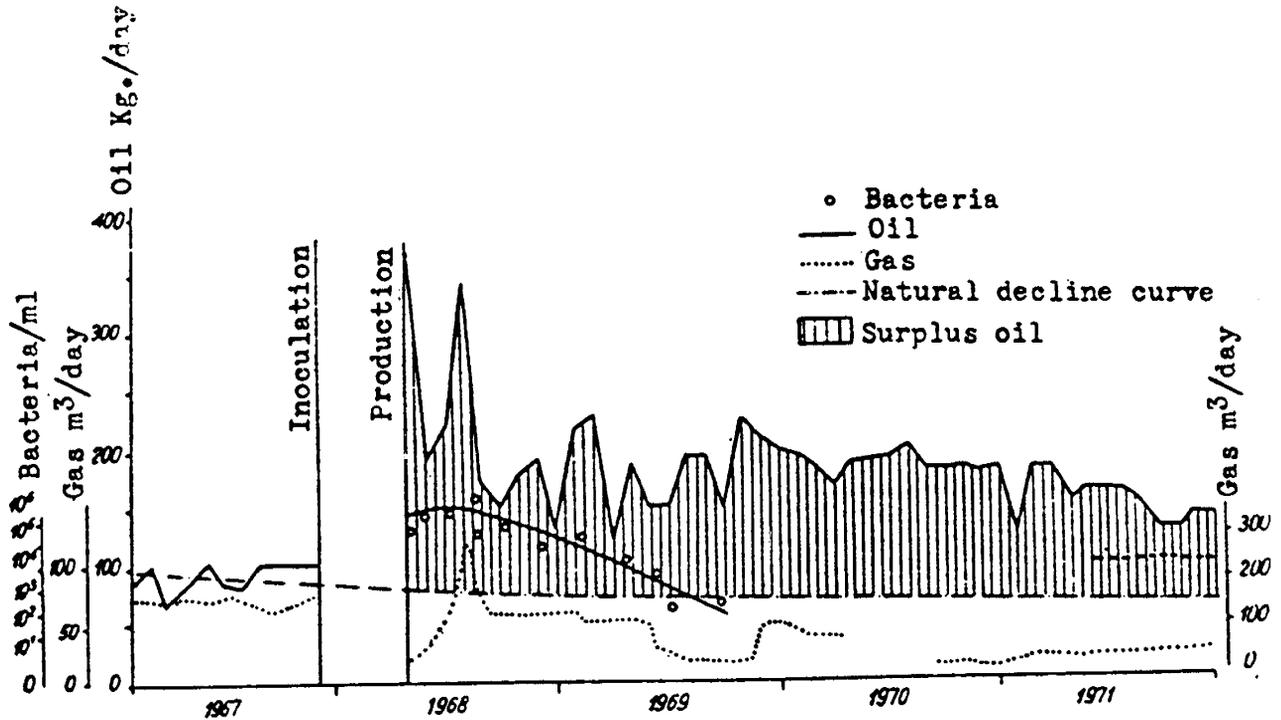


FIG. P-XI-2—The effect of bacterial inoculation on production in Well B118.

POLISH FIELD TEST XII

Year of test: 1968. Location: Bobrka Field—Well B117

Prior production history—Well B117 has produced since May 1956. In 1969(?) from the 802 m level it produced 3200 kg oil and 2400 kg water monthly.

Geological data—See data Bobrka test XI (B118 well). Well B117 is in the southern area. Depth: 802 m.

Microbial well treatment—Well B117 was inoculated on May 3, 1968 and closed for 85 days.

Culture employed: Unknown.

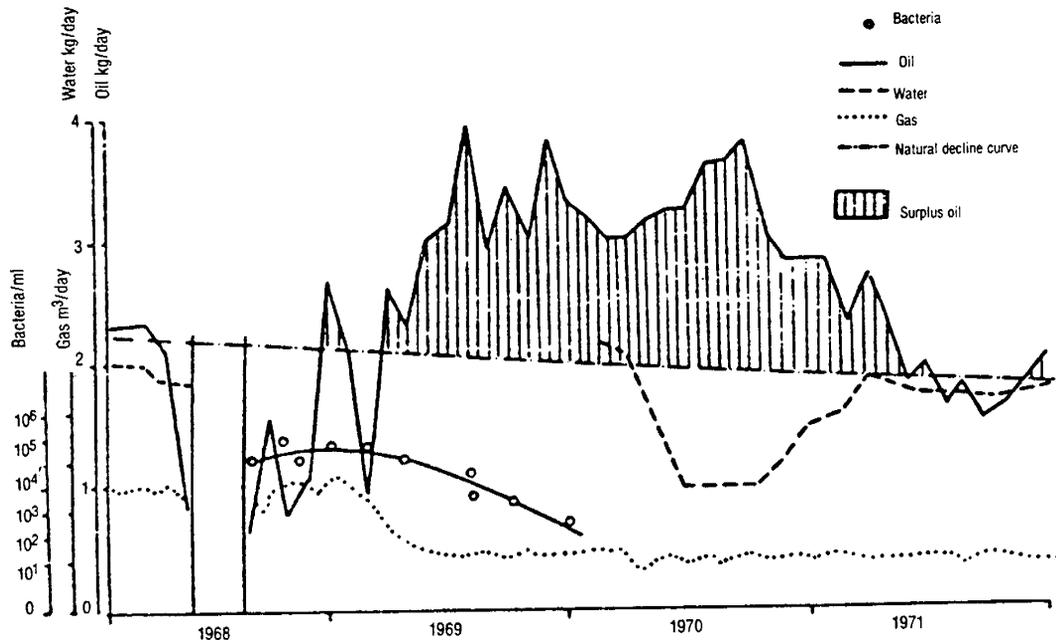


FIG. P-XII-1—The effect of bacterial inoculation on production in Well B117.

Response—Upon resuming production of Well B117 the following production was obtained:

Well B117—Tons Oil/Yr		
Year	Normal	Surplus
1969	71.09	33.85
1970	70.35	50.29
1971	55.00	9.60

By 1971 a total of 93.74 tons of excess oil (yearly increase 46 percent) was produced. After 2 yr the oil production returned to normal (Fig. P-XII-1).

The microbial count increase occurred in the first 6 mo of production then decreased. The viscosity decreased only 1.0 cSt in the first year. No change in water composition was observed but the pH decreased from 8.4 to 7.5 in the first 6 mo. There was some production of gas. The oil composition changed with an increase in the lighter fractions (Fig. P-XII-2).

Remarks—The well responded after the microbial growth had reached its peak and did not have as long a sustained production increase as Well B118.

POLISH FIELD TEST XIII
Year: 1968. Location: Rowne Field

Geological data—The West August area is bounded from the west by a lateral dislocation which runs past the Bobrka A35 well. The area is divided by two lengthwise fractures which separate the south, center, and northern areas. The surface of the center area is Type I Eocene variegated slate. The structure is rather smooth, falling to the south at an angle of 40-50° and more steeply to the north. The West August field is a Type II Cieszkowice sandstone. Poor oil horizons are also in Types I and III. The depth of the III is between 450-500 m and is 120 m thick. The oil zones are grouped in the second ridge of sandstone. The lower series is producing but hydrated. No cores are available (Fig. P-XIII-1).

The Fractional Composition of Oil from Well B117
(Engler Distillation)

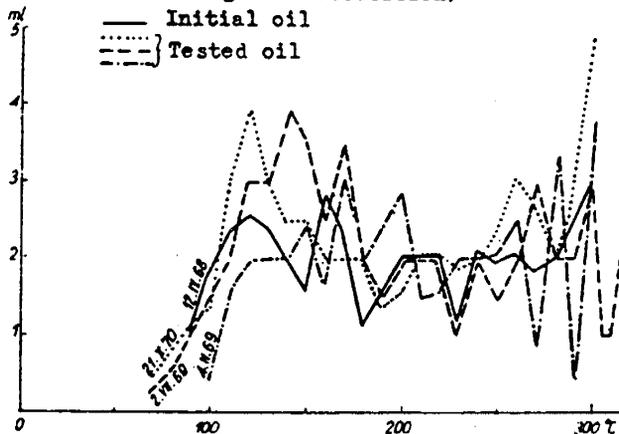


FIG. P-XII-2—The fractional composition of oil from Well B117 (Engler distillation).

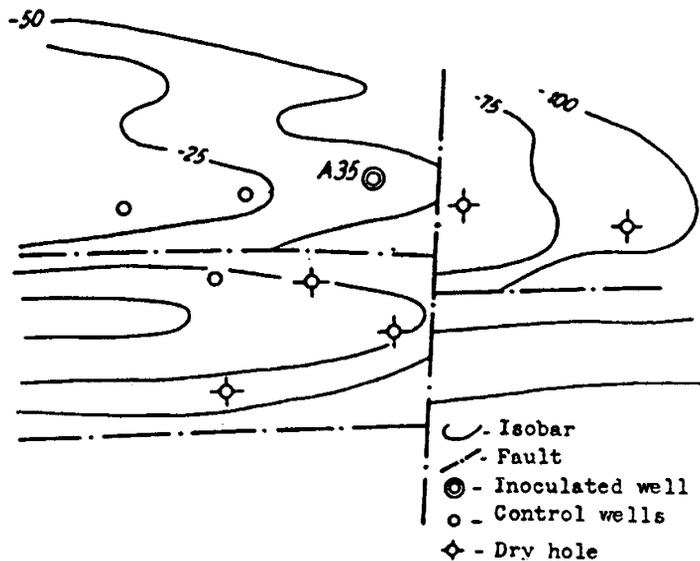


FIG. P-XIII-1—Structural map of the Cieszkowickiego sandstone (Type III) and location of Well A35.

Depth: 578 m. Viscosity: 4 cSt.

Prior production history—Well A35 has produced since October 1960. In March 1968 it produced 3100 kg oil, 4600 kg water, and 200 m³ gas.

Microbial well treatment—Well A35 was inoculated on July 22, 1968 with 500 l of inoculum, 1600 kg molasses and 45,000 l of formation water. The well was shut in for 100 days.

Culture employed: Unknown.

Response—In the period from 1968-71 the well had produced 85.56 tons of excess oil (average increase 68 percent) (Table P-XIII-1).

In 1970 production started to decline and declined further in 1971 (Fig. P-XIII-2).

The pH declined from 8.5 to 7.5 with an increase in microbial counts. The water production increased. During the shutdown period the wellhead pressure increased to 2.5 atm and the water was gassy. The viscosity declined as shown (Table P-XIII-2).

The oil in A35 is nonparaffinic so even a minimal decrease in viscosity could have a large effect on production. The distillation of the oil showed an increase in the lighter fractions of the oil (Fig. P-XIII-3).

Remarks—Microbial growth caused an increase in oil and gas production.

TABLE P-XIII-1—Production from Well A35 Following Bacterial Inoculation

Well A35 Production	Normal	Surplus
1968	19.20	6.35
1969	32.90	41.75
1970	34.84	27.21
1971	31.85	9.75

MEOR Field Applications

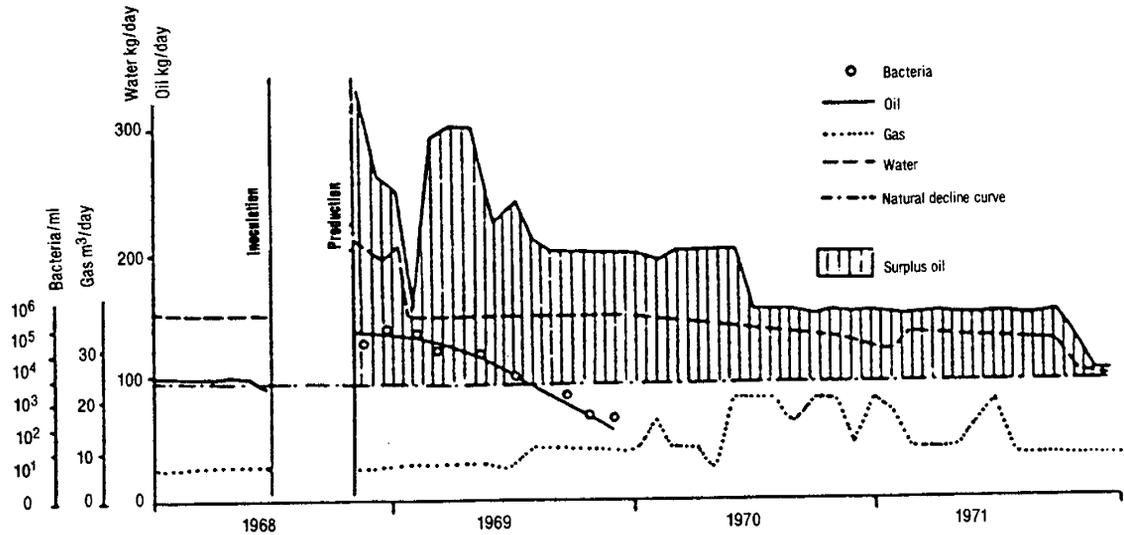


FIG. P-XIII-2—The effect of bacterial inoculation on production from Well A35.

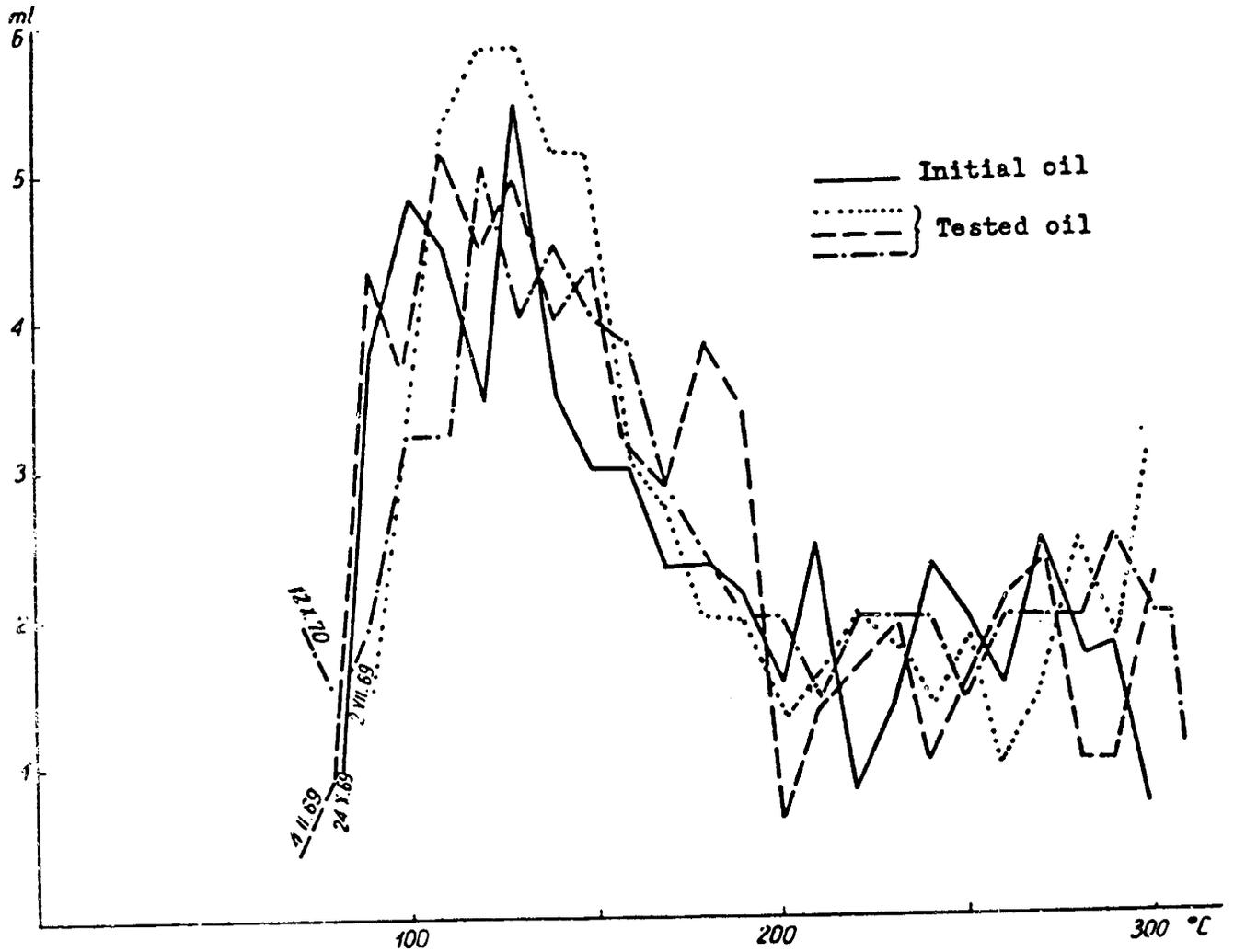


FIG. P-XIII-3—The fractional composition of oil from Well A35 (Engler distillation).

TABLE P-XIII-2—Effect of Bacterial Inoculation on Viscosity and Specific Gravity in Well A35

Date Sampled	Viscosity (cSt at 20°C)	Specific Gravity (g/cm ³ at 20°C)
June 20, 1968.....	4.57	0.8429
July 22, 1968 (inoculation)		
Oct. 24, 1968	3.90	0.8418
Oct. 30, 1968	3.90	0.8358
Dec. 19, 1968	3.99	0.8369
Jan. 23, 1969	3.84	0.8348
Feb. 4, 1969	3.77	0.8334
Feb. 20, 1969	3.84	0.8348
June 4, 1969.....	3.83	0.8365
July 2, 1969	4.74	0.8519
Sept. 19, 1970.....	4.70	0.8520
Nov. 20, 1970.....	4.18	0.8500

POLISH FIELD TESTS XIV-XVI

Year of tests: 1969. Location: Weglowka II Field

Geological data—The structural map of the lower Chalkpit ridge in the Weglowka field is shown (Fig. P-XIV-1).

Since the test wells are all within this plotted area, they are described together (Table P-XIV-1).

Well W176 is bounded on the south by a lateral fault in the profile of Well W280. On this productive block there is only the first vein (thickness 16 m), porosity 11 percent, with a saturation coefficient of 0.19. The oil-water interface is at 810 m. The depth of the first vein is 980–1130 m.

Well W266 is bounded on the south by a lateral fault running near W176 and W280. The parameters are the same as for W176.

Well W271 is situated in the same fashion as W176 and W266 in tectonic block 176 and has the same parameters.

Well W288, also in block 176 which is bounded on the south by a lateral fault from the profile of Well W230. It has the same parameters as Well W176.

Well W273 is a control well in tectonic block 276 separated by tectonic faults from the wells used in the test. It possesses the same parameters as the above wells. It was closed for 3 mo but not inoculated.

Microbial treatment of test wells—All inoculated using technology employed in Weglowka I test on Well W169.

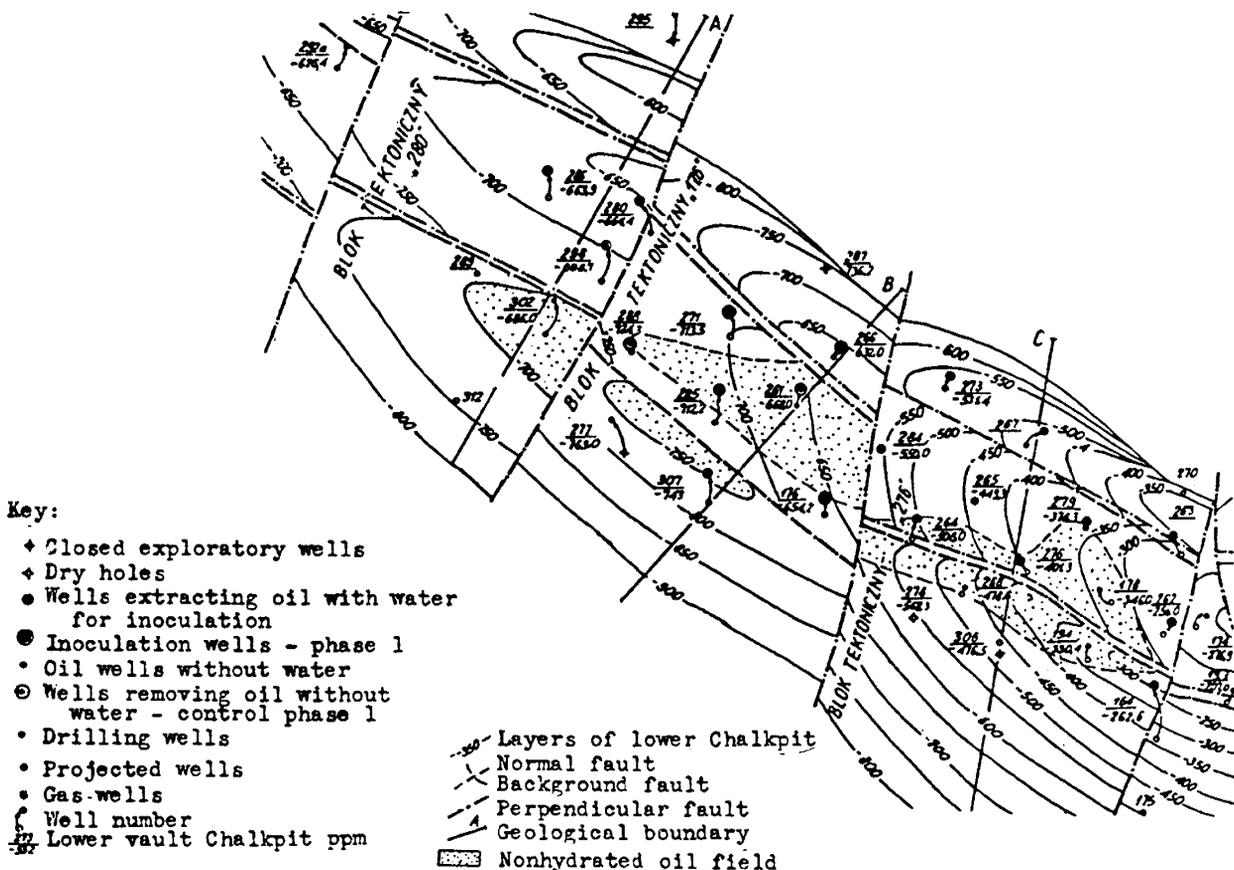


FIG. P-XIV-1—Structural map of the Lower Chalkpit ridge and the location of wells of the Weglowka II (see key).

TABLE P-XIV-1—Data on Test Area in Weglowka II Area

Well	Date Drilled	Production m Depth*	Oil Sat. Coeff.	Prior Monthly Production			
				Date	kg Oil	kg Water	m ³ Gas
W176	Oct. 1959	1100	0.19	Feb. 1969	5500	37500	15450
W266	May 1961	1144.2	0.19	Feb. 1969	22700	25900	10500
W271	Apr. 1960	1124.4	0.9	Feb. 1969	9400	14400	11000
W288	Sept. 1963	1125		Feb. 1963	7100	2800	6000
W273	Control well	1100			8000	3200	3800

*Thickness of production zone in the wells was 16 m, the porosity was 11 percent, and the oil-water interface was at 810 m.

Well	Date Inoculated	Shut In Period (Days)
W176	Apr. 10, 1969	90
W266	Apr. 9, 1969	90
W271	Apr. 5, 1969	90
W273	Control well only shut in, not inoculated	90

Well W176 was perforated on Sept. 2, 1970 so oil production after that date is due to both treatments. Well W266 was hydraulically fractured on Oct. 15, 1970.

Response—W176: In 1969 reported to have an excess oil production of 55,900 kg (increase of 32 percent) and in 1970 an excess of 70,730 kg (20.7 percent increase) (Fig. P-XIV-2). However during the 90 day shut in period 95,000 kg was lost so the surplus was only 31,600 kg. The data in 1970 include production resulting from the perforation treatment.

Well W266: In 1.5 yr the excess oil was reported as 6200 kg. With the fracturing on Oct. 15, 1970 the excess oil by the end of 1970 was reported as 139.39 tons. This marked increase was claimed to be the result of the combined microbial and fracturing treatment—especially of formations of lower permeability.

Well W271: No excess oil was reported. The reason cited was the low permeability. At the time of inoculation it was necessary to use 130 atm for 2 days to inject the inoculum.

Well W273: Control well—due to shut in period, 90 tons of oil was lost. There was no increase in production observed for 2 yr.

Remarks—The injection of bacteria into the formation was both successful and unsuccessful. The perforating and fracturing of wells which had been inoculated showed marked success. Wells in areas of low permeability showed no effect of microbial treatment. A control well showed no response due to bacterial injection.

POLISH REFERENCES

Karaskiewicz, J. (1962). Versuche der Mikrobiologischen Aktivisierung von Erdollagerstätten. Wissenschaftliche Tagung für Erdolbergbau, Budapest, 13 Oct. 1962. pp. 566-577. (Experiments in the Microbiological Activation of Crude Oil Deposits, Proceedings of the Third International Conference for Geochemistry, Microbiology and Crude Oil Chemistry.)

Karaskiewicz, J. (1974). Zastosowanie Metod Mikrobiologicznych W Intensyfikacji Karpackich Ztoz Ropy, Naftowej. (The Application of Microbiological Methods for Secondary Oil Recovery from the Carpathian Crude Oil Reservoirs.) Prace Instytutu Naftowego, pp. 1-67.

Karaskiewicz, K. (1975). Investigations Concerning the Intensification of the Oil Recovery from Carpathian Crude Oil Reservoirs By Means of Bacteria. Nafta (Pol.) 31, No. 3-4, pp. 144-150.

Karaskiewicz, J. (1968). Recovery of Crude Oil from Its Reservoirs with the Aid of Bacteria. Nafta (Pol.) 24, No. 7, pp. 198-202.

Karaskiewicz, J. (1964). Attempts to Microbiologically Liberate Oil from Reservoirs. Nafta (Pol.) 20, No. 3, pp. 61-66.

Karaskiewicz, J. (1968). Experiments on the Use of Bacteria in the Recovery of Crude from Areas Adjacent to Oil Wells. Nafta (Pol.) 1, pp. 1-3.

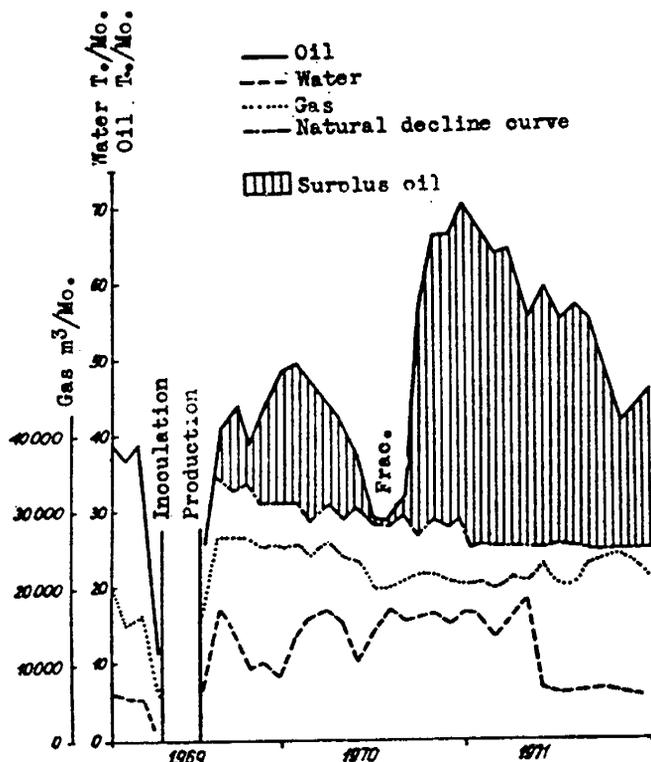


FIG. P-XIV-2—The effect of bacterial inoculation and perforating on production from Well W176.

TABLE C-I-1—Number of Sulfate Reducing Bacteria/ml, Days After Phase 1 Inoculation

Well	0	5	18	26	64
Z2	10°	10°	1 × 10 ¹	8 × 10 ¹	2 × 10 ²
Z4	10°	10°	1 × 10 ¹	2 × 10 ¹	2 × 10 ²
Z5	10°	10°	10°	2 × 10 ¹	3 × 10 ²
Z7	10°	10°	10°	10°	1 × 10 ²

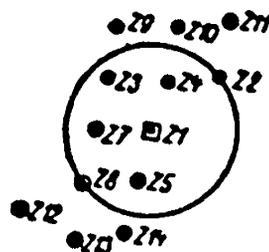


FIG. C-I-1—Location of wells in Deposit Z test.

CZECHOSLOVAKIA FIELD TESTS I AND II

Year of tests: 1954. Location: Southern Moravia

Geological data—Deposit Z is composed of coarse Tortonean sands of variable granularity. The upper boundary of the well (depth about 50 m) is regular while the lower boundary was not defined. Only the main part of the sand contains oil with the water content increasing with depth. The oil is characterized as a heavy asphaltic oil of high sulfur content (average 79.6 mg/l) with a small content of H₂S.

Depth: 50 m. *Porosity:* 24-32 percent. *Permeability:* 3000-8000 mD. *Residual oil saturation:* 30 percent.

Prior production history—The Well Z1 is on the boundary of a flooded-out area. The daily oil recovery is in the tens of kg/well. The pH of the water is 8.3.

Microbial well treatment—There were two phases to the test. In 1954 Well Z1 was inoculated with 40 liters of a mixed culture of sulfate reducing bacteria and hydrocarbon bacteria without the addition of nutrient. In the second phase in 1955, the same inoculum was used (40 l) but in addition 30 kg molasses wastes were added and pushed with water at 4.9 atm. The culture was inoculated into Well Z1 with the surrounding wells serving as controls (Fig. C-I-1).

Response—Phase 1 (1954): Inoculation without nutrient addition. The microbial count increased in the wells (Table C-I-1).

Phase 2 (1955): Inoculation with added molasses. The presence of nutrient stimulated microbial growth (Table C-I-2).

The effect of the added nutrient is evident between Phase 1 (no nutrient) and Phase 2 (added nutrient) since in Phase 1 the count increased only one order while in Phase 2 the count increase was 6 orders. The exhaustion of nutrient was shown by drop in counts 100 days after inoculation (some loss of nutrient was attributed to water production from the wells).

The growth of bacteria and the response in oil production are shown in Fig. C-I-2.

Although the observed oil recovery was not impressive the point is made that the increase did correlate to the time of maximum microbial growth. The microbial increase was faster and was maintained for a longer time in the structural high (Well Z2) and was less in the lower part of the structure (Well Z3).

Remarks—Microbial counts increased in the reservoir after inoculation with added nutrients stimulating microbial growth. The oil production increases paralleled microbial increases.

TABLE C-I-2—Number of Sulfate Reducing Bacteria/ml, Days After Phase 2 Inoculation

Well	0	20	48	68	97	117	145
Z2	10°	10°	10°	4 × 10 ⁸	4 × 10 ⁸	2 × 10 ⁷	3 × 10 ⁵
Z3	10°	10°	—	1 × 10 ⁹	—	7 × 10 ¹	—
Z4	10°	10°	—	2 × 10 ⁵	5 × 10 ⁴	—	2 × 10 ³
Z5	10°	10°	2 × 10 ¹	2 × 10 ³	2 × 10 ⁴	1 × 10 ⁵	2 × 10 ⁴
Z7	10°	10°	2 × 10 ¹	3 × 10 ³	1 × 10 ³	4 × 10 ³	4 × 10 ⁴
Z8	10°	10°	10°	7 × 10 ¹	8 × 10 ²	1 × 10 ³	6 × 10 ³

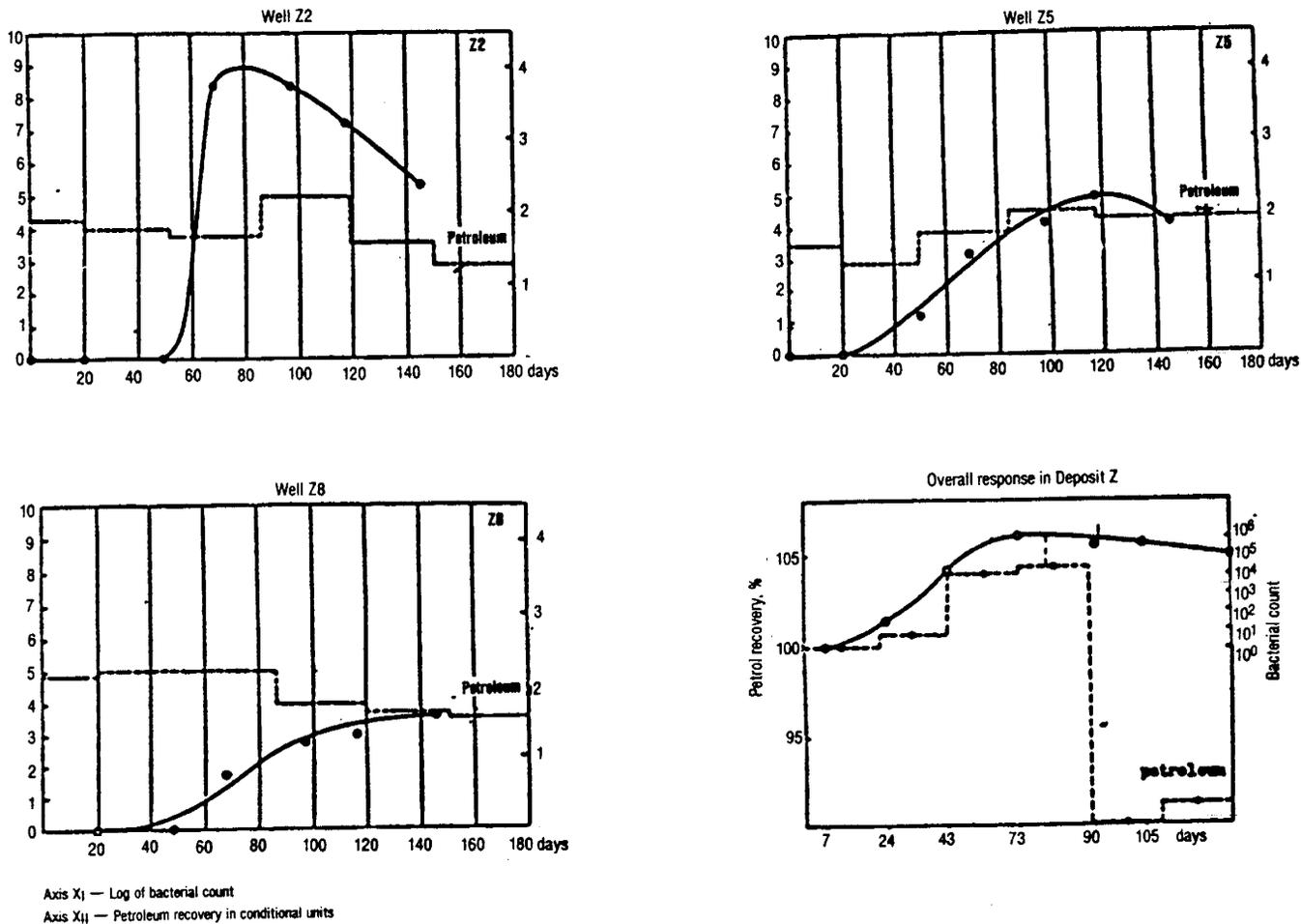


FIG. C-1-2—Microbial growth and oil production in deposit and selected wells.

CZECHOSLOVAKIA FIELD TEST III
Year of test: 1955. Location: Deposit H

Geological data—The area is in the Moravian part of the Vienna Basin. The geology and reservoir parameters are reported to be similar to the Hodonin deposit (see Field Test IV). The wells involved in the test are shown in Fig. C-III-1.

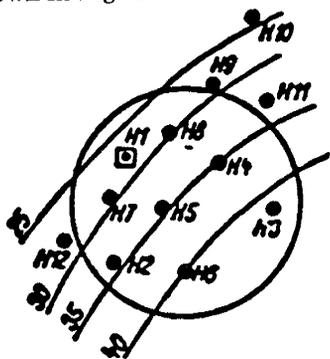


FIG. C-III-1—Location of wells in Deposit H test.

Microbial well treatment—The Well H1 was first cleaned and the water level lowered. The well was inoculated with 40 l of culture and 30 kg of molasses wastes. The inoculum and nutrients were injected at 9.4 atm pressure.

Culture injected: A stable mixed culture which contained sulfate reducing bacteria (10^9 /ml) and *Pseudomonas hydrocarbon* bacteria (10^9 /ml). (Same as Deposit 2.)

Response—The growth of the sulfate reducing bacteria was evident (Table C-III-1) within 48 days following inoculation.

The growth of sulfate reducing bacteria compared to oil and water production for selected wells and for the deposit as a whole are shown in Fig. C-III-2.

The oil recovery was calculated only on the basis of the wells in the test program and not for the entire deposit. During the test about 5,000,000 liters of water were produced from the test wells.

TABLE C-III-1—Multiplication of Sulfate Reducing Bacteria (Number/ml) in Deposit H

Well No.	Days of Test							
	0	7	28	48	73	99	118	169
H2	10 ⁰	10 ⁰	—	4 × 10 ¹	1 × 10 ³	3 × 10 ⁴	—	—
H3	10 ⁰	10 ⁰	10 ⁰	1 × 10 ³	—	1 × 10 ⁵	—	2 × 10 ⁵
H4	10 ⁰	10 ⁰	10 ⁰	1 × 10 ⁵	7 × 10 ⁶	4 × 10 ⁶	3 × 10 ⁷	1 × 10 ³
H5	10 ⁰	10 ⁰	10 ⁰	1 × 10 ³	6 × 10 ³	8 × 10 ⁴	5 × 10 ⁴	1 × 10 ³
H6	10 ⁰	10 ⁰	—	1 × 10 ²	8 × 10 ²	1.2 × 10 ⁴	1 × 10 ⁴	—
H7	10 ⁰	10 ⁰	4 × 10 ¹	1 × 10 ⁴	1 × 10 ⁵	1 × 10 ⁵	1 × 10 ⁵	1 × 10 ⁴
H8	10 ⁰	—	10 ⁰	1.7 × 10 ³	3 × 10 ⁴	2.5 × 10 ⁵	—	2 × 10 ³

The viscosity of the oil was measured before and during the changes of microbial growth (time unknown). The results show (see Table 4) that only in Well

H3 was there a noticeable decrease and they report this well had the highest microbial activity during the test.

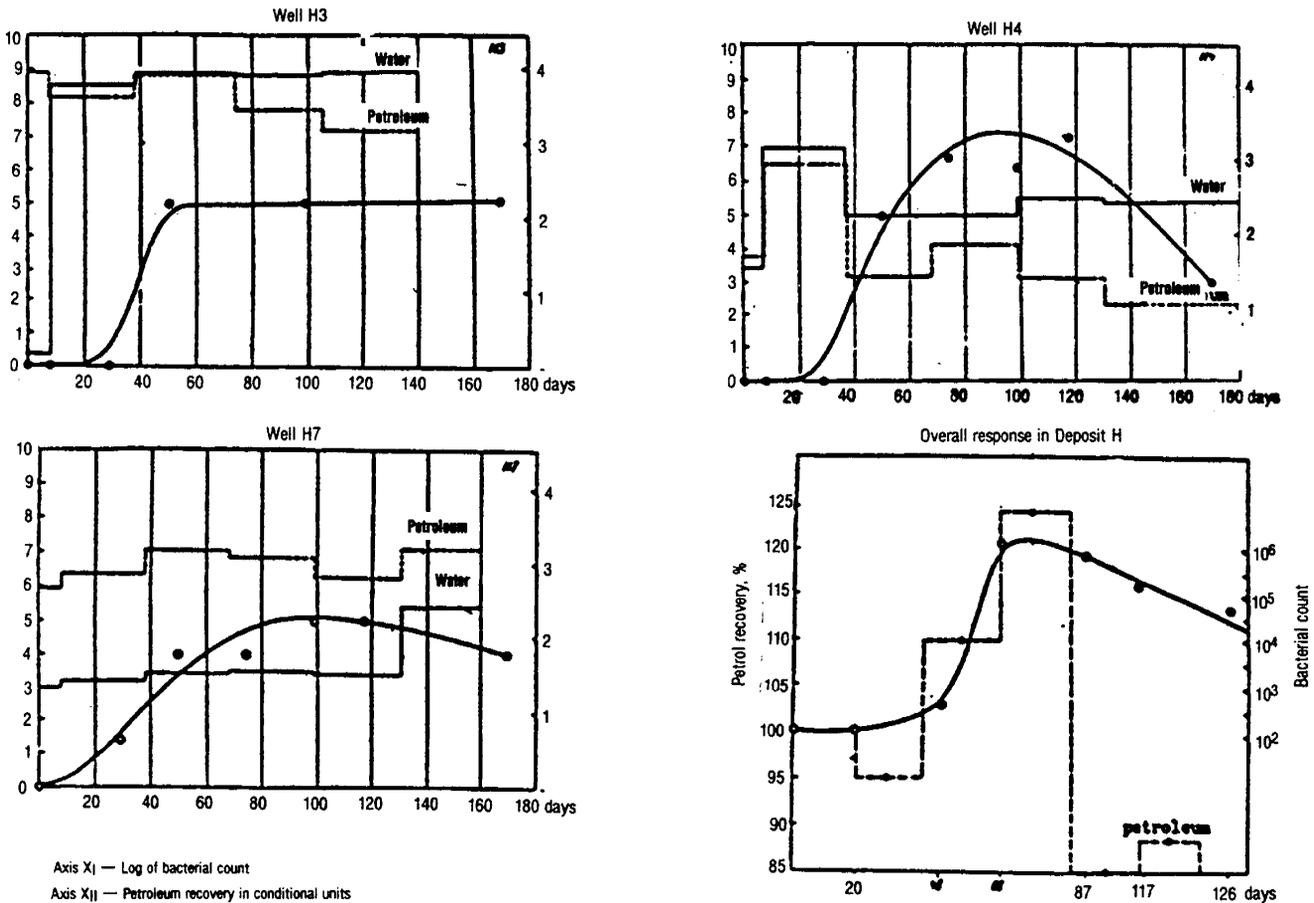


FIG. C-III-2—Microbial growth and oil and water production in Deposit H and selected wells.

CZECHOSLOVAKIA FIELD TEST IV

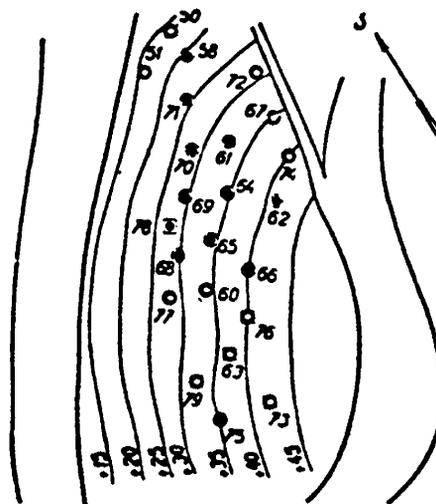
Year of test: 1957-58. Location: Sarmatian Region, Vienna Basin (Hodonin Deposit)

Geological data—The field is the smallest deposit in a portion of the Vienna Basin which is situated in an intermediate block confined by two faults: one to the east, one to the west. Both faults dip eastward at an angle of 55°. The strata within the block creates a semi-vault apexing at the bend of the eastern fault and the strata dips westward. The productive Sarmatian horizon occurs at the Pannonian-Sarmatian interface. The upper boundary of the apex portion is 150 m deep with the relative difference in elevation being 30 m. The zone originally had a free gas cap, now depleted. Production has caused the whole zone to have a waterbed. Water enters across the western fault or from the north where the fault does not seal (Fig. C-IV-1). The water is saline of the Na bicarbonate type, pH 6.5-7.5, with a sulfate content of 9.5-14.4 mg/l.

Depth: 150 m (first Sarmatian zone). *Porosity:* 36 percent (fine to medium granular sand). *Viscosity:* 100 cSt (specific gravity of oil 0.941 g/cm³). *Permeability:* 3300 to 8100 mD. *Residual oil saturation:* 40 percent after waterflooding (composed of naphthenic 53 percent, paraffinic 30 percent, aromatic 17 percent).

Prior production history—The test area had previously been injected with molasses through Well 78.

Microbial well treatment—The injection Well 78 was first cleaned and the water level lowered. The well was inoculated with 60 liters of a 4-day-old culture of *Desulfovibrio* and *Pseudomonas* cultures (contained 2 × 10⁹ cells/ml). Simultaneously with the culture was injected 50 kg of distillery effluents at a pressure of 5.4 atm. The pressure was increased after 24 hr to ensure the inoculum was injected into the formation. After 10 days the water level was normal.



- Production well
- ⊙ Terminated well
- Nonproducing well
- ◻ Injection well
- ▬ Fault

FIG. C-IV-1—Structural map of the deposit and the location of the producing wells.

Culture injected: Stable mixture of *Desulfovibrio* and *Pseudomonas* with decomposed hydrocarbons. Culture grown at 32°C.

Response—Well 78 was inoculated and tests were made on 10 control wells. An increase in microbial numbers was observed throughout the test area (Table C-IV-1). Sulfate reducing bacteria counts were determined by the lead acetate paper technique. The microbial numbers reported are only for sulfate reducing bacteria since the *Pseudomonas* did not follow the pattern nor did viscosity measurements indicate their activity.

TABLE C-IV-1—Variations of the Number of Sulfate-Reducing Bacteria in Oil Water After Injection of Culture

Days	Well Number										
	61	62	64	65	66	68	69	70	71	78	58
Before experiment	4.6 × 10 ²	2.8 × 10 ³	2.5 × 10 ³	1.5 × 10 ²	5 × 10 ²	1 × 10 ³	1.3 × 10 ³	3 × 10 ²	1.8 × 10 ³	1 × 10 ³	0
3	2 × 10 ²	6 × 10 ²	—	9 × 10 ²	2 × 10 ³	3 × 10 ³	3 × 10 ³	4 × 10 ³	2 × 10 ³	1.3 × 10 ³	0
7	2 × 10 ²	8 × 10 ²	1 × 10 ²	0	0	0					
15	7 × 10 ²	4 × 10 ³	3 × 10 ³	1 × 10 ²	6 × 10 ³	9 × 10 ³	1 × 10 ²	1 × 10 ²	3 × 10 ⁴	1 × 10 ⁶	0
22	0	2 × 10 ³	2 × 10 ³	1 × 10 ³	4 × 10 ⁴	2 × 10 ³	6 × 10 ⁴	1 × 10 ⁶	0		
29	5 × 10 ⁴	3 × 10 ⁵	5 × 10 ³	1.5 × 10 ³	3 × 10 ⁵	2 × 10 ⁴	5 × 10 ³	5 × 10 ⁴	4 × 10 ⁵	1 × 10 ⁶	0
36	4 × 10 ⁴	4 × 10 ⁵	4 × 10 ⁴	4 × 10 ³	4 × 10 ³	1.2 × 10 ⁵	1 × 10 ⁶	4 × 10 ⁴			
43	7 × 10 ⁴	2 × 10 ⁵	6 × 10 ⁴	6 × 10 ⁴	3 × 10 ⁵	7 × 10 ⁴	6 × 10 ⁵	4 × 10 ⁵	2 × 10 ⁵	1 × 10 ⁶	6 × 10 ⁴
52	1 × 10 ⁵	1 × 10 ⁵	1 × 10 ⁵	—	1.7 × 10 ⁵	5 × 10 ⁵	5 × 10 ⁵	5 × 10 ⁵	8 × 10 ⁵	1 × 10 ⁶	8 × 10 ⁴
63	2 × 10 ⁵	5 × 10 ⁴	5 × 10 ⁵	6 × 10 ⁵	8 × 10 ⁵	8 × 10 ⁵	7 × 10 ⁵	5 × 10 ⁴	0	1 × 10 ⁶	5 × 10 ⁴
99	1 × 10 ⁴	1.2 × 10 ⁴	3 × 10 ⁵	1.4 × 10 ⁵	3 × 10 ⁵	1.3 × 10 ⁵	2 × 10 ⁵	1 × 10 ⁴	6 × 10 ⁴	1 × 10 ⁶	8 × 10 ⁴
148	1 × 10 ³	5 × 10 ³	4 × 10 ⁵	5 × 10 ³	1 × 10 ⁶	5 × 10 ⁴	1 × 10 ⁴	1 × 10 ⁴	1 × 10 ⁴	1 × 10 ⁵	1 × 10 ⁵
168	2 × 10 ³	2 × 10 ³	2 × 10 ²	3 × 10 ²	9 × 10 ³	5 × 10 ³	4 × 10 ³	1 × 10 ³	2 × 10 ³	0	2 × 10 ⁴

Note: 0 = Not analyzed — = Cannot be evaluated

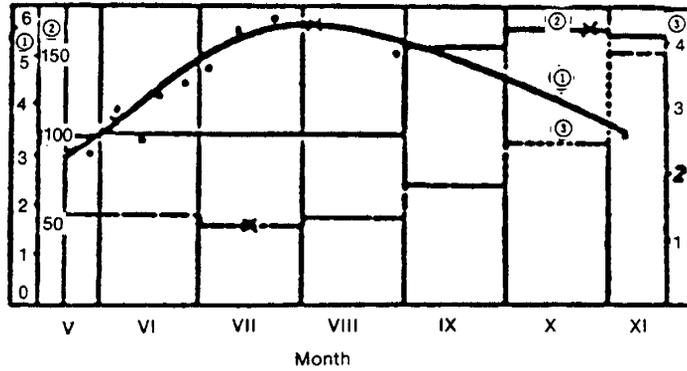


FIG. C-IV-2—Changes in sulfate reducing bacteria counts and oil production in Well 68.

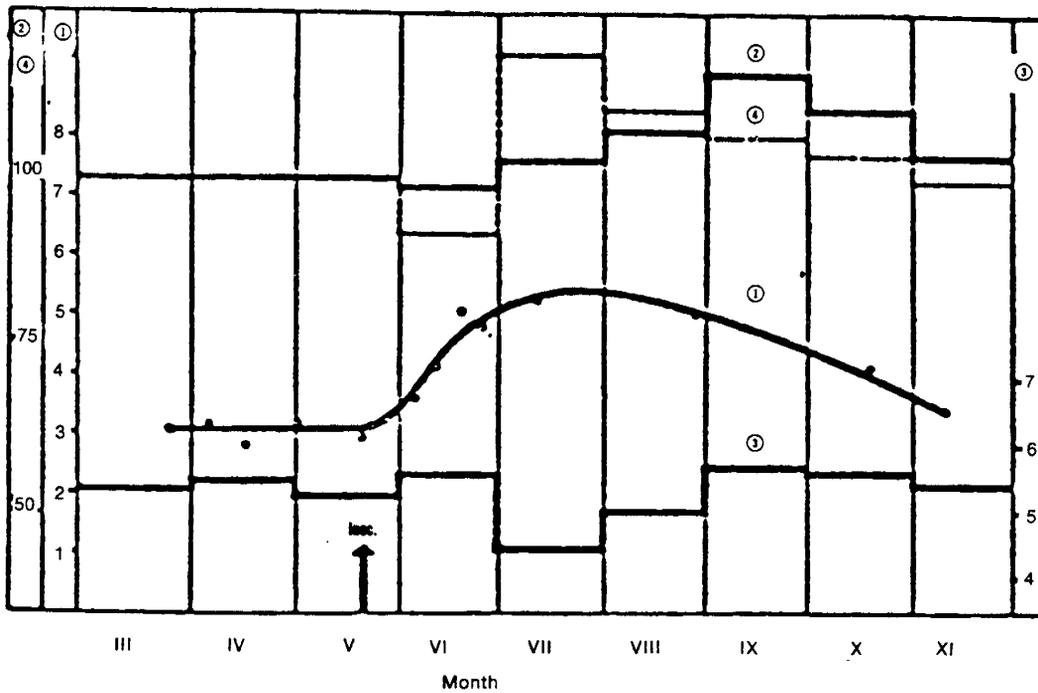


FIG. C-IV-2—Changes in sulfate reducing bacteria counts and oil production in entire test area.

The number of sulfate reducing bacteria was compared to the oil and water production and the oil/water ratio (Fig. C-IV-2, Table C-IV-2).

The oil production increases and the change in oil/water ratios show that in 7 wells the oil production increased 12-36 percent while for the deposit as a whole oil production increased by 6.85 percent.

There was reported a dramatic increase in the wells in the lower part of the strata which produced the most water while in the structure high the favorable results were not observed.

The growth of bacteria was calculated for both the oil/water ratios (Fig. C-IV-3) and for maximum rise in production (Fig. C-IV-4).

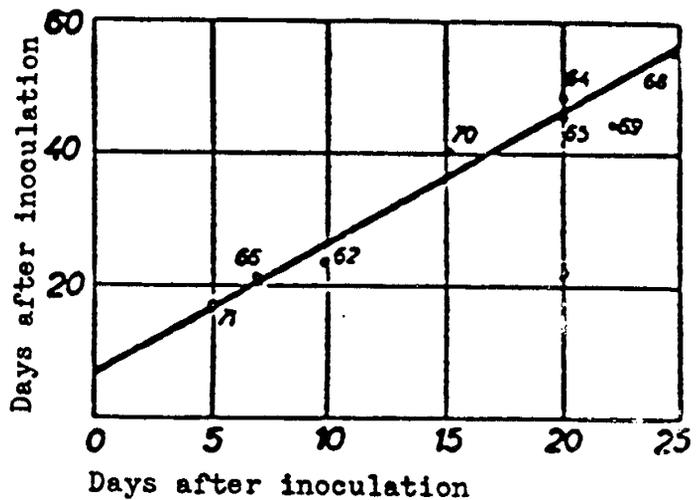


FIG. C-IV-3—Correlation of time between beginning of bacteria growth and maximum drop in oil/water ratio.

TABLE C-IV-2—Average Daily Production and the Oil/Water Ratio for the Individual Wells

Well	Production of Oil and Water, Percent							
	Before Test	In the Individual Months of the Test						Throughout the Test
		VI	VII	VIII	IX	X	XI	
N	100	112.67	111.97	115.49	112.67	109.85	112.67	112.55
61 V	100	100	131.25	144.14	144.14	141.40	144.14	134.17
N/V	0.027	0.031	0.024	0.022	0.021	0.021	0.022	
N	100	85.63	82.97	88.82	117.55	126.59	101.06	100.43
62 V	100	100	53.28	48.43	48.43	66.78	87.28	67.36
N/V	0.029	0.025	0.045	0.054	0.071	0.056	0.034	
N	100	90.27	118.05	151.38	202.77	220.20	198.61	163.54
64 V	100	39.01	58.14	55.52	55.42	47.73	38.08	49.00
N/V	0.009	0.020	0.018	0.025	0.033	0.042	0.047	
N	100	97.07	96.53	97.94	97.40	95.77	90.80	95.91
65 V	100	97.14	97.25	92.30	88.79	88.00	88.79	92.04
N/V	0.101	0.101	0.100	0.108	0.111	0.109	0.103	
N	100	96.46	101.82	104.18	88.63	69.66	65.38	87.68
66 V	100	100.16	102.00	102.00	70.33	25.88	20.00	70.06
N/V	0.156	0.149	0.155	0.159	0.196	0.418	0.508	
N	100	100.17	100.70	100.70	150.70	166.07	160.95	129.88
68 V	100	66.67	89.79	83.49	89.79	72.33	47.64	74.95
N/V	0.143	0.142	0.122	0.131	0.181	0.248	0.365	
N	100	111.18	115.38	136.36	139.68	134.96	124.47	127.03
69 V	100	116.86	394.98	376.01	372.62	373.81	372.40	334.40
N/V	0.047	0.046	0.014	0.017	0.018	0.017	0.016	
N	100	125.00	128.12	155.46	153.90	139.84	96.09	133.06
70 V	100	129.50	294.11	192.20	187.64	190.50	184.68	196.40
N/V	0.030	0.028	0.013	0.023	0.023	0.021	0.015	
N	100	107.08	127.55	126.77	173.22	130.70	132.28	132.93
71 V	100	106.62	108.84	108.84	108.84	108.21	72.49	102.30
N/V	0.012	0.012	0.014	0.014	0.019	0.015	0.020	
N	100	94.48	115.74	125.98	125.98	133.85	125.98	120.33
58 V	100	101.39	92.17	89.76	89.76	142.95	106.28	103.71
N/V	0.022	0.019	0.027	0.029	0.029	0.027	0.018	

N = oil V = water N/V = oil/water ratio (calculated from production in tons)

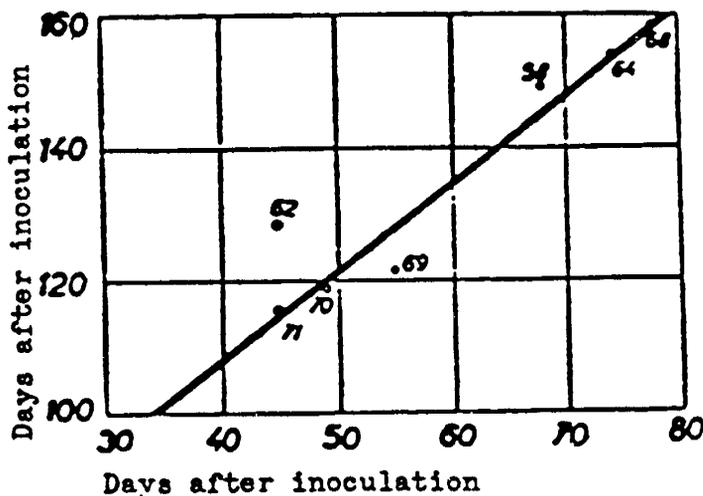


FIG. C-IV-4—Correlation of time with maximum growth of bacteria with maximum production.

X axis = beginning microbial growth; Y axis = maximum decrease in oil/water ratio; Wells 58 and 61 could not be evaluated.

X axis = maximum growth of bacteria; Y axis = maximum rise in oil production; Wells 61, 65, and 66 could not be evaluated.

The growth curves (Fig. C-IV-2) show a log and stationary curve and indicate the nutrients added were gradually exhausted or washed out in the produced water. In the area of the injection well the pattern differs in a steep log phase and longer stationary phase. The maximum increase in microbial numbers generally occurred between the 50th and 78th days of the test except in Wells 62, 66 and 71 where nutrients penetrated faster. On the basis of microbial counts the test could be divided into two stages: (1) time from first growth to maximum growth, and (2) the stationary or decline period of counts.

Period	Number of Bacteria	Oil Production, Percent	Water Production, Percent	Oil/Water Ratio
Before test	1×10^3	100	100	.058
Stage 1	1.4×10^5	101.22	108.13	.056
Stage 2	2.3×10^4	112.70	101.97	.085

In the second stage the production of oil increased in 8 of the 10 wells observed (no increase in Wells 62, 65, 66). In most wells they observed a pattern that would follow the following trend:

Stage 1—Water production up—oil production unchanged or decreased.

Stage 2—Water production down to original level—oil production up.

Remarks—The increase in oil production was attributed to the production of surface active substances and changes in the surface tension of the oil-water rock surfaces. This would cause an inversion of wettability of the rock surface changing it from oil to water wet. They had observed changes in deabsorption of oil in laboratory experiments. If this surface tension theory is correct, they explain the observed effects in the two stages as:

Stage 1: The oil/water ratio dropped. More water influx compared to oil. Bacteria increase the effective permeability of the reservoir to water and the oil is assumed to be released by the microbial growth.

Stage 2: The oil/water ratio increased. Oil released from smaller pores. This release of oil does not affect the permeability of the reservoir.

CZECHOSLOVAKIA FIELD TEST V

Year of test: ? Location: Hodonin Deposit

Geological data—See Field Test IV. *Depth:* 150 m. *Porosity:* Fine to medium grained sand. *Permeability:* 3300–8100 mD.

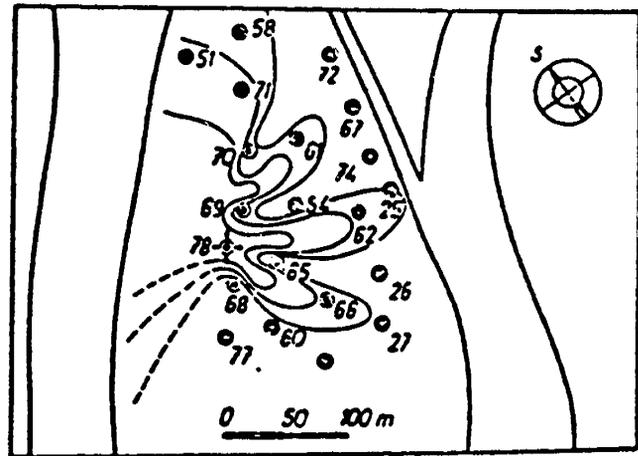


FIG. C-V-1—Map of time distribution of changes in activity of the microflora in the deposit after the injection of nutrients. Hodonin deposit. Isolines of infiltration of nutrients from the injection bore after 10 days.

Prior production history—It is not known if this test is before or after Field Test IV or even if it is different. However, since there are reported differences in the procedure and results, it is assumed to be a separate test.

Microbial well treatment—Well 78 was injected under pressure with a 60 l inoculum of sulfate-reducing bacteria and 100 kg of molasses. There were 10 control wells sampled.

Culture injected: Sulfate-reducing bacteria.

Response—A significant increase in microbial counts was observed in all wells (Table C-V-1).

This unequal distribution of the nutrient penetration of the formation is also shown in Fig. C-V-1.

Microbial counts increased most rapidly in Wells 61, 62 and 66 which was explained by the drainage pattern in the oil sand. It should be noted that variations in the number of bacteria in the water before this test do not exceed the limits of one order. Well 78 is the exception and was used previously for stationary culture tests. After these tests, microbial counts reached values 3

TABLE C-V-1—Changes in the Number of Sulfate-Reducing Bacteria After Nutrient Injection, the “Mean Proliferation Time” and the Rate of Infiltration of Nutrients Through the Hodonin Deposit

Well	Log of Numbers of SRB/ml		MPT Days	Distance from Injection Well (m)	Rate of Infiltration m/10 Days
	Before Injection	After Injection			
78	5.00±0.37(6)	5.80±0.28(10)	4	0	0
71	3.25±0.35(7)	4.60±0.83(10)	17	140	82
66	2.70±0.40(7)	4.65±1.20(12)	22	95	43
62	3.45±0.52(4)	4.37±0.90(12)	22	125	57
61	2.66±0.39(6)	3.98±0.96(11)	24	110	46
58	3.00±0.64(3)	4.74±0.25(7)	24	165	68
70	2.48±0.38(4)	4.02±1.29(12)	27	85	34
69	3.11±0.62(6)	4.41±0.37(12)	28	30	11
65	2.18±0.81(7)	3.76±0.70(11)	31	70	22
68	3.00±0.80(5)	4.45±0.70(12)	35	35	10
64	3.40±0.90(7)	4.40±1.05(12)	36	80	22

MEOR Field Applications

orders higher with the highest values in the injection well and nearest wells. Wells over 400 m distant did not show a large increase.

Results—The results show microbial numbers can be used to ascertain the direction of the most rapid and slowest fluid flow and to estimate the permeability.

CZECHOSLOVAKIA FIELD TEST VI
Year of test: ? Location: Petrova Ves Deposit
in Vienna Basin

Geological data—In the Petrova Ves deposit production is from the basal Helvetian zone (3-10 m thick) at a depth of about 700 m and is inclined at an angle of about 18°. The structurally lower parts of the deposit are filled with water.

Depth: 700 m. *Porosity:* Fine-grained sand. *Permeability:* Ranges in tens of mD.

Prior production history—Unknown.

Microbial well treatment—Well 404 was first pumped to reduce the water volume to a minimum followed by injection of 200 kg of distillery molasses.

Culture employed: None.

Response—*First test in Petrova Ves deposit:* Well 404 was injected with only nutrients and the number of sulfate reducing bacteria determined in 15 control wells by the spot test method (see Fig. C-VI-1). The first control well was 600 m from the injection well (see Fig. C-VI-2). A significant increase in count was found in 13 of the control wells while in Wells 445 and 461, both over 500 m away, the count increase not significant (Table C-VI-1).

The spread of bacteria (Fig. C-VI-2) shows that in

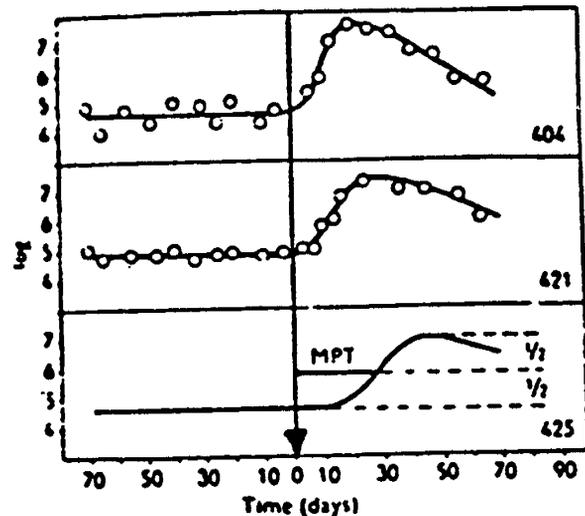


FIG. C-VI-1—Response of sulfate-reducing bacteria (No/ml) after injection of nutrients (Wells 404, 421, and 425). — Nutrient injection into Well 404. MPT = mean proliferation time.

areas near Wells 429 and 430 the spread of nutrients was retarded while near Wells 421, 427 and 402 the spread was most rapid. When these results were compared to a permeability map of the same area (Fig. C-VI-3) it was found that Wells 429 and 430 lie in area of lowest permeability while Wells 421 and 427 are in highest permeability (over 40 mD). The spread of nutrients to Well 444 shows that the fault is not tight in this area.

Remarks—The spread of nutrients and microbial response generally follows the areas of highest permeability. The natural microflora of the reservoir can be stimulated by the injection of nutrients.

TABLE C-VI-1—Changes in the Number of Sulfate-Reducing Bacteria After the Injection of Nutrients into the Deposit, the “Mean Proliferation Time” and the Rate of Infiltration of the Nutrients Through the Deposit. Petrova Ves Deposit, First Experiment

Bore	Log of Number of Sulfate Reducing Bacteria in 1 ml Water		P	MPT, Days	Distance from Injection Bore, Meters	Rate of Infiltration Nutrients, m/5 Days
	Before Injection $\bar{x} \pm s_x(n)$	After Injection $\bar{x} \pm s_x(n)$				
404	4.62±0.35(10)	6.52±0.86(10)	< 0.01	8	0	—
416	3.95±0.40(8)	6.30±0.90(9)	< 0.01	14	100	36
421	4.84±0.11(10)	6.26±0.84(10)	< 0.01	14	110	39
427	4.00±0.74(9)	6.10±1.65(9)	< 0.01	14	110	39
443	4.62±0.49(6)	6.00±0.90(9)	< 0.01	14	170	60
424	5.00±0.84(7)	6.30±1.10(6)	< 0.05	23	170	38
402	4.62±0.60(7)	5.84±0.80(8)	< 0.05	23	205	45
428	4.76±0.46(9)	5.44±0.77(12)	< 0.05	28	255	45
425	4.60±0.32(10)	6.00±0.79(10)	< 0.01	28	200	36
444	3.95±0.40(10)	5.78±0.90(7)	< 0.01	28	200	36
405	4.00±0.83(8)	5.20±1.34(6)	< 0.10	28	290	52
429	4.60±0.63(9)	5.45±1.03(7)	< 0.10	51	260	25
430	4.00±0.71(10)	5.12±1.26(8)	< 0.05	51	180	18
437	4.30±1.36(8)	5.30±1.90(4)	< 0.50	56	420	37
445	4.60±1.20(8)	5.00±2.20(6)	> 0.50	—	505	—
461	4.43±0.53(7)	4.70±1.10(6)	> 0.50	—	615	—

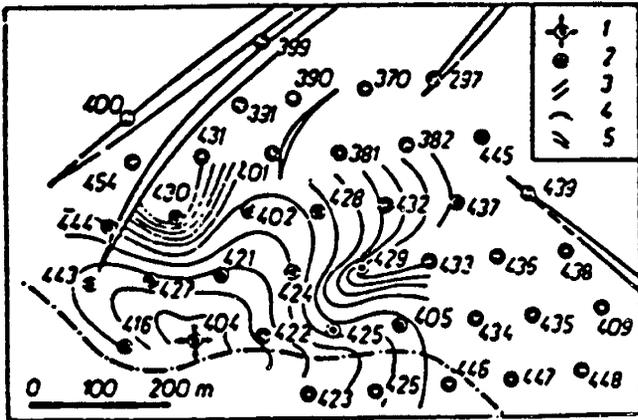


FIG. C-VI-2—Map of time distribution of bacteria activity after nutrient injection. Petrova Ves deposit, first test. 1 = injection well, 2 = test wells, 3 = fault, 4 = water-oil contact line, 5 = isosolines of infiltration after 5 days.

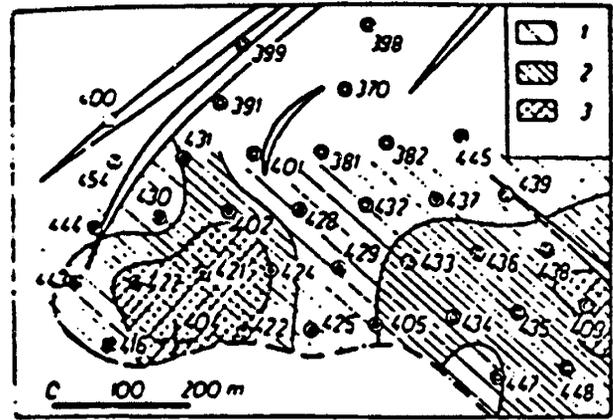


FIG. C-VI-3—Map of effective permeability in Petrova Ves deposit. Permeability: 1 = 0-20 mD, 2 = 20-40 mD, 3 = over 40 mD.

CZECHOSLOVAKIA FIELD TEST VII

Year of test: ? Location: Petrova Ves Deposit in Vienna Basin

Geological data—The test was made in the same deposit as the first test but in the area lying near the water-oil line further to the southeast (Fig. C-VII-1). This area is highly faulted. It can be assumed the parameters are similar to those in Field Test VI.

Microbial well treatment—Well 481 (open to zone 2a) was used for injection of nutrient while 9 wells served as control wells. Seven of these opened into zone 2a into which the inoculum was injected while 2 (Wells 477 and 482) were in the higher zone 2b. These 2 wells served as a control on zone 2b.

Culture employed: Unknown and it is unclear if one was really injected.

Response—In this second test in the Petrova Ves deposit, there was a significant increase in bacteria

count in 4 wells (420, 470, 476, 486) in zone 2a. No increase was noted in the other 3 wells in zone 2a nor in the zone 2b wells (see Table C-VII-1).

The growth of sulfate reducing bacteria in response to the injection of the nutrient was interpreted together with the fault pattern (Fig. C-VII-1).

The results show that the fault transecting communication between Wells 481 and 486 is not tight and nutrient passed between the two wells. However, the fault between 481 and 483 is impermeable. Communication existed between 481 and 470 but not between 481 and 471 and 472. There was no communication between Zones 2a and 2b.

Remarks—The test showed that sulfate reducing bacteria counts increased when a nutrient solution was injected. The results suggest permeability is the key to the movement of nutrients (microorganisms) and communication between wells can be determined.

CZECHOSLOVAKIA REFERENCES

Dostalek, M. (1961). Bacterial Release of Oil III

TABLE C-VII-1—Changes in the Number of Sulfate-Reducing Bacteria After the Injection of Nutrients into the Deposit, the “Mean Proliferation Time” and the Rate of Infiltration of Nutrients Through the Deposit. Petrova Ves Deposit, Second Experiment

Bore	Zone	Log of Number of Sulfate Reducing Bacteria in 1 ml Water		P	MPT, Days	Distance from Injection Bore, Meters	Rate of Infiltration of Nutrients, m/10 Days
		Before Injection	After Injection				
481	2a	4.62±0.49(6)	6.40±0.90(9)	< 0.01	9	0	—
420	2a	5.10±0.19(9)	5.50±0.47(9)	< 0.05	22	105	47
476	2a	5.06±0.26(8)	5.82±0.38(6)	< 0.05	25	100	40
486	2a	5.21±0.25(7)	6.03±0.54(7)	< 0.01	30	180	60
470	2a	4.76±0.46(9)	5.44±0.77(12)	< 0.05	46	230	50
471	2a	4.83±0.76(9)	5.05±0.78(10)	< 0.50	—	165	—
472	2a	5.27±0.21(6)	5.35±0.06(7)	> 0.50	—	200	—
483	2a	5.27±0.23(5)	5.19±0.26(6)	> 0.50	—	300	—
477	2b	5.02±0.33(9)	5.13±0.38(10)	> 0.50	—	180	—
482	2b	4.70±0.34(8)	4.82±0.40(8)	> 0.50	—	205	—

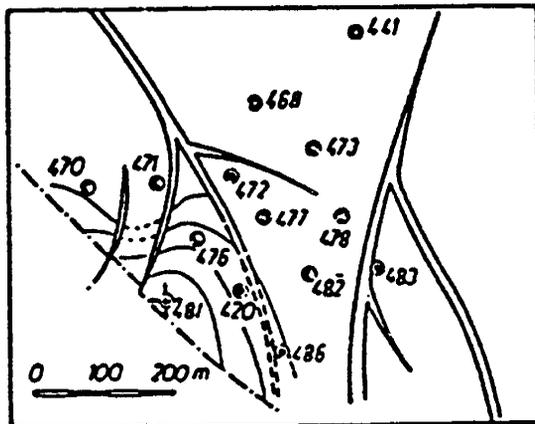


FIG. C-VII-1—Map of time distribution of bacterial activity after nutrient injection. Petrova Ves deposit—second test. Isolines of infiltration after 10 days.

Areal Distribution of the Effect of Nutrient Injection into the Deposit. *Folia Microbiologica* 6, pp. 10-16.

Dostalek, M., Spurny, M. (1957). Release of Oil by Microorganisms. I Pilot Experiment in an Oil Deposit. *Czechoslovenska Mikrobiologie*. 2 No. 5, pp. 300-306.

Dostalek, M., Spurny, M. and Rosypalova, A. (1958). Action of Bacteria on Petroleum Loosening in Collectors. *Prace Ustav pro Naftory Vyskum* 9, pp. 29-46.

Spurny, M., Dostalek, M., and Ulehla, J. (1957). A Method of Quantitative Determination of Sulfate-Reducing Bacteria. *Fol. Biol. (Praha)*. 3, pp. 202.

Dostalek, M., Spurny, M. (1958). Bacterial Release of Oil. I Preliminary Trials in an Oil Deposit. *Fol. Biol. (Praha)* 4, pp. 166.

Dostalek, M., Spurny, M. (1957). Release of Oil Through the Action of Microorganisms. II Influence of Physical Physical-Chemical Conditions in Oil-bearing Rock. *Cesk. Mikrobiol.* 2, pp. 307-317.

HUNGARIAN FIELD TEST I
Year of test: ? Location: Unknown

Geological data—Test was made in sandstone reservoir. *Depth*: 200 m. *Reservoir temp.*: 25-30°C.

Prior production history—Unknown.

Microbial well treatment—Unknown except reported supplemental injections were made. *Culture employed*: Unknown.

Response—The bacteria migrated from the injection well to the surrounding control wells. The viscosity of the oil was reduced and the pH decreased. There was a reported 12-26 percent increase in oil production for 18 mo.

Remarks—Little is known of this test but increased oil and bacterial migration occurred.

HUNGARIAN FIELD TEST II
Year of test: 1961. Location: Lendvaujfalú area.

Geological data—Oil of intermediate naphthenic type—color blackish brown. *Depth*: 700 m. *Reservoir temp.*: 50°C. *Viscosity*: 42.57 cSt. *Permeability*: 600-700 mD.

Prior production history—Unknown.

Microbial well treatment—Inoculated well with 50 l of thermophilic mixed culture (containing $13 \times 10^{7-8}$ cells/ml) + 2 tons molasses + 100 kg KNO₃, 10 kg superphosphate mixed in 60 m³ of formation water and injected at 120 atms. The well was closed for 5 mo. The control well was 100 m distant.

Culture employed: Anaerobic thermophilic mixed sewage inoculum grown on molasses at 50°C in formation water.

Response—The microbial count increased in both the test and control wells.

Microbial Count (No/ml)

Well	Before Test	14 Days After Test	16 Days After Test
Inoculated well	5×10^2	10^4	2×10^6
Control well	5×10^2	Fluctuated between 10^2 and 10^6	

The pH of the water prior to the test was 9.0 which decreased to 6 (Table H-II-2). The water production remained unchanged. The viscosity decreased from 42 to 18.6 in the inoculated wells while the control well decreased from 42 to 26. This same effect of greater changes in the inoculated well than the control well was observed in the specific gravity measurements (Table H-II-2). These effects were reportedly due to increased microbial action which was explained as: more gas dissolved in the oil, added microbial products, or the breakdown of the oil. This bacterial effect persisted for 8 mo after which the viscosity returned to normal.

Samples from the reservoir were checked for interfacial tension of the oil, water, and the oil/water mixture (Table H-II-1). The results showed a noticeable decrease in the water surface tension.

TABLE H-II-1—Surface Tension at 20°C (dynes/cm)

Date Sampled	Oil	Water	Oil/Water
August, 1960	33.33	61.71	14.81
June, 1961	34.74	55.96	1.24

TABLE H-II-2—Changes of Composition of Oil and Water in Test and Control Well

Inoculated Well Date Sampled	Viscosity cSt at 40°	Sp. Grav. G/cm ³	pH	Carbonate °DH	CO ₂ mg/l
Feb. 19, 1961	19.7	0.885			
Apr. 10, 1961	21.9	0.888			
June 8, 1961	22.8	0.892			
June 16, 1961	24.5	0.890			
July 4, 1961	—	—		208.0	352
July 10, 1961	24.2	0.890	6.1	208.9	339
July 21, 1961	18.6	0.888	6.0	208.3	
Aug. 1, 1961	—	—	7.8		
Oct. 9, 1961	29.1	0.888			
Control Well					
Sept. 29, 1960	26.7	0.892			
Dec. 20, 1960	25.6	0.891	7.5	189.9	
Feb. 19, 1961	26.3	0.889			
Apr. 10, 1961	25.1	0.891			
May 3, 1961	27.8	0.893			
June 8, 1961	25.5	0.891			
June 15, 1961	27.2	0.892			
June 19, 1961	27.6	0.893			
July 4, 1961	—	—		197.4	371
July 10, 1961	—	—	6.2	198	372
July 21, 1961	25.7	0.892	6.1	197.1	
Aug. 1, 1961	—	—	7.6		
Oct. 9, 1961	30.0	0.890			

Prior to the test the inoculated well did not have a free gas cap and its oil contained relatively little dissolved gas so there was no analysis of volume or composition of produced gas. Seven days after inoculation the waters from the control were very gassy and by the 9th day the first free gas sample was taken. The CO₂ was reported after 4 mo to amount to 40 m³/day (reaching 11 percent CO₂) and the CO₂ dissolved in the water was 370 mg/l. The composition of the gas analyzed (Table H-II-3) showed:

While laboratory studies had shown high concentrations of H₂ produced there was no H₂ measured in field samples. There was no H₂S produced. It was estimated that 42 m³ of gas leaked from the reservoir each

TABLE H-II-3—Composition of Gas Samples (Vol. %)

Date Sampled	C ₁	C ₂	C ₃ +	CO ₂	N ₂
Aug. 1, 1960	93.75	0.96	0.56	4.51	0.22
Aug. 4, 1960	91.56	1.06	0.71	5.90	0.77
Oct. 20, 1960	89.84	0.87	0.71	8.33	0.25
Nov. 4, 1960	89.85	1.06	0.80	8.13	0.16
Nov. 14, 1960	88.06	0.87	0.86	9.88	0.33
Feb. 3, 1961	87.63	7.10	2.08	2.49	0.70*
Feb. 13, 1961	87.00	0.80	0.22	11.76	0.22
Mar. 1, 1961	87.06	0.95	0.25	11.40	0.34
Mar. 16, 1961	87.52	1.06	0.25	11.03	0.18
Apr. 5, 1961	87.35	1.01	0.23	11.23	0.11
May 3, 1961	88.47	0.95	0.22	10.66	0.30
June 8, 1961	88.73	0.86	0.25	9.86	0.30
June 15, 1961	88.46	0.77	0.26	10.27	0.24
June 19, 1961	88.33	0.97	0.26	10.01	0.43
July 4, 1961	88.37	0.84	0.25	10.45	0.09

*Note—low conc. of CO₂ presumably due to destruction of hydrocarbons.

day during the measured period and the reservoir produced 2100 m³ gas. This is considered low because some gas leaked before measurements could be made. The gas volume fluctuated but the reason was unknown and it was assumed it related to molasses utilization.

The changes noted by bacterial action reportedly affected an area of 60,000 m². The production increase continued for 8 mo after which the well production fell below that reported before the treatment. There was a temporary improvement upon an additional injection.

Remarks—The results showed an increased gas production (especially CO₂) and a decreased pH, viscosity, and surface tension. These changes were all attributed to bacterial activity. The increase was short term and additional treatment gave only a temporary improvement.

HUNGARIAN FIELD TEST III

Year of test: ? Location: Unknown

Geological data—Sandstone reservoir. *Depth*: 1400 m. *Reservoir temp.*: 50°C. *Permeability*: 10-70 mD.

Microbial well treatment—Unknown.

Response—No significant effects were observed.

Remarks—The permeability was probably too low for microbial action.

HUNGARIAN FIELD TEST IV

Year of test: ? Location: Unknown

Geological data—Sandstone reservoir, light paraffinic oil. *Depth*: 994 m. *Reservoir temp.*: 70°C. *Permeability*: 150-30 mD.

Prior production history—Unknown.

Microbial well treatment—Unknown.

Response—A comparison of the reservoir parameters before treatment to those observed 3 mo after treatment show:

	Before Treatment	After 3 Months
Oil production	—	Increased 10 percent
Oil viscosity	3	Unchanged
CO ₂ content of gas	—	Increased
pH	7	6

Remarks—A medium permeability reservoir at 70°C showed a positive response to microbial action.

HUNGARIAN FIELD TEST V

Year of test: ? Location: Unknown

Geological data—Sandstone reservoir with highly paraffinic oil (solidification point 30°C). *Depth*: 1392 m. *Reservoir temp.*: 72°C. *Permeability*: Low.

Prior production history—Unknown.

Microbial well treatment—Unknown. The injection well was 300 m from the producing well.

Response—No change in oil production, oil viscosity, pH, or gas output was noted in a period of 18 mo following injection.

Remarks—No response to treatment probably due to low permeability.

HUNGARIAN FIELD TEST VI

Year of test: ? Location: Nagylengyel Region

Geological data—Unknown. *Depth*: 2457 m. *Reservoir temp.*: 97°C. *Pressure*: 228 atms.

Prior production history—The well was not operated for 2 yr prior to the test. The production wells were 288–1700 m from the injection well.

Microbial well treatment—The well was injected with 100 l of culture, 40 tons of molasses, 120 kg KNO₃, 100 kg sugar and 120 m³ water. The well was shut in for 3 mo.

Culture employed: Mixed sewage sludge culture—*Desulfovibrio desulfuricans*.

Response—The bacteria spread throughout the formation with a total increase in numbers and especially *Desulfovibrio desulfuricans*. The treatment also stimulated the indigenous microflora. The pH decreased from 9 to pH 6 while the oil viscosity decreased from 600 to 300. The oil production increased 60 percent.

Remarks—This is a very deep and hot reservoir which responded to microbial treatment.

HUNGARIAN FIELD TEST VII

Year of test: ? Location: Unknown

Geological data—The reservoir was reported to be a limestone type with a naphthenic oil. *Depth*: 700 m. *Reservoir temp.*: 50°C.

Prior production history—The wells had various oil/water production or were unproductive for 7 yr.

Microbial well treatment—Unknown.

Response—The effects observed in the wells lasted from 2 weeks to 18 months depending on the well but no values were offered. A comparison was made between observation before and 8 mo after treatment of

the following parameters. It is not known if these are for one well or more than one well.

	Before Treatment	After 8 Months
Viscosity, cSt at 40°C ...	600	300
pH	7	6
CO ₂ content of produced gas, percent	12.4	60.5
Oil production		Increased 60 percent
Oil/water ratio		Decreased

Remarks—This test in a limestone reservoir showed a positive effect due to microbial action.

HUNGARIAN FIELD TESTS VIII AND IX

Year of tests: 1969. Location: Demjen Area (East)

Geological data—The field was discovered in the 1950's and includes 3 sectors separated (100 m to several kilometers) by nonproductive areas. Demjen East is the main sector (80 percent of reserves) while Demjen West and Demjen Punkosdhegy contain 20 percent. All sectors have the same geology composed of sandstone of the Oligocene which form the rocks of the Demjen carbonate (?) reservoirs. The minimum strata dip is 4 percent, maximum 15 percent (averaging 7–8 percent dip). The sandstone grains are fine to average (physical parameters vary widely) and contain argillaceous, clayey marl and calciferous binding materials. The reservoirs are tectonic fractured containing more than 100 blocks. The faults form obstacles and shut off hydrodynamic connections horizontally but water can rise vertically to areas of reduced pressure.

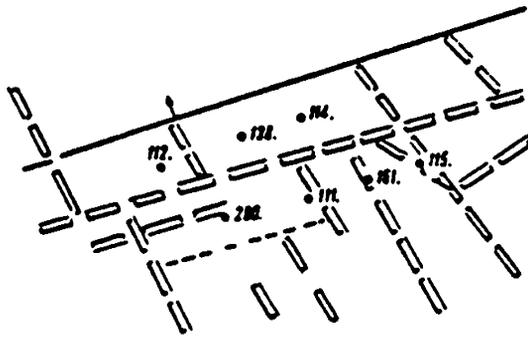
Depth: 300–1000 m. (up to 15 stratum in a single reservoir). *Reservoir temperature*: 25–30°C. *Porosity*: 20–28 percent (depends on stratum). *Viscosity*: 5 cps at 30°C. No gas cap but oil is at bubble point (oil viscosity is 3 times that of water). *Permeability*: 104 mD horizontal, 67 mD vertically. *Residual oil saturation*: Primary oil production 10–20 percent (main obstacle is fracturing and faults water saturation 30 percent).

Prior production history—Unknown.

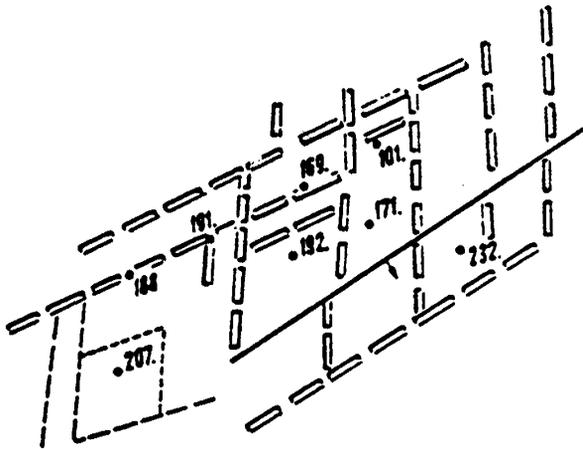
Microbial well treatment—Inoculated 2 wells (DK 114 and DK 192) by the following procedure: Injected 20 m³ of formation water followed by 1/2 of a solution containing 2 tons of molasses, 70 kg KNO₃, 10–20 kg of sodium phosphate and 50 kg of sucrose in 100 m³ of formation water. This was followed by 100 l of the inoculum and the remaining 1/2 of the nutrient solution, followed by 50 m³ of formation water. The wells were closed and remained closed except to add more nutrients when noted.

Well DK 114: Was first inoculated in August 1969. Additional nutrients were added to this well in October 1969 and also in July 1970. The control wells were DK 111, 112, 115, 138, 161 and 280. The distances to these wells and their locations in regard to faults can be seen in Fig. H-VII-1.

Well DK 192: Inoculated July 1970. Control wells



Zone of Well DK-114



Zone of Well DK-192

FIG. H-VIII-1—Location of wells and faults in Demjen East area.

were DK 101, 169, 171, 188, 191, 207. In this area was also well DK 232 (see Fig. H-VIII-1).

Culture Employed: The mixed culture was derived from 70-100 strains found in sewage sludge which required minimum nutrients and that could convert residual oil to CO₂ and acids.

TEST WELL DK 114 AREA

Response—The count of inoculated type organisms increased as well as organisms native to the reservoir. By Sept. 10, 1969 bacteria were first seen (at 10⁵/ml) in wells DK 138 and 112. Increases were later observed in wells DK 111, 115 and 280. By 1971 counts in wells DK 111, 115 and 138 were present at 10³/ml. The pH of water at DK 114 and its control wells decreased. To improve microbial movement nutrients were added to Well DK 114. If the nutrient was low or absent counts remained low or decreased.

The viscosity of oil in Well DK 114 decreased and the viscosity of the control wells was altered with time and flow pattern (see Fig. H-VIII-2).

In Well DK 111 the changes observed were less evident because high volumes of oil concealed the reduced viscosity caused by microbial action. In DK 280 the viscosity remained below its initial value. The viscosity increased as the nutrients were depleted and the microbial action decreased.

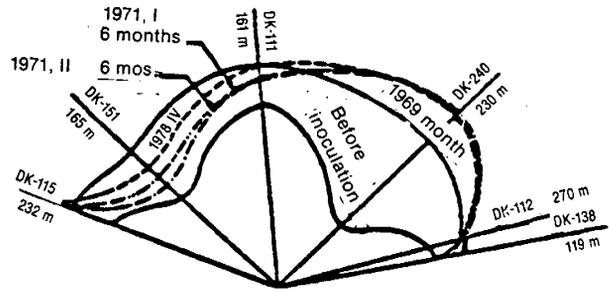


FIG. H-VIII-2—Viscosity curves of the DK-114 well zone.

Fluid production in an illustrated 2 well zone in the DK 114 area showed that the daily total yield during the inoculation period decreased slightly. The oil increase during the test period exceeded the amount before inoculation but declined to the normal decline curve value. The water production declined following inoculation but returned to its original value in the test period (see Fig. H-VIII-3).

TEST WELL DK 192 AREA

Counts increased in Well DK 192 but by 1971 the count had decreased to less than 10³/ml because well DK 192 received no nutrient additions as had been injected in well DK 114. There is no pH data for Well DK 192.

The flow patterns and distances to control wells together with the viscosity changes are shown (Fig. H-IX-1). The viscosity in well DK 192 initially decreased but as the nutrients were consumed the viscosity increased.

The oil and water production during the test period July 1970 to December 1971 show a slight decline in oil production but remains above the normal decline curve. The water production declined (Fig. H-IX-2).

Production during the test period of 2009 days in the control wells were compared and showed:

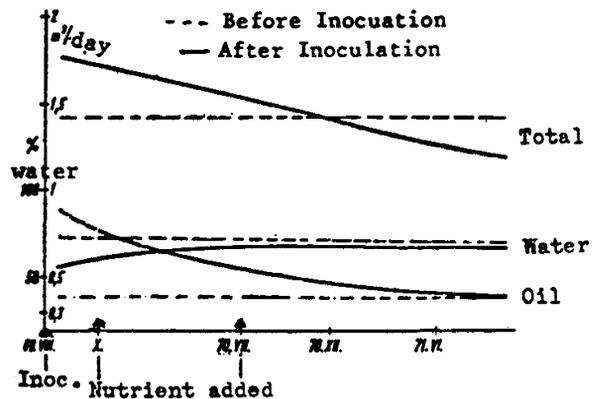


FIG. H-VIII-3—Recovery status of the DK-114 well zone.

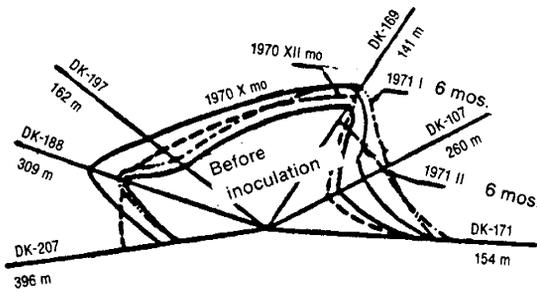


FIG. H-IX-1—Viscosity curves of the DK-192 well zone.

	Daily Oil Yield (m ³)
Production before inoculation	0.4668
Expected normal decline production	0.4914
Production after inoculation	0.5475 (117 percent increase)

A conclusion reached was that one inoculation stimulated the control wells for 15 months.

Well DK 232: Well DK 232 is in the area of inoculated Well DK 192 but was separated by a main fault line (see Fig. H-VIII-1). It was not expected to respond to the test program but it was found that bacterial counts increased to the highest level (10⁴-10⁵/ml) in this well and there was a large increase in oil and gas recovery. For these reasons the well was included in the test program. This well responded for the longest period of time but by the end of 1971 the microbial count declined as had been observed in the other control wells. The conclusion drawn was that an unsuspected hydrodynamic link existed between wells DK 192 and DK 232.

Overall production increases: The comparison was made on 12 control wells selected in the vicinity of both wells DK 192 and DK 114. The production from a total of 5967 days after inoculation was compared to the production for 1010 days before inoculation. The daily oil production increased from 0.4264 m³ before inoculation to 0.5359 m³ after inoculation (126 percent increase). The total net oil increase was 651.73 m³ for the two areas while the water production dropped from 63.26 percent to 57.2 percent. These favorable results were achieved at the expense of 8 tons of molasses and

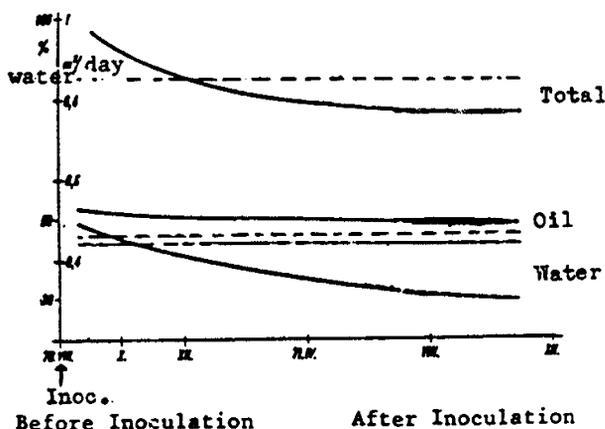


FIG. H-IX-2—Production status of the wells in the zone of the DK-192.

costs of the operations.

Remarks—An increase in oil production was attributed to microbial action. The periodic addition of nutrients enhances the microbial action. As nutrients are depleted microbial growth ceases. It is suggested however, that microbial products could be produced which are inhibitory to growth and that waiting 1-2 years may be necessary to clean the reservoir before reinoculation.

HUNGARIAN FIELD TEST X

Year of test: 1970. Location: Demjen area (West)

Geological data—See information from Demjen East. Marl deposits between sandstone layers are cracked and do not seal well.

Depth: 200 m. **Reservoir temperature:** 25-30°C. **Porosity:** 20-28 percent depending on stratum. **Viscosity:** 30-40 cp at reservoir temperature. **Specific gravity** is 0.86 (practically gas free). **Permeability:** See Demjen East.

Prior production history—Unknown.

Microbial well treatment—Inoculated Well De 61 in August 1970 using method of Demjen East wells. Control wells were De 19, 20, 21, 23 and 60.

Culture employed: Same as in Demjen East test.

Response—Counts increased in De 61. However Well De 61 and its control wells were not evaluated because underground combustion tests in Well De 62 involved injection of air as well as a combustible testing mixture, which interfered with the test results.

REFERENCES

- Jaranyi, I., Kiss, L., Szalanczy, G., Szolnoki, J., (1963) Veranderung einiger Charakteristiken von Erdolsonden Durch Einwirkung von Mikrobiologischer Behandlung. (Change in Some Characteristics of Crude Oil Wells Through the Effect of Microbiological Treatment). Wissenschaftliche Tagung fur Erdolbergbau. (Scientific Meeting for Crude Oil Exploration 1963) pp. 633-650.
- Dienes, M., Jaranyi, I., (1973) Increase of Oil Recovery by Introducing Anaerobic Bacteria into the Formation, Demjen Field, Hungary. Koolaj es Foldgaz v. 106, No. 7, pp. 205-208.
- Bubela, B., (1978). Role of Geomicrobiology in Enhanced Recovery of Oil: Status Quo. APEA Journal 1978, pp. 161-166.
- Jaranyi, I., (1962). Report on Microbiological Experiments for the Purpose of Secondary Oil Production in the Upper Layer of Lovaszi and Lendvarujfalu. Hungarian Eotvas Lerond Geophysical Institute.

TABLE USSR-I-1—Water Factor by Year in Wells

Year	Water Factor							By the Reservoir
	By Wells							
	1016	167	1015	957	309	310	1451	
1964	—	—	—	—	—	0.5	—	0.1
1965	—	—	—	—	0.1	0.7	—	0.2
1966	—	—	0.1	0.8	1.0	1.3	14.1	0.7
1967	—	—	0.8	0.8	1.4	7.6	4.8	1.6
1968	—	—	1.7	3.0	1.2	7.3	8.2	2.3
1969	—	0.1	2.3	7.4	1.1	7.6	8.5	2.8
1970	—	0.3	1.2	6.7	1.2	6.0	7.5	2.0
1971	0.3	0.5	2.5	8.3	1.2	5.1	8.5	1.7
1972	0.8	0.7	4.0	0.8	3.0	6.2	9.0	1.9
1973	0.1	1.0	2.2	0.4	3.2	2.2	14.7	2.1
1974	0.3	1.5	8.2	0.4	3.5	4.7	8.5	3.1
1975	0.03	1.0	9.3	5.2	2.6	4.2	14.5	2.9

3 years for a distance of at least 600 meters, the distance between Well 1455 and 957.

The density and viscosity of the oil decreased. The gas ratio increased from 17 to 70 m³/ton with more N₂, CO₂, and higher hydrocarbon gases. The changes in gas composition show (Table USSR-I-3) an increase in the heavy hydrocarbons with the ratios of C₃>C₁>C₂ to C₃>C₂>C₁, being different in the Arlansk and Nikolo-Berezovsk areas. The observed decrease in methane was reportedly due either to an outflow in the Nikolo-Berezovsk area or an influx of gas in the Arlansk area. There is a general tendency that gas production increases as the formation pressure increases (wells 310, 957, 1015 and 1451) with regular fluctuations governed by injection of fresh water in Well 1455.

Remarks—Although the results are clouded by the water injection it was observed there were changes in the gravity and the composition of the dissolved gases.

The greatest effects coincide with fresh water injection and microbial growth. In the Arlansk field the recovery coefficient was 66 percent which is 12–18 percent higher than in a similar field without treatment.

USSR FIELD TEST II

Year of test: Unknown. Location: Sernovodsk USSR

Geological data—Unknown.

Depth: 1000 m. *Viscosity:* 40.3 cSt.

Prior production history—Unknown.

Microbial well treatment—The well was inoculated with a mixed bacteria culture and 54,000 liters of 4 percent molasses. After inoculation the well was closed for 6 months.

Culture employed: Mixed culture of bacteria growing on molasses under anaerobic conditions.

Response—Oil viscosity increased to 49.3 cSt. The wellhead pressure increased by 1.5 atm. The nitrogen content of produced gas increased from 20 to 35 percent, the CO₂ and propane content increased but methane decreased. The production increase and duration reported were:

	Production/Month		
	Oil (Tons)	Water Content	Gas Factor (m ³ /ton)
Before test	37	7.5 percent	11–15 percent
After test	40	5–6 percent	16 percent
4 months later	36.4	4 percent	16 percent

Remarks—A positive response in oil and gas increase noted but the oil viscosity did not decrease as expected.

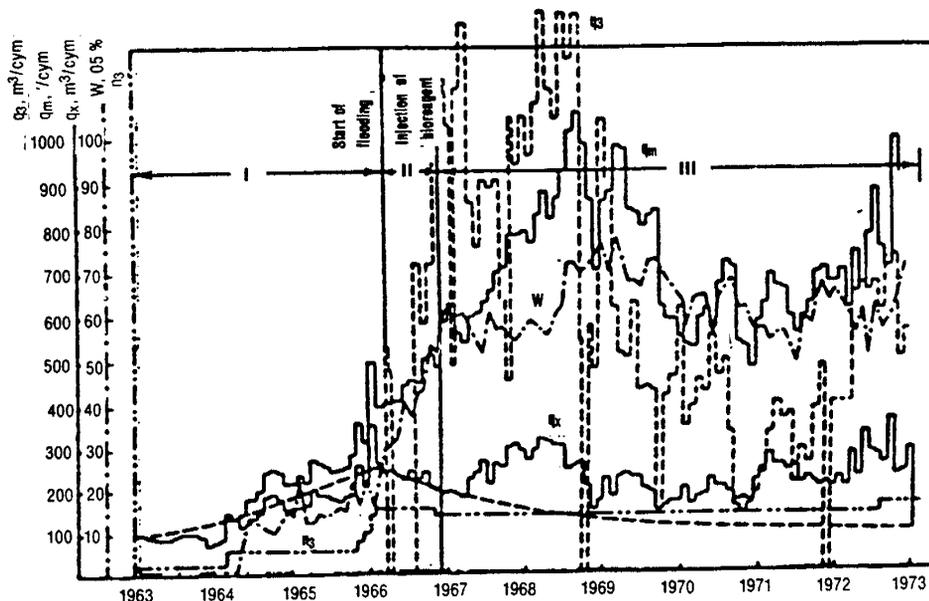


FIG. USSR-I-3—Diagram of the development of the Aleksinsk horizon, Arlansk field.

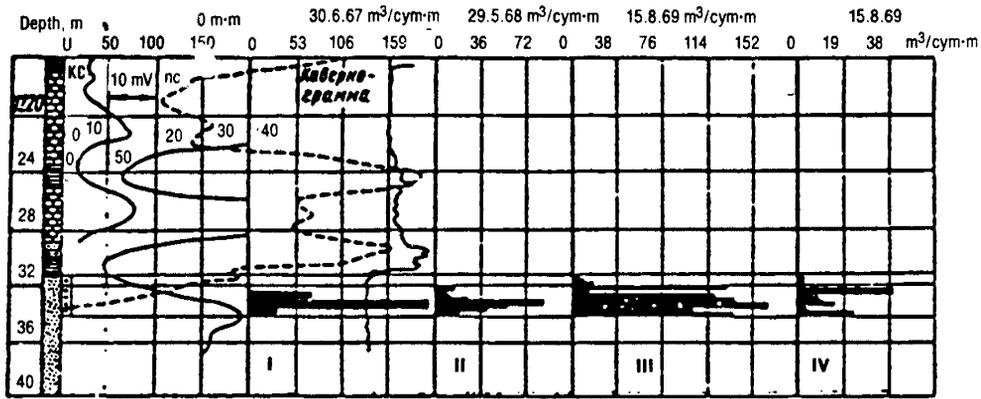


Рис. 2. Профиль injectивности скв. 1455.
 I — $q = 399 \text{ м}^3/\text{сут.}$ $\rho = 81 \text{ кгс/см}^2$; II — $q = 335 \text{ м}^3/\text{сут.}$ $\rho = 105 \text{ кгс/см}^2$; III — $q = 907 \text{ м}^3/\text{сут.}$ $\rho = 95 \text{ кгс/см}^2$; IV — $q = 120 \text{ м}^3/\text{сут.}$ $\rho = 71 \text{ кгс/см}^2$

FIG. USSR-I-4—Injectivity profile of Well 1455.

TABLE USSR-I-2—Results of Microbiological Investigations of Water from Wells of the Aleksin Horizon and of Fresh Water (KNS No. 4) Used for Injecting the Stratum

Sampling Date	Well No.	Exploited Horizon	Degree of Flooding, pct	Specific Gravity of Water at 20°C	Sulfate-reducing	Denitrifying	Bacteria Growing on Media												
							With Oil						With Paraffin	With Mannite	With salts of Mineral Acids	Cellulose-digesting	Methane-producing	Hydrogen-producing	Sulfur-oxidizing
							In Anaerobic Conditions		In conditions With Limited Oxygen Availability										
							-H	H	H-A	M-H	H	H-A							
2/10/67	1455	C ₁ ²	100	1.01	-	+	-	+	+	+	+	+	+	+	-	-	-	+	
12/22/67	1455	»	100	1.01	-	-	+	-	-	-	+	+	+	+	+	-	-	+	
6/29/68	1455	»	100	1.01	+	+	+	+	+	+	+	+	+	+	-	-	-	-	
6/29/68	957	»	62	1.18	-	-	-	-	-	+	+	-	-	-	-	-	-	-	
6/29/68	KNS No. 4	Q	100	1.00	-	-	+	+	+	+	-	-	+	+	-	-	-	-	
2/25/69	1455	C ₁ ²	100	1.01	+	+	+	+	+	-	-	+	+	+	+	-	-	+	
2/25/69	309	»	75	1.18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
2/25/69	310	»	90	1.18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
2/25/69	957	»	90	1.05	+	-	-	+	+	-	-	-	-	-	+	-	-	-	
2/25/69	1015	»	65	1.08	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
2/25/69	1451	»	85	1.18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

Note: 1. +) good bacterial growth; -) no growth. 2. M-N) petroleum of methane-naphthenic composition with $d_4^{20} = 0.85$; N) naphthenic petroleum $d_4^{20} = 0.85$; N-A) naphthenic aromatic petroleum $d_4^{20} = 0.04$.

Well No.	Sampling Date	Specific Gravity at 20°C	pH	Composition of Water, mg-equiv. in 100 ml of Solution							Composition of Water in Equiv.-Pct							Ratios						
				Cl ⁻	SO ₄ ²⁻	HCO ₃	Ca ²⁺	Mg ²⁺	Na ⁺ +K ⁺	Σ	Cl ⁻	SO ₄ ²⁻	HCO ₃	Ca ²⁺	Mg ²⁺	Na ⁺ +K ⁺	Palmer Characteristic S ₁ S ₂ A ₂	Na ⁺ Cl ⁻	Cl ⁻ -Na ⁺ Mg ²⁺	Ca ²⁺ Mg ²⁺	SO ₄ ²⁻ Cl ⁻	Ca ²⁺ +Mg ²⁺ Na ⁺ +K ⁺		
167	7/5/66	1.1840	6.7	402.4	1.79	0.09	39.06	18.74	346.3	808.52	49.8	0.2	0.01	4.8	2.3	42.9	85.7	14.3	0.02	0.86	2.98	2.08	0.0004	0.17
309	11/9/66	1.1828	6.5	398.8	1.89	0.22	34.7	17.60	348.8	801.98	49.7	0.2	0.03	4.3	2.2	43.5	87.0	13.0	0.06	0.87	2.85	1.97	0.0005	0.15
310	11/9/66	1.1827	6.5	398.8	1.93	0.17	35.08	16.73	349.2	802.0	49.7	0.2	0.02	4.4	2.1	43.5	87.1	12.9	0.04	0.87	2.96	2.09	0.005	0.15
957	7/18/66	1.0800	7.9	229.8	5.64	0.56	20.3	10.99	204.7	472.0	48.7	1.2	0.02	4.3	2.3	43.4	86.7	13.0	0.24	0.89	2.34	1.85	0.020	0.15
957	2/25/69	1.0546	-	124.7	5.65	0.29	15.8	4.41	116.5	261.3	47.7	2.1	0.01	5.0	1.7	43.5	87.0	12.6	0.02	0.80	3.40	3.59	0.050	0.17
1015	2/25/69	1.1151	-	149.4	6.09	0.49	13.81	9.91	132.2	311.9	48.0	1.94	0.15	4.4	3.2	42.3	82.6	15.28	0.30	0.85	2.19	1.40	0.04	0.18
1451	5/27/66	1.1826	5.8	400.6	0.08	0.09	37.31	18.26	345.2	801.5	50.0	0.01	0.01	4.7	2.3	43.1	86.1	13.8	0.02	0.86	3.0	2.04	0.0002	0.16
1451	7/14/67	1.1735	7.4	380.5	0.38	0.05	36.2	16.00	328.7	762.0	50.0	0.05	0.01	4.8	2.1	43.2	86.3	13.7	0.02	0.86	3.2	2.26	0.001	0.16
1451	7/18/68	1.1831	8.1	403.1	1.93	0.17	35.4	17.73	352.0	810.0	49.7	0.24	0.02	4.4	2.2	43.4	86.9	13.1	0.04	0.88	2.6	2.00	0.005	0.15
1451	2/25/69	1.1769	-	454.3	2.41	0.10	39.2	9.03	408.6	913.7	49.3	0.28	0.01	4.3	1.5	44.6	89.2	9.92	0.02	0.89	4.9	4.34	0.005	0.12
1455	3/24/66	1.1821	5.0	398.8	1.78	0.21	36.6	17.08	347.0	801.5	49.8	0.22	0.03	4.6	2.1	43.3	86.6	13.3	0.06	0.87	3.0	2.14	0.004	0.15
KNS No. 4	7/23/68	1.00	-	0.08	0.07	0.20	0.20	0.05	0.10	0.70	11.4	10.0	28.6	28.6	7.1	14.3	28.6	14.3	57.14	1.25	-0.4	4.00	0.88	2.5

Note: pH values not determined.

TABLE USSR-I-3—Composition of Gas in the Aleksinsk Horizon (Reservoir C-O)

Gas Factor m ³ /m ³	Components: Percent (Volume) Before and After Inoculation											
	N ₂		C ₁		C ₂ + Higher		CO ₂		Asphotic Content of Gas			
	Before	After	Before	After	Before	After	Before	After	Before	After		
<i>Nikolo-Berezovsk area</i>												
Well No. 167	12	27	52	49.5	8.5	3.1	38.1	44.9	1.4	2.5	4.5	14.5
1016	23	34	56.6	40	8.0	9.5	33.8	48.2	1.6	2.3	4.2	5.1
1015	10	32	52.2	17.5	9.4	8.1	36.8	71.3	1.6	3.1	3.9	8.8
<i>Arlansk area</i>												
Well No. 957	8	18	47.3	45.4	13.3	8.5	37.4	43.6	2.0	2.5	2.8	5.1
310	18	66	43.8	37.4	15.5	10.9	39.2	50.1	1.7	1.6	2.5	4.6

REFERENCES

1. Kuznetsov, S. I. (1955) Razrabotka Metodov Mikrobiologicheskogo Vozdeystviya na Plast s Tsel'yu Intensifikatsii Neftedohychi i Uveluheniya Neftotdachi, Sb. "Metody Uvelicheniya Neftotdachi Plastov" (The Development of Methods of Microbiological Reaction Upon a Stratum in Order to Intensify the Extraction and Increase the Oil Yield of Strata). Materialy Vses. Soveshchaniya Ministerstva Neft. Prom. SSSR.

2. Kuznetsov, S. I., Ivanov M. V. Lyalikova, N. N. (1963) Introduction to Geological Microbiology. McGraw-Hill.

3. Yulbarisov, E. M. (1976) Evaluation of the Effectiveness of the Biological Method for Enhancing Oil Recovery of a Reservoir. Neftyanoye Khozyaystvo. No. 11, pp. 27-30.

4. Senyukov, V. M., Yulbarisov, E. M., Taldykina N. N., Shishenina, E. P. (1970) Mikrobiologiya 39, pp. 612-616. Translated from Mikrobiologiya 39 No. 4 pp. 705-710 July-Aug. 1970.

5. Cowey, F. K. (1976) Enhanced Recovery of Petroleum Using Microorganisms—Literature Survey. In: The Genesis of Petroleum and Microbiological Means for its Recovery. (Group Symposium on Oct. 6, 1976, Institute of Petroleum, London), pp. 57-75.

6. Lazar, I. (1979) Microbiological Methods in Secondary Oil Recovery. European Symposium on Enhanced Oil Recovery. Edinburgh, Scotland, July 5-7, 1978, pp. 279-287.

7. Rozanova, E. P. (1978) Sulfate Reduction and Water-Soluble Organic Substances in a Flooded Oil Reservoir. Mikrobiologiya 47, pp. 401-405. (Translated from Mikrobiologiya 47 No. 3 pp. 495-500 May-June 1978).

8. Yulbarisov, E. M. (1972) Results from Analysis of Petroleum Gas on Introduction of Biochemical Processes in the Oil Formation: Gazovoe Delo. No. 4 pp. 26-28.

ROMANIAN FIELD TEST

Years of test: 1975-1978 (9 wells inoculated).

Location: Romania

Geological data—Sandstone reservoirs.

Depth: 500-1550 m. Reservoir temperature: 27-56°C. Permeability: 80-1000 mD. Residual oil saturation: Wells produced 30-100 percent water. (Salinity from 5-200 g/l NaCl.)

Prior production history—Three types of wells inoculated: (1) those which still produced oil (0.2 to 1 T/day), (2) those producing only water, (3) those used for waterflooding.

Microbial well treatment—First well inoculated June 1975 and in same year 4 more wells were inoculated. For types (1) and (2) wells the Hungarian process was used and for type (3) wells a method described by Hitzman was used. The nutrients added thru 1977 were similar to Hungarian test nutrients. Only unsupplemented 2-4 percent molasses injection is now used.

Culture employed: Adapted mixed bacteria populations obtained from enrichment cultures. It is suggested bacteria that have the ability to metabolize the oil be used.

Response—Have observed modifications in physicochemical properties of produced fluids and some significant increase in oil production (still observing).

Remarks—It is reported the greatest release of oil occurs when sand is 78-82 percent and oil is 11-20 percent.

REFERENCES

1. Lazar, I. (1979) Microbiological Methods in Secondary Oil Recovery. Presented at the "European Symposium on Enhanced Oil Recovery," Edinburgh, Scotland 5-7 July 1978, pp. 279-287.

2. Lazar, I. (1976) Use of Bacteria in the Methods of Oil Liberation and Migration from Deposits. Mine, Petrol. Gaze 27 (10) pp. 475-480.

3. Lazar, I., Zamfirescu, I. (1978) Instalatii si modalitati de obtinere la scara industrială de bacterii adaptate conditiilor din zăcământul de titei. (Installations

and Methods for Obtaining Industrial Scale Quantities of Bacteria Adapted to the Conditions Existing in Petroleum Deposits). In Proceedings of the Symposium of Industrial Microbiology, 18-19 Dec. 1976, Iasi Romania pp. 284-289.

4. Lazar, I., Zamfirescu, I., Dumitru, L., Grigoriu, A., Mihoc, A. (1978) Cercitari privind obtinerea de bacterii adaptate conditiilor din zacamintele de titei (Studies on the Method for Obtaining Bacteria Adapted to the Conditions—Existing in Oil Deposits). In Proceedings of the Symposium of Industrial Microbiology, 18-19 Dec. 1976 Iasi Romania pp. 290-296.

5. Lazar, I., Dumitru, L., Zamfirescu, I., Mihoc, A., Grigoriu, A. (1978) Cercetari asupra capacitatii bacteriilor in eliberarea si migrarea titeiului dintr-un colector (Studies on the Capacity of Bacteria to Cause the Release and Migration of Petroleum from a Collector). In Proceedings of the Symposium of Industrial Microbiology, 18-19 Dec. 1976, Iasi, Romania, pp. 297-302.

6. Hitzman, D. O. (1967) Oil Recovery Process Using Aqueous Microbiological Drive Fluids, U.S. Patent 3,340,930.

DUTCH FIELD TEST I

N.V. de Bataafsche Petroleum Company
Year of test: 1956(?). Location: Unknown.

Geological data—Unknown.

Depth: Formation 10 m thick. *Viscosity*: 300 cp.

Prior production history—Unknown.

Microbial well treatment—Injected 1000 l of inoculated sucrose-molasses medium (10 percent sugar content) whose viscosity had increased to 2 cp, followed by a solution of the same composition but uninoculated.

Culture employed: *Betacoccus dextranicus*.

Response—Continuous production from the well showed oil to water ratio of 95:5 and total oil production then amounted to 70 percent of the total pore volume of the formation (if only water had been injected total oil production would have been only 40 percent of its total pore volume).

Remarks—The increased oil production is suggested to be the result of increasing the viscosity of the water to that of oil so as to equalize the permeability of the formation by forming viscous plugs in regions of greater permeability. (Even 3-5 cp improvement shows some effectiveness).

DUTCH FIELD TEST II

N.V. de Bataafsche Petroleum Company
Year of test: Unknown. Location: Unknown.

Geological data—Formation characterized by non-uniformity, 30 m thick, variable permeability.

Prior production history—Unknown.

Microbial well treatment—Injected 3000 l of inoculated medium (same as in Dutch Test I) and 100 m³

of a 50 wt percent molasses solution into the oil bearing sand. After a 5 day waiting period, water was pumped in the well.

Culture employed: Slime forming bacteria.

Response—The oil to water ratio changed to 1:20 compared to 1:50 when untreated.

Remarks—Improved oil to water ratio by viscosity improvement.

REFERENCES

1. Von Heiningen, J., Jan de Haan, H. and Jensen, J. D. (1958) Process for the Recovery of Petroleum from Rocks. Netherland Patent 89,580 (N.V. de Bataafsche Petroleum Maalschapij).

USA I

Mobil Field Test

Year of test: 1954. Location: Union Co., Arkansas

Geological data—Nacotoch formation 30 ft section.

Depth: 2000 ft (608 m). *Reservoir temperature*: 34°C. *Porosity*: 30 percent. *Permeability*: 1 to 5700 mD. *Residual oil saturation*: <10 percent (4-9 percent of pore volume).

Prior production history—Waterflood operations started in 1949 using brine of 20-25,000 ppm salinity. In May 1954, fresh water injection started. Prior to test the oil production had declined steadily. Estimated decline curve predicted mean production rate of about 0.6 bbl/day for period November 1954 to May 1955.

Microbial well treatment—In July 1954 started inoculation of culture and a 2 percent beet molasses solution into one injection well. A total of 4000 gal of a dense inoculum added in 18 separate injections. Process continued for 5.5 months (to Nov.) at an injection rate of 100-500 bbl/day.

Culture employed: *Clostridium acetobutylicum*; selected from a number of species.

Response—Only the control well nearest the inoculated well (400 ft) showed a response and bubbled like soda water. Water production remained fairly constant so the reportedly increased production was not attributed to a water flow increase. Fresh water breakthrough occurred after 70 days and fermentation products and sugars appeared at the production wells 80-90 days after first inoculation of the injection well. Products observed consisted of C₁-C₇ fatty acids, ethyl alcohol, acetone, butanol, CO₂ and CH₄ which were converted from 50 percent of the molasses injected. No significant increase in H₂. Products observed were unlike those of a pure culture so probably contamination occurred.

Isotake: Analysis of gases showed 80 percent of CO₂ was recent (from molasses), 20 percent of the CO₂ was ancient (neutralization of acids by carbonate formation), and 20 percent of the CH₄ was recent.

Production rate: Actual production rates started to rise in September 1, 1954 and remained above pro-

MEOR Field Applications

jected curve until end of test in May 1955. Mean oil production during this period was 2.1 bbl/day.

Production increase: Increased from 1 bbl/day to 3 bbl/day (200 percent increase).

Production duration: After 8+ months the test stopped due to the low cost of oil.

Remarks—Successfully conducted an underground fermentation. The test period was too short and too much molasses was said to have been used. No further tests were made as at that time there was no prospect for an economically viable process and bacteria were not expected to penetrate formation of low permeability.

USA II

Pure Oil Co. Fracturing Field Test

Year of test: 1957(?). Location: Unknown U.S. location.

Geological data—Sandstone reservoir, 20 foot section. *Depth:* 3000 ft (912 m).

Prior production history—Oil production prior to test was 15 bbl/day.

Microbial well treatment—5000 gal of fracturing medium consisting of Ca or Na lactate medium (composition [g/l]—Ca or Na lactate 3.5, ascorbic acid 0.5, yeast extract 1.0, MgSO₄ 0.2, K₂HPO₄ 0.5, NaCl 10.0) 2 percent agar gel agent (or CMC) and a propping agent, sand, etc. was injected into the well and formation inoculated with *D. hydrocarbonoclastus*. A packer was set immediately above the oil bearing interval and approximately 400 gal of gel filled the tubing and space adjacent to the producing zone. Applied 900 psi and fractured with the pressure drop to 100 psi and the remaining portion of the gel injected. Well shut in 3 months, then normal production resumed.

Culture employed: *Desulfovibrio hydrocarbonoclastus*.

Response—Production rate increased to 25 bbl/day. Conventional fracturing jobs on other wells in this area had little or no effect on their production rate. Rate of production on this and similar wells in the same formation was unchanged by shutting in wells.

Remarks—Microorganisms in fracturing fluids increased oil production.

USA III

A. C. Johnson Process

Years of test: 1977-1981. Location: 150 wells in U.S.A.

Geological data—Varied; all stripper wells.

Depth: 60-500 m. *Reservoir temperature:* 38°C (optimum). *Porosity:* Variable.

Prior production history—The wells were mainly stripper wells which produced less than 10 bbl oil per day (average 2 bbl/day), most had almost no wellhead pressure.

Microbial well treatment—The wells were injected with mixed cultures together with an aqueous solution of fermentable carbohydrate (usually crude

molasses and a suitable [proprietary] mineral nutrient). The size of the inoculum slug was dependent on the type of recovery process which was being used. The recovery processes were divided into 4 types. These types are: "huff and puff," well bore clean-up, partial repressurization, or in conjunction with a waterflood. The well was normally shut in 10-15 days following treatment.

Culture employed: Mixed cultures of *Bacillus* and *Clostridium* not utilizing hydrocarbons in their normal metabolism.

Response—The results varied depending on the treatment and well but reportedly if the proper oil type and reservoir conditions were chosen an average increase in oil production in excess of 350 percent could be expected (can recover an additional 20-30 percent of the oil in place). The treatment was found to be most effective: if the process used was on 15-30° API gravity (viscosity less than 400 cp) reservoir, if the salt content was less than 100,000 ppm, if it was a carbonate reservoir, and if the temperature was about 38°C. The mechanisms proposed for oil release are the production of gases (CO₂, CH₄ and N₂), and the production of acetic acid, (releasing CO₂ from carbonates), surfactants, acetone, higher alcohols.

Remarks—The number of wells tested and the successes reported are impressive. The effect of CO₂ production is stressed.

USA IV

Petrogen, Inc.

Years of test: 1977-1982.

Location: Mainly mid-continental U.S.

Geological data—Varied—6 different formations. *Depth:* 300 to 4600 ft (90-1400 m).

Microbial well treatment—Unknown—24 wells treated.

Culture employed: Unknown.

Response—Of 24 wells treated, 75 percent showed pressure increase of 10 to over 200 psi, the 25 percent remaining had no pressure increase for known (i.e., cracks in casing wall) or unknown reasons. In addition to the 24 injected wells, 30 additional wells were influenced by pressure or increased production or both.

Of the 24 wells: 4 wells doubled production for over 6 months; 12 wells increased production 50 percent for 3 months; and in one 5-spot pattern production levels increased 6 fold for a short time (most recent test) and this test is still being monitored.

For all tests the overall production increase averaged 42 percent (the level would have been higher if the data had been selected from wells where the specific goal was to increase production).

Remarks—All reported results are preliminary. Suggest at least 100 wells need to be treated under strict criteria to confirm results.

REFERENCES

1. Bond, D. C. (1961) Bacteriological Method of Oil Recovery. U.S. Patent 2,975,835.
2. Coty, V. F. (1975) XVII. Microorganisms of Oil Recovery: Status of Microbial Oil Recovery. The Role of Microorganisms in the Recovery of Oil. National Science Foundation, Easton, Maryland, Nov. 9-14, 1975, pp. 77-80.
3. Johnson, A. C. (1979) Microbial Oil Release Technique for Enhanced Recovery. Conf. on Microbial Processes Useful in Enhanced Oil Recovery. U.S. Dept. of Energy, San Diego, Calif. Aug. 29-Sept. 1, 1979. pp. 30-34.
4. Letter, A. G. Swan (Apr. 9, 1982). Limited Results from Science Research Center at Hardin-Simmons Univ. performing on a grant from Petrogen, Inc.

Overall Summary

SUMMARY

1. Biopolymers are already in use by the petroleum industry for a wide variety of applications. The biopolymer from *Xanthomonas campestris* is used extensively for drilling mud preparations and it has been field tested as a waterflood additive. An extracellular polysaccharide prepared from aerobic fermentation of crude oil has been developed in China and is proposed as a waterflood additive to increase the sweep efficiency of oil recovery. A biosurfactant product (a glycoprotein) from *Acinetobacter calcoaceticus* RAG-1 is being prepared in commercial quantities for use as an emulsifier of crude oil and may be important for cleaning tanks and pipelines. The biosurfactant produced by *Corynebacterium lepus* has been demonstrated to be effective for the release of bitumen from tar sands. Recently a mixed culture was discovered that produces a biosurfactant when fermented aerobically with crude oil; it causes a decrease of the oil phase viscosity of 95 percent and produces a non-wetting (on glass or steel) emulsion.

2. Salt tolerant bacteria of the genus *Clostridium* have been isolated and shown to produce large amounts of carbon dioxide and solvents in 5-6 percent salt solutions. Spores of these bacteria do not adsorb on sandstone rock samples, thus the spores can penetrate deep within a petroleum reservoir to germinate where most needed. In addition, a field test in the United States with *Clostridium acetobutylicum* resulted in a two and a half increase in production from a two-spot injection test and production of 35,000 kg of acids and 5,700 m³ of carbon dioxide during the nine month test period.

3. Mixed bacteria cultures (Gram-negative, facultative rods) have been isolated that produce copious amounts of gas and are designated for possible repressurization of oil fields in Canada. A nutrient solution also was developed that aids in the injection of the bacteria by inhibiting adsorption. A second growth medium activates the bacteria to produce a biopolymer *in situ*.

4. Living cells have been found to be strongly adsorbed on sandstone which restricts the injection of non-spore forming bacteria unless carrier solutions are developed that inhibit cell adsorption and thus allow deep migration of the cells into the oil reservoir.

5. Bacteria were isolated from the Wilmington oil field in California at a depth of 1,000 meters that are capable of degrading polyacrylamides that are used for mobility control and formation of biopolymer that may inhibit treatment by biocides.

6. Two of seven field tests in Romania with mixed cultures of bacteria that were acclimated to reservoir conditions resulted in a two-fold increase.

CONCLUSIONS

The presented papers and discussions at this Conference revealed many positive aspects in the use of microorganisms in enhanced oil recovery. Numerous field tests throughout the world have in general been successful, although the release of residual oil in these tests was not maximized.

Biological production of chemicals, such as biopolymers and biosurfactants, for enhanced oil production shows promise for both *in situ* production and for surface production. The use of microorganisms and their products for recovery or treatment of heavy oil and tar sands has been demonstrated. The potential for the use of crude oil as a substrate for anaerobic growth of MEOR organisms continues to be investigated and the possibility exists that a consortium of microorganisms might successfully be used whereas a pure culture of microorganisms would probably not grow under these conditions.

From the papers and discussions, it is obvious that there is still much to be learned before MEOR is an established procedure for use in the oil field. The workers in this field of research gave realistic appraisals of the vast amount of knowledge that is still missing, but which can be attained. This was not an expression of negative attitudes toward the potential for MEOR. Instead it was an expression of an awareness of the amount of work yet to be done. With more knowledge and development, MEOR will be a viable tool in the oil field. It may not become a universal means of recovering residual oil, but it will become another tool in the arsenal of tools available for enhanced oil recovery, and under certain reservoir conditions, it should be the tool of choice.

