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Bio-Engineering High Performance Microbial Strains for MEOR by Directed Protein Evolution Technology

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- Sections written by Qinhong Wang, Xiangdong Fang and Patrick Shuler

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Bio-Engineering High Performance Microbial Strains for MEOR by Directed Protein Evolution Technology

DOE Project: DE-FC26-04NT15525

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California Institute of Technology

Year Two Annual Report October 2005 – September 2006

1. EXECUTIVE SUMMARY

This report summarizes the research work performed and results of that work for Year 2 of this 3-year project. The main objectives are to employ the latest bioengineering technologies to develop manufacturing methods at the surface that can create more cost-effective surfactants for the chemical flood EOR process; and to improve the surfactant activity and the thermal stability of bio-surfactant systems deployed subsurface for MEOR. Other objectives are to develop improved laboratory methods and tools that screen quickly candidate bio-systems for EOR. These detailed laboratory procedures to identify effectively and quickly mutated genes for their bio-surfactant activity and thermal stability is a substantial effort. Documenting these procedures will accelerate greatly the development of yet newer bio-systems for improved oil recovery applications in similar, future studies.

During the second year of the project period,

1. We successfully engineered the new mutant strains *P. aeruginosa* PEER02 and *E. coli* TnERAB so they can produce rhamnolipid biosurfactans. LC-MS spectrum showed the structure of purified

rhamnolipids from *P. aeruginosa* PEER02 was similar to those from other *P. aeruginosa* strains, but have different percentage for each component. The main component of purified rhamnolipids from *E. coli* TnERAB is C10-C10 with monorhamnose (2 alkyl chains, each with 10 carbons, and a single rhamnose head group).

2. Core flooding tests showed that rhamnolipids produced by our engineered bacteria are effective agents for enhanced oil recovery (EOR). At 250ppm rhamnolipid concentration from *P. aeruginosa* PEER02, 42% of the remaining oil after waterflood was recovered. These results were therefore important for considering the exploration of the studied rhamnolipids as EOR agents.

3. Our engineered *P. aeruginosa* PEER02 strain can produce rhamnolipids with different carbon sources as substrate. Interfacial tension analysis (IFT) showed that different rhamnolipids from different substrates gave different performance. Those rhamnolipids with plant oil as substrate showed as low an IFT as 0.05mN/m in the buffer solution with pH5.0 and 2% NaCl. Fed-batch fermentation with soybean oil as substrate greatly enhanced the production of rhamnolipids, and the yield reached as high as 25g/L. These results showed some potential for producing high-performance rhamnolipids with high yield from low-cost renewable resources.

4. Through the methodology of synthetic biology and metabolic engineering, we engineered *E. coli* strains harboring various gene combinations from *P. aeruginosa* and successfully produced either mono-rhamnolipids or di-rhamnolipids (one or two head groups). Engineered rhamnolipids showed different performance in interfacial tension and antimicrobial activity. LC-MS analysis confirmed they mainly contained C_{10}-C_{10} and C_{10}-C_8 carbon chain as well as carbon chains with other lengths in smaller percentages.

5. A mutant with different product selectivity was found via directed evolution and subsequent high-throughput screening. Compared with the wild-type strain, the mutant almost produced rhamnolipids with only C_{10}-C_{10} carbon chain.

2. BACKGROUND

In MEOR processes it is thought that the bacteria in place may mobilize trapped oil by several mechanisms. Some of the best candidate microbes produce gases, low molecular weight acids, and polymers in addition to surfactants (Bryant, 1987, 1989, 1996, Premuzic, 1991). This proposed work will focus on the biological generation of surfactants because the ability of these chemicals to reduce interfacial tension (IFT) between the water and oil phases offers the best chance of

mobilizing substantial amounts of hydrocarbons not otherwise recovered by waterflooding. It has been reported that a 30% increase in total oil recovery occurs from sandstone cores by using trehalolipids from *Nocardia rhodochrous*. More typically, bio-systems in MEOR laboratory tests recover of the order of 10 – 20% of post water flood incremental oil (Yonebayashi, 1997).

The chemical material cost is perhaps the main factor controlling the profitability of conventional surfactant flooding EOR (Wu 1996, Taber 1996). An inherent potential advantage of a biological approach is that we can make surfactants whose cost is decoupled from the price of crude oil. The nutrients and other raw materials to create bio-surfactants do not have to be petroleum-based, and in fact potentially may come from waste streams. Commercial synthetic surfactants invariably are more expensive than the crude oil from which they are derived.

For EOR applications, chemical surfactants cost in range of $1 to $3 per pound, whereas lignin-based sulfonates are cheaper. But the current cost of bio-surfactants may be about 3 to 10 times higher using current technology (Desai and Banat, 1997). Attempts have been made to improve overall process economics in bio-surfactant production. Those efforts have been focused on searching for cheap substrates, increasing the productivity by manipulating physiological conditions, mixing multiple microbial cultures, and modifying the downstream recovery processes. Potential overall improvements via these strategies are quite limited.

Results from our study will open the door to manufacture of bio-surfactants suitable for EOR at a reasonable price. First and foremost, we will employ advanced bioengineering methods to have bacteria with the capability to create surfactant with orders of magnitude greater activity than at present. Secondly, because these materials are to be used in the oilfield, there is much less concern with the purity, color, etc. of the produced product. We can save on much of the expense normally associated with product separation and purification by accepting a less refined product for oilfield use. In particular, if the bio-surfactant manufacture is at or near a target oil field, then the supernatant solution containing the chemical may be a sufficiently concentrated product as is (there is little or no transportation cost, hence excess water in the product is not a factor for this strategy.) Thirdly, one of our strategies is to engineer the bacteria to be more robust in their nutritional requirements. Potentially, the nutrients for the bacteria creating the bio-surfactant may come from waste streams such as waters containing dissolved and dispersed oil components, further driving down the cost of their manufacture.

Another major challenge to deploying MEOR technology where the bacteria are introduced into the subsurface is that they have only limited activity towards the production of bio-surfactant (and other desirable products). A second concern is also the "care and feeding" of injected bacteria. Once these bacteria are introduced they must receive continued nutrition to survive. They also are subject to decreased activity or even extinction under the high reservoir temperatures or other changes in their environment.

Our proposed approach will address both of these concerns. We will clone the mutated gene responsible for extraordinary growth of bio-surfactant from a host organism into candidate bacteria that have a demonstrated ability to thrive in oil reservoir environments. Thermophillic, chemotropic organisms are prime host candidates as they 1) function at high temperatures, 2) use constituents indigenous to the substrate, 3) tolerant of pH and salinity extremes.

3. RESULTS AND DISCUSSION

3.1 Summary of the Tasks for this Reporting Period

Task 1.0 Directed evolution studies for *P. aeruginosa* and *B. subtilis* (24 months)
These studies will identify the first-round of "super-mutants" with one or more desirable characteristics, 1) "super-activity", 2) thermal stability, or 3) use of cheap substrates. We will include in our reports laboratory procedures to arrive at the successful mutant candidates.

Subtask 1.2 (month 9 to 22) -- Second round mutation studies (via in vitro DNA shuffling) to upgrade to a smaller number of better candidate mutants. Perform batch fermentation on selected mutated strains to demonstrate they achieve a desired effect.

Task 2.0 Genetic cloning of "supermutants" into thermophilic oilfield strains (29 months)
The purpose of these studies is to clone the mutants with high activity and thermal stability into microbes adaptable to in-situ, oilfield reservoir conditions. Part of this task is to document the detailed laboratory procedures and results in our efforts to create a robust MEOR microbe.

Subtask 2.1 (Month 7 - 20) -- Develop laboratory methods to add bio-surfactant function to these target microbes. Initially use wild, natural versions of *P. aeruginosa* and *B. subtilis* as the genome source.

Subtask 2.2 (9 months) -- Clone the mutant with the best features for in-situ MEOR (high activity and thermal stability) into the oilfield thermophilic host microorganism(s).

Task 3.0 Evaluation of Bio-Surfactants and Bio-Systems for EOR (21 months)

The purpose of these studies is to 1) characterize and optimize the target bio-surfactants (rhamnolipids and surfactin) as candidate chemicals for conventional chemical EOR, and 2) evaluate in-situ, MEOR performance of oil reservoir microbes now mutated to produce prodigious amounts of rhamnolipids or surfactin. One activity will be to document the surfactant performance screening procedures and results in a report.

Subtask 3.2 (9 months) -- Use whole cell lysate from fermentation tests from the best "supermutants" to spot check the bio-surfactant phase behavior and IFT versus a series of n-alkanes, plus re-test the solid adsorption behavior (expect same results as in Subtask 3.1). Conduct detailed tertiary oil displacement performance tests of generated biosurfactant in sand packs and Berea core floods. Optimize oil-displacement performance for at least one of the two candidate bio-surfactant systems for an example light crude oil system. Compare and contrast with a synthetic alkyl aryl sulfonate surfactant system.

3.2 Engineering Bacteria for production of rhamnolipids as agent for enhanced oil recovery

Abstract

Rhamnolipids as potent natural biosurfactant have wild potential biotechnological applications such as enhancing oil recovery, biodegradation and bioremediation. Rhamnolipids are composed of rhamnose sugar molecules and ß-hydroxyalkanoic acids. Rhamnosyltransferase complex (RhlAB) is the key enzyme which is responsible for transferring the rhamnose moiety to ß-hydroxyalkanoic acid moiety and biosynthesizing rhamnolipids in *P. aeruginosa.* Through transposon-mediated chromosome integration, RhlAB gene was randomly inserted into the chromosome of the *P. aeruginosa* mutant PAO1-rhlA$^-$ and *Escherichia coli* BL21(DE3), both of which could not produce rhamnolipids. After chromosome integration of the RhlAB genes, the new mutant strains *P. aeruginosa* PEER02 and *E. coli* TnERAB can produce rhamnolipids. LC-MS spectrum showed the structure of purified rhamnolipids from *P. aeruginosa* PEER02 was similar to those from other *P. aeruginosa* strains, but having different percentage for each component. The main component of

purified rhamnolipids from *E. coli* TnERAB is C10-C10 (both alkyl chains with 10 carbons) with monorhamnose (one sugar head group). Surfactant performance of rhamnolipids was evaluated by measurement of interfacial tension (IFT) and oil recovery via core flooding. IFT analysis revealed that pH and salt concentration in the buffer would significantly affect the properties of IFT. At only 250 ppm rhamnolipid concentration from *P. aeruginosa* PEER02, 42% of the remaining oil after waterflooding was recovered. These results are therefore important for considering the exploration of the studied rhamnolipids as an enhanced oil recovery agent.

Introduction

Surfactants pervade our everyday lives. Some surfactants, known as biosurfactant are biologically produced by bacteria or yeast from various substrates including sugars, glycerol, oils, hydrocarbons and agricultural wastes (Lin, 1996). Biosurfactant are classified as glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids, polymeric and particulate compounds (Desai *et al*., 1997). Most of these compounds are either anionic or neutral. Only a few are cationic such as those containing amine groups. The hydrophobic portion of the molecule is based on long-chain fatty acids, hydroxyl fatty acids or α-alkyl-β-hydroxy fatty acids. The hydrophilic moiety can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol.

Biosurfactants have been receiving increasing attention as a result of their unique properties (i.e., mild production conditions, lower toxicity, and higher biodegradability) compared to their synthetic chemical counterparts (Rosenberg *et al*., 1999). It may thus be possible to reduce the environmental risk of an application by replacing chemical additives with biosurfactants. The interest in biosurfactant has increased considerably because of their broad range of potential applications in various fields. Biosurfactants reduce surface tension and interfacial tension in both aqueous solutions and hydrocarbon mixtures. These properties lead to the formation of microemulsions, where hydrocarbons can solubilize in water, or water in hydrocarbons. The initial focus and most of the industrial interest in biosurfactants has been towards application in the oil industry to aid the clean up of oil spills as well as to enhance oil recovery from oil reservoirs. Chemically synthesized surfactants have been used for the same purpose, but they pose environmental problems because of their greater toxicity. Biosurfactants can have equivalent emulsification properties and are biodegradable (Mulligan, 2005).

The rhamnolipid, mainly from *Pseudomonas aeruginosa*, is a group of biosurfactants that has been studied extensively (Liang *et al*., 1999). Rhamnolipids are produced as mixtures in various

proportions, including two rhamnoses attached to β-hydroxyalkanoic acid or one rhamnose connected to the identical fatty acid (Fig. 1) (Soberon-Chavez et al., 2005). *Pseudomonas aeruginosa* can produce rhamnolipids from many different substrates including C11 and C12 alkanes, succinate, pyruvate, citrate, fructose, glycerol, olive oil, and glucose (Robert *et al*., 1989). The type of rhamnolipid produced depends on the bacterial strain, the carbon source used, and the process strategy (Mulligan *et al*., 1993). The lengths of the fatty acid chains of rhamnolipids can vary significantly, resulting in a multitude of different rhamnolipd compositions. Fatty acyl chains can have 8, 10, 12, and 14 carbons in length, as well as 12- or 14-carbon chains with double bonds. Rhmanolipids reduce remarkably the surface tension of water from 72 to values below 30mN/m and the interfacial tension of water/oil systems from 43mN/m to values below 1mN/m. They also have excellent emulsifying power with a variety of hydrocarbons and vegetable oils (Abalos *et al*., 2001).

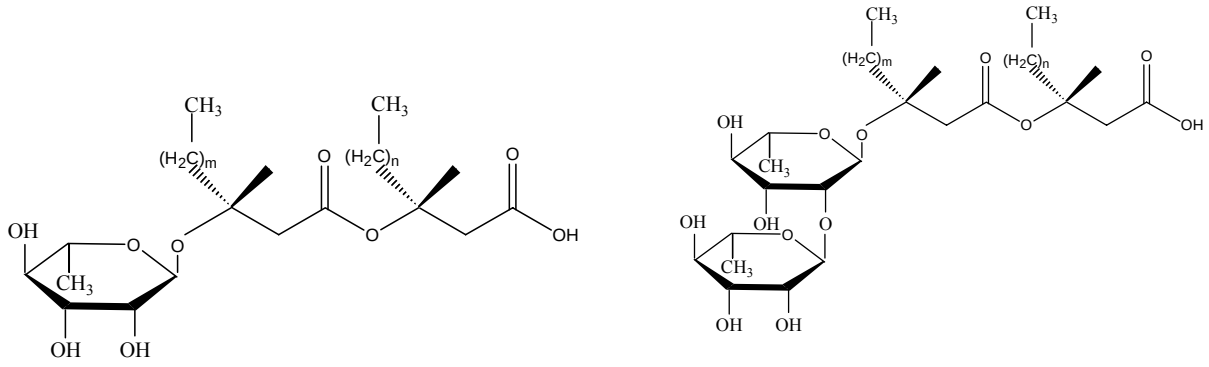


Fig 1. Structure of rhamnolipids produced by *P. aeruginosa.* Left: mono-rhamnolipid; Right: di-rhamnolipid. Alkyl chain length may vary. (In shorthand, mono-rhamnolipid: Rha-C_{m+4}-C_{n+4}, di-rhamnolipid: Rha- Rha-C_{m+4}-C_{n+4}, m, n=4-8)

Increasing ecological concerns have led to the increased interest in the application of rhamnolipids as substitutes for their synthetic chemical surfactant counterparts, although production costs would need to be reduced. The use of renewable and low-cost substrate, such as soybean oil, could provide rhamnolipids production (Nitschke *et al*., 2005) with acceptable economics. Many bacteria, especially for the Pseudomonads, can efficiently utilize renewable and low-cost substrates for supporting cell growth, but lack the ability to biosynthesize the rhamnolipids, or have very low yield of rhamnolipids. Here, Starting from *P. aeruginosa* PEER01 and *Escherichia coli* BL21(DE3) (see Material and Methods) which are unable to produce rhamnolipids, we attempt to integrate the key genes of rhamnolipids biosynthesis by state-of-the-art transposome-mediated chromosome

insertion in order to program bacteria to produce rhamnolipids. The overall objectives of the present study include: (1) to engineer *P. aeruginosa* and *E coli* strains to improve or modify rhamnolipids production; (2) to characterize the compounds from engineered strains by thin layer chromatography (TLC) and HPLC-MS; (3) to study the interfacial activity of these compounds from engineered strains; and (4) to evaluate the possibility of using these as agents for enhanced oil recovery in the field of petroleum industry.

MATERIALS AND METHODS

Bacterial strain, media and chemicals

P. aeroginosa PAO1-RhlA$^-$ (Rahim et al, 2001) (a gift from Dr. Joseph S. Lam, University of Guelph) and *E. coli* BL21(DE) (*F^- ompT gal dcm lon hsdS$_B$(r_B^- m_B^-) λ(DE3)*) which can not produce rhamnolipids were as the parental strain for engineering rhamnolipids-producing strain. *E. coli* DH5α (F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–) was as the host strain for constructing various recombinant plasmids. The *E. coli* strains were commercially available. Except for rhamnolipids fermentation, all these bacteria were grown on LB media supplementing suitable antibiotics at 37 ºC (Sambrook and Russell, 2001). Unless noted otherwise, antibiotics were used at the following concentration: chloramphenicol, 100μg/mL for *P. aeruginosa* and 25μg/mL for *E. coli*; ampicillin, 50μg/mL for *E. coli*; Kanamycin, 40μg/mL for *E. coli*. All enzymes used for DNA manipulation were purchased from New England Biolabs (Beverly, MA). Pfx DNA polymerase and dNTP mixture for PCR amplification were purchased from Invitrogen (Carlsbad, CA). EZ::TN™ Transposase was purchase from EPICENTRE Biotechnologies (Madison, WI).

Fermentation media and conditions

Nutrient broth from BD (Franklin Lakes, NJ) was used for seed culture of *P. aeruginosa* and LB for seed culture of *E. coli*. The mineral salts medium and a 0.4% or 2% glucose or 2% soybean oil as carbon source was the rhamnolipd fermentation media. The mineral salts medium contained (g/L): $NaNO_3$, 15; KCl, 1.1; NaCl, 1.1; $FeSO_4.7H_2O$, 0.00028; KH_2PO_4, 3.4; K_2HPO_4, 4.4; $MgSO_4.7H_2O$, 0.5; yeast extract 0.5; and 5 ml of a trace elements solution containing (g/L): $ZnSO_4.7H_2O$, 0.29;

$CaCl_2.4H_2O$, 0.24; $CuSO_4$. $5H_2O$, 0.25; $MnSO_4.H_2O$, 0.17 (Lindhardt et al, 1989). The trace element solution was filter-sterilized through a 0.22-µm membrane filter (Millipore, type GS) and then added to the medium, which had been autoclaved and allowed to cool.

P. aeruginosa wild-type and mutant were first grown in nutrient broth for 24h at 30ºC with shaking and then diluted 1:10 into mineral salts medium and 2% glucose or soybean oil and incubated for 4 days. *E. coli* wild-type and mutant were first grown in LB for 24h at 30ºC with shaking and then diluted 1:10 into mineral salts medium or LB with 0.4% glucose supplementing 50µM IPTG (isopropyl-beta-D-thiogalactopyranoside) as inducer and incubated for 24h. Incubation was carried out in 250mL Erlenmeyer flaks with 25mL medium and at 30ºC with orbital shaking at 250rpm.

Plasmids and strains construction

pMOD-2C which derived from pMOD-2 (EPICENTRE Biotechnologies Madison, WI) was as our transposon construction vector. Briefly, pACYC184 from New England Biolabs (Beverly, MA) was digested with *Xba*I and *Sty*I to produce a chloramphenicol cassette. After blunted with T4 DNA polymerase, this chloramphenicol cassette was inserted into *Sma*I site of pMOD2 to produce pMOD-2C. Rhamnosyltransferase 1 complex gene with native operon promoter (containing upstream 400bp sequence of structural genes), RhlABa, was amplified with primer RhlAB-1a (5'-CCCAATCTCTAGATGCCTTTTCCGCCAACCCCTCGCTG-3') and RhlAB-2 (5' - AAC CAA GCT TTC AGG ACG CAG CCT TCA GCC ATC G - 3') and *P. aeruginosa* PA01genomic DNA as template; PCR product of RhlABa was digested with *Xba*I and *Hind*III and cloned into pMOD-2C to produce the recombinant plasmid pMOD-2CRABa (Fig 3a). 3.5kb chimeric transposon TnRABa with native operon promoter was produced by digesting pMOD-2CRABa with *Psh*AI. Rhamnosyltransferase 1 complex structural genes RhlABb was amplified with primer RhlAB-1b(5'- AGTTGGTACCATGCGGCGCGAAAGTCTGTTGG -3') and RhlAB-2 (5' - AAC CAA GCT TTC AGG ACG CAG CCT TCA GCC ATC G - 3') and *P. aeruginosa* PA01genomic DNA as template; PCR product of RhlABb was digested with *Kpn*I and *Hind*III and cloned into pET30a(+) to produce the recombinant plasmid pETRAB. 2.4kb SphI-HindIII from pETRAB was cloned into pMOD-2C to produce the recombinant plasmid pMOD-2CRABb (In this case, *Sph*I and *Xba*I in pMOD-2C were blunted, then ligated). Chimeric transposon TnRABb with T7 promoter (from pET30a(+)) was produced by digesting pMOD-2CRABb with *Psh*AI (Fig3a). Chimeric transponon TnRABa or TnRABb was incubated with EZ::TN™ Transposase in the absence of Mg2+ to

produce chimeric transposome TnRABasome or TnRABbsome (Fig3b). TnRABasome and TnRABbsome was transformed into *P. aeruginosa* PAO1-RhlA$^-$ or *E. coili* BL21(DE3) by electroporation (Smith and Iglewski, 1989), respectively. After genotypic and phenotypic analysis, engineered strain *P. aeruginosa* PEER02 and *E. coli* TnRAB were constructed.

Analytical method for rhamnolipids

Rhamnolipids were quantified in triplicate by weight and by the colorimetric determination of sugars with orcinol (Candrasekaran and Bemiller 1980). Rhamnolipids were purified by first separating the cells from supernatant by centrifugation (10000 ×g). The supernatant was then extracted with chloroform and ethanol. 0.5mL rhamnolipid sample was extracted with 1mL chloroform:ethanol (2:1, v/v). The organic phase was evaporated to dryness and 0.5mL of H_2O was added. To 0.1mL of each sample with suitable dilution, 0.9mL of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added. After heating for 30min at 80ºC the samples were cooled at room temperature and the OD_{421} was measured. The rhamnolipid concentrations were calculated from standard curves prepared with L-rhamnose (0-50mg/L) and expressed as rhamnose equivalents.

Rhamnolipids purification, Thin-layer chromatography and LC-MS analysis

The rhamnolipids were precipitated by acidifying culture supernatant to pH 2 with concentrated HCl and kept at 4°C overnight, and recovered by centrifugation at 10000g for 1h and dissolved in deionized water. 2 volumes of chloroform:ethanol (2:1, v/v) was added to rhamnolipids solution and shaken 30 min for extraction. The organic phase was evaporated to dryness, and the rhamnolipid residue was then dissolved to 500mg/l final concentration. The purified rhamnolipids were separated, visualized, and compared to known rhamnolipids samples (JBR425, Jeneil BIosurfactant Co., LLC) using thin layer chromoatography (TLC; silica gel 60 plates). Next, a 10μL sample was loaded into silica gel 60 plates. After being dried at room temperature, the silica gel was developed with a solution of chroroform-methanol-water (65:15:2 by volume), then visualized using a 50:1:0.05 mixture of the solution glacial acetic acid-sulfuric acid-anisaldehyde at 90°C for 30min. The HPLC-MS analyses were performed with a Gemini C18 column (2 mm x 50 mm, 5μm particle) from Phenomenex and a ThermoFinnigan LCQ Classic ion trap mass spectrometer. The LC gradient was: starting at 8%B and holding for 1 minute, then ramp to 75%B

in 20 minutes, hold at 75%B for 10 minutes, back to 8%B in 1 minute and hold at 8%B for 5 minutes. Solvent B is 10:90 water:acetonitrile with 0.1% acetic acid. Solvent A is 98:2 (v/v) water:acetonitrile with 0.1% acetic acid. The mass spectrometer was operated in the negative ion mode scanning 250-950 m/z range. Data dependent MS/MS was set to analyze MS/MS of the more abundant ions in the mass range.

Interfacial tension analysis of Rhamnolipids

The IFT was determined by using a spinning drop tensiometer (Temco Inc.) as detailed by Cayais (1975). Aqueous rhamnolipid solution was loaded into glass tube (Inner diameter, 2mm and Outer diameter, 5mm), followed by injection of 1.5μL octane. The glass tube with solution was spun in the tensiometer, and IFT was determined from the octane drop geometry. After 10min-spinning, the data were collected for analysis. In these studies, we measured the "fresh" IFT values without pre-mixing rhamnolipid solution and octane together to reach phase equilibrium. To adjust pH of rhamnolipid solution, 50mM citrate-Na_2HPO_4 buffer was used.

Sand-packed core flooding test

Dried playground sand manufactured by Paragon Building Products Inc (Norco, CA) was packed into a stainless iron tube (2×60cm) as a flooding core. The sand-pack was evacuated via a vacuum pump and saturated with a 50mM citrate-Na_2HPO_4, 2% NaCl, pH5.0 buffer solution (Brine). This buffer-brine was selected because the rhamnolipid from PEER02 generated its lowest IFT versus n-octane in this solution at room temperature. Pore volume (PV), porosity as well as brine permeability (approximately 50 Darcies) of the pack core at three brine flow rates were calculated. Then n-octane (the selected oil phase) displaced brine until no more water comes out. The oil-flooded pack was aged for 24h. Then, brine was injected into aged oil-saturated core to displace the oil which was not trapped strongly by sand and recovered with brine until no more oil came out. At this point, the core was ready for biosurfactant (rhamnolipids) flooding. 3 PV rhamnolipid solution (in 50mM citrate-Na_2HPO_4, 2% NaCl added, pH5.0) was injected, and oil recovery and water cut (percentage of non-oil phase in each elution fraction) were recorded. Next, the core with rhamnolipids was shut-in overnight at room temperature. Another 3 PV of rhamnolipid solution was injected into at second day. Similarly, oil recovery and water cut were recorded. After rhamnolipids flooding, 6 PV brine (50mM citrate-Na_2HPO_4, 2% NaCl, pH5.0 buffer) was injected, and oil

recovery and water cut in this stage were also recorded. The IFT for eluted solution was measured with the method mentioned above.

Results

Engineering *P. aeruginosa* and *E. coli* for rhamnolipids production

From the biosynthetic pathway (Maier and Soberon-Chavez, 2000), the synthesis of rhamnolipids proceeds by sequential glycosyl transfer reactions, each catalyzed by a specific rhamnosyltransferase with TDP-rhamnose acting as a rhamnosyl donor and 3-hydroxyalkanoyl-3-hydroxyalkanoate acting as acceptor. L-Rhamnosyl-L-rhamnosyl- 3-hydroxyalkanoyl 3-hydroxyalkanoate and L-rhamnosyl-3-hydroxyalkanoyl-3- hydroxyalkanoate, referred to as rhamnolipids 1 (mono-rhamnolipids) and 2 (di-rhamnolipds), respectively, are the principal glycolipids produced in liquid cultures. Rhamnosyltransferase 1 is encoded by the *rhlAB* genes, which are organized in an operon and responsible for biosynthesis mono-rhamnolipds. The active enzyme complex is located in the cytoplasmic membrane, with the RhlA protein being localized in the periplasm and the catalytically active RhlB component crossing the membrane (Ochsner et al., 1994).

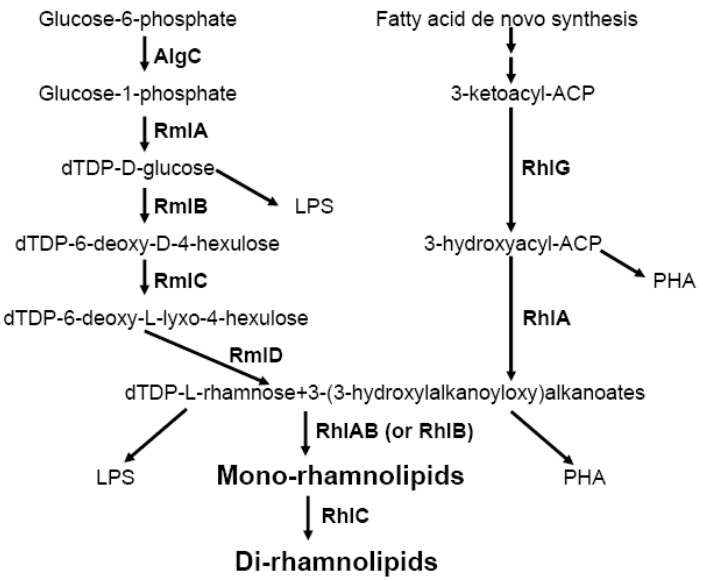
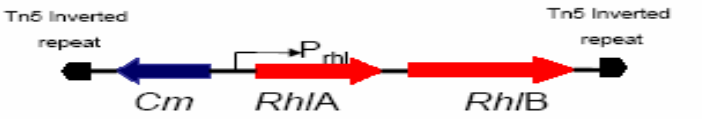


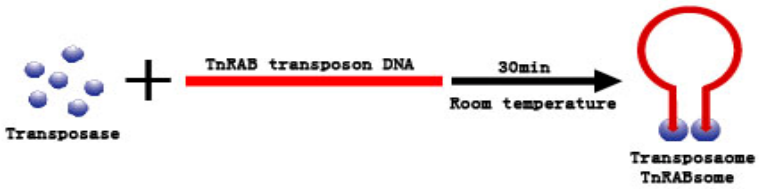
Fig 2 Rhamnolipids biosynthetic pathway from *P. aeruginosa*

RmlA-D: dTDP-L-rhamnose biosynthesis gene cluster; RhlAB: rhamnosyltransferase 1 gene ; RhlC PhaC: rhamnosyltransferase 2 gene; RhlG: ketoacyl reductase gene.

RhlAB is the key enzyme of rhamnolipids biosynthesis, but this biosynthesis was modulated by the complex transcriptional regulatory network in *P. aeruginosa* (Soberon-Chavez and Aguirre-Ramirez, 2005). However, to achieve the rhamnolipids production in the strains which can not produce rhamnolipids, integrating RhlAB is indispensable. In this circumstance, convenient molecular biotechniques would give great insights. Here, we show the state-of-the-art transposome-mediated chromosomal integration (Fig. 3) to modify metabolic pathway of rhamnolipid biosynthesis. Briefly, RhlAB with native promoter and selection maker gene Cm (chloramphenicol resistance gene) were cloned into Tn5 derived transposon plamid pMOD-2 (Epcentre Biotechnologies, Madison, WI) to produce the recombinant plasmid pMOD-2CRAB. pMOD-2CRAB was digested with PshAI to obtain chimeric transposon TnRAB (Fig 3a) which can bind transposase to produce transposome TnRABsome (Fig 3b). TnRABsome was electroporated into *P. aeruginosa* PAO1-rhlA$^-$ (Fig 3c) wild-type strain. Consequently, we successfully constructed rhamnolipid-producing *P. aeruginosa* from the wild-type strain (which did not have the ability for rhamnolipid biosynthesis) by transposome-mediated chromosomal integration of RhlAB after genotypic and phenotypic analysis. RhlAB with native promoter was inserted into the chromosome of rhamnolipid-deficient strain (*P. areuginosa* PAO1-rhlA$^-$), and the mutant strain *P. areuginosa* PEER02, which was confirmed to have RhlAB by PCR amplification and subsequent sequencing (data not shown) can effectively produce rhamnolipid in saline media with either glucose, or soybean oil as substrate. As showed in Table 1, soybean oil was a better substrate for rhamnolipid production than glucose, with the yield of rhamnolipid in soybean oil being two fold more than in glucose.



(a)



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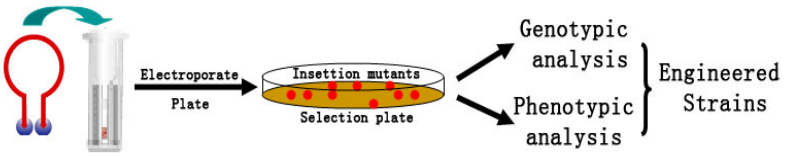


(c)

Fig. 3 Diagram of Engineered Rhamnolipids-producing *P. aeruginosa* Construction

(a) Structure of rhamnolipid biosynthetic gene RhlAB-containing Transposon (TnRABa or TnRABb); Cm: chloramphenicol resistance gene cassette; P: RhlAB native promoter or T7 promoter; TnRABa with the native promoter of RhlAB; TnRABb with T7 promoter fused with RhlAB. (b) Transposome TnRABasome or TnRABbsome construction;

(c) Transposome eletroporation and insertion mutants selection.

Table 1 Rhamnolipid fermentation by *Pseudonomas aeruginosa* mutant

Stains	Rhamnolipid (mg/L)	
	Glucose	Soybean oil
P. areuginosa PAO1-RhlA$^-$	ND	ND
P. areuginosa PEER02	785.4	1819.1

ND: none detected

In addition, to overcome the complex environmental regulation of rhamnolipid biosynthesis in *P. aeruginosa*, we attempted to achieve rhamnolipd formation in *E. coli* by same transposome-mediated chromosomal integration of RhlAB. T7 promoter replaces native promoter of RhlAB in the transposon TnRAB to produce another chimeric transposon TnRAB7. This new transposon was inserted into the chromosome of *E. coli* BL21(DE3) by transposome-mediated integration to engineer a strain *E. col*i TnERAB which was also confirmed by PCR (data not shown). With IPTG induction, *E. col*i TnERAB can produce rhamnolipid both in rich (LB) and minimal (MS) media and glucose as substrate. Because *E. coli* can grow better in LB media than minimal media, rhamnolipid yield is higher in LB media (Table 2).

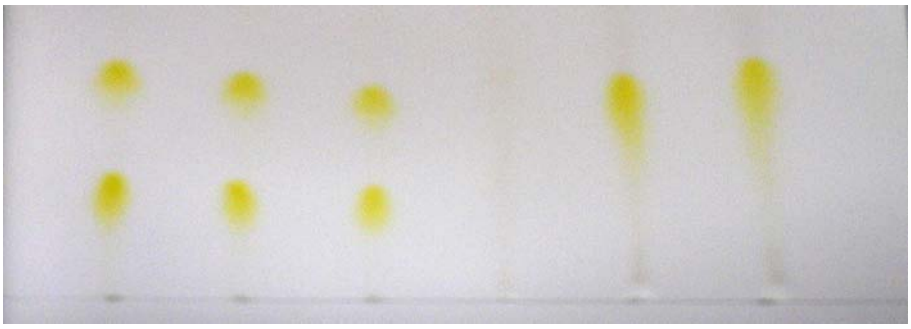
Table 2 Rhamnolipid fermentation by engineered *E. coli* in rich or minimal media

Stains	Rhamnolipid (mg/L)	
	LB plus 0.4% glucose	MS plus 0.4% glucose
E. coli BL21(DE3)	ND	ND
E. coli TnERAB	175.3	75.6

ND: none detected

Rhamnolipids characterization

Rhamnolipids, as one of the best known biosurfactants, consist of one or two units of rhamnose linked to one or two fatty acid chains with C8-C12 carbon atoms, which may or may not be saturated (Fig. 1). TLC results suggested that the isolated surface-active products from *P. aeruginosa* PEER 02 and E. coli TnERAB were composed of rhamnolipids (Fig. 4). The products were separated on TLC plates alongside a sample of a commercially available purified rhamnolipid (JBR425, Jeneil Biosurfactant Company). When these samples were visualized, the products from *P. aeruginosa* PEER02 have two spots which were similar to the commercial product. The lower spot consisted of di-rhamnolipids, while the higher spot consisted of mono-rhamnolipids. The products from *E. coli* TnERAB only have the higher spots which are mono-rhamnolipids. Thus, we also showed that RhlAB only synthezied mono-rhamnolipids. Di-rhamnolipids biosynthesis needs the presence from other gene(s). *P. aeruginosa* PEER02 can produce di-rhamnolipids because it contain gene RhlC which was confirmed by PCR and sequencing (data not shown).

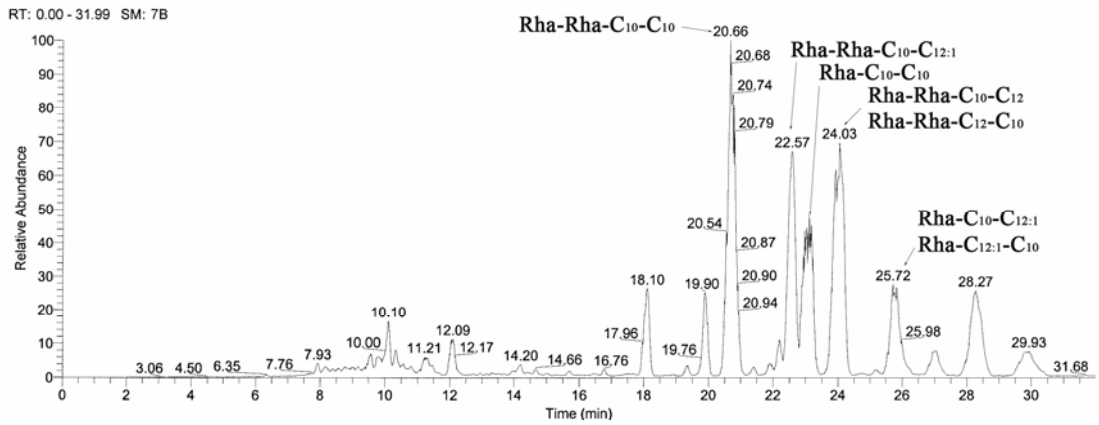


(a) (b) (c) (d) (e)

Fig 4 TLC analysis of rhamnolipids from engineered strains. (a) Commercial rhamnolipids JBR425; (b) Rhamnolipids produced by *P. aeruginosa* PEER02 with soybean oil as glucose; (c) Rhamnolipids produced by *P. aeruginosa* PEER02 with soybean oil as substrate; (d) Rhamnolipids produced by *E. coli* TnERAB in LB plus glucose media; (e) Rhamnolipids

produced by *E. coli* TnERAB in MS plus glucose media; (10 μL sample of 500mg/L for each lane).

Then, the products from our engineered strains were next submitted to HPLC/MS analysis to further confirm the presence of rhmanolipids. Rhamnolipids in crude fermentative broth was precipitated after acidifying with concentrated HCl, and extracted with chloroform:ethanol (2:1,v/v). Organic phase was collected and air dried. After drying, the yellow oil paste was suspended in water. The sample was ready for LC-MS analysis as well as for IFT analysis. Rhamnolipid structural information was obtained through the use of mass detector equipment with electrospray ionization (ESI) probe. Figure 5 presents the base peak ion intensity chromatograms for the rhamnolipid samples produced by our engineered strains with soybean oil or glucose as carbon source. The profiles of these chromatograms differ mostly in the late eluting region which is composed mainly of rhamnolipids containing only one fatty acid moiety. The proportions of the various rhamnolipids listed in Table 3 were obtained from the relative intensities of their corresponding pseudomolecular ions. For rhamnolipids of the same molecular weight which were chromatographically resolved, this was simply performed by integration of the intensities of their pseudomolecular ion. For isomers that were not chromatographically resolved, their relative proportion was determined by the relative intensities of different fragments ions produced by cleavage of the two molecules at the same position. For example, the rhamnolipid Rha-C8-C10 was not sufficiently separated from Rha-C10-C8 to allow direct quantification on the basis of the intensity of their pseudomolecular ion at m/z 475. However, their mass spectra differ by the presence of an m/z 305 ion for the former and an m/z ion 333 for the latter. These two ions arise from cleavage at the 3-carbon-oxygen bond in both molecules. The relative intensities of the two isomers were calculated by measuring the relative intensities of both ions, in an averaged spectrum obtained from all the spectra presenting the proper pseudomolecular ion, and multiplying these two values with the intensity of their common pseudomolecular. This method allows the analysis of very closely related rhamnolipids without resorting to long and difficult chromatographic separation.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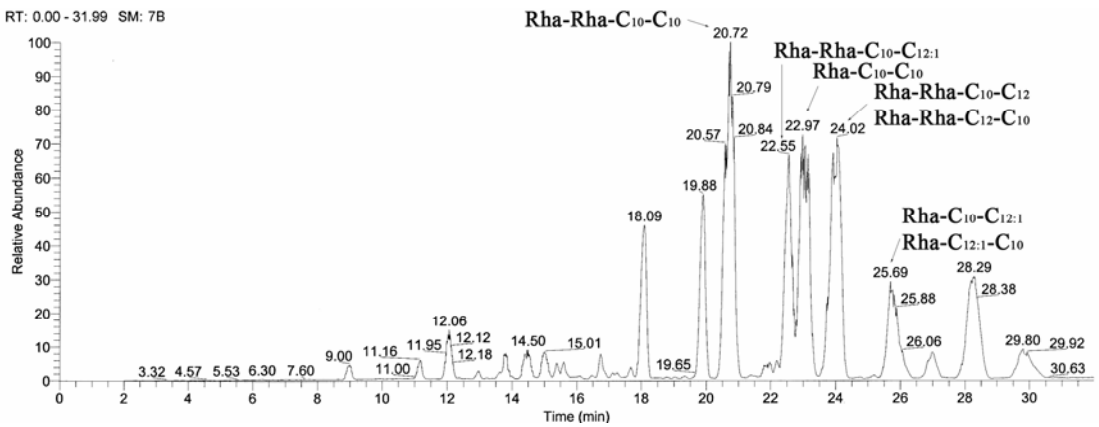
Rha-Rha-C10-C10
Rha-Rha-C10-C12:1
Rha-C10-C10
Rha-Rha-C10-C12
Rha-Rha-C12-C10
Rha-C10-C12:1
Rha-C12:1-C10
Relative Abundance
Time (min)

(a)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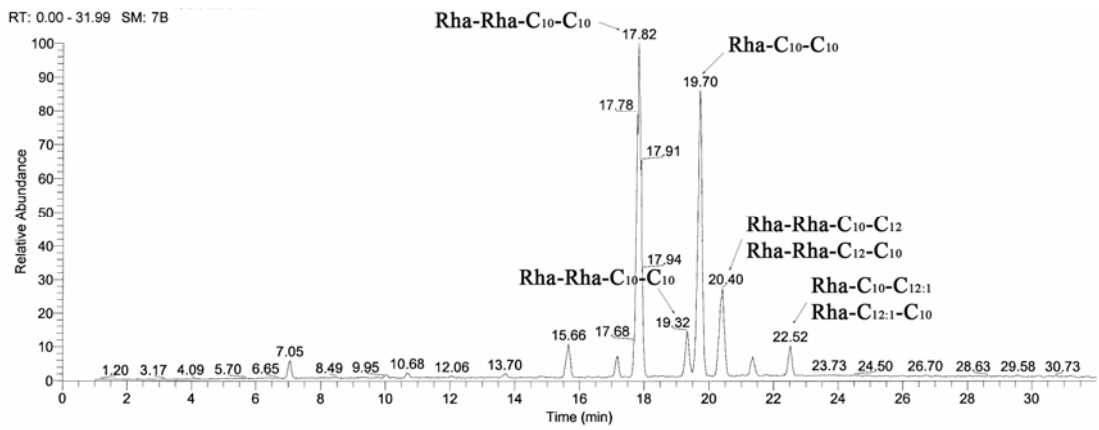
Rha-Rha-C10-C10
Rha-Rha-C10-C12:1
Rha-C10-C10
Rha-Rha-C10-C12
Rha-Rha-C12-C10
Rha-C10-C12:1
Rha-C12:1-C10
Relative Abundance
Time (min)

(b)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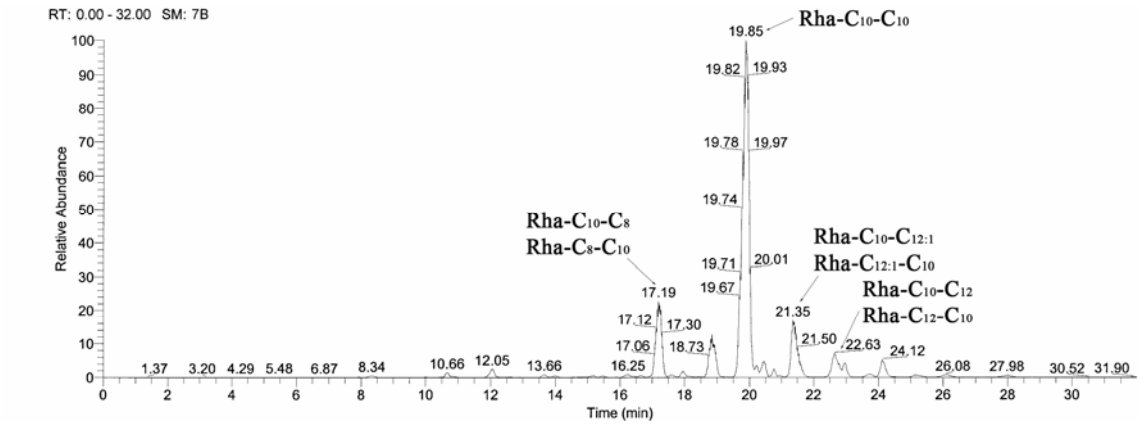
Rha-C10-C10
Rha-Rha-C10-C12
Rha-Rha-C12-C10
Rha-Rha-C10-C10
Rha-C10-C12:1
Rha-C12:1-C10
Relative Abundance
Time (min)

(c)



(d)

Fig. 5. Base peak ion intensity chromatograms of rhamnolipids from various sources.

(a) rhamnolipids produced from minimal media supplemented soybean oil by *P. aeruginosa* PEER02; (b) rhamnolipids produced from minimal media supplemented glucose by *P. aeruginosa* PEER02; (c) commercial rhamnolipids JBR425; (d) rhamnolipids produced from minimal media supplemented glucose by *E. coli* TnERAB;

In the ion intensity chromatograms (Fig. 5), the main structural composition of rhamnolipids from each samples were labeled. For the samples from *E. coli*, the main composition is Rha-C10-C10 which almost occupied 40~60% of total amount according the samples from different media. The main compositions of the samples from engineered *P. aeruginosa* also have C10-C10 carbon chain: Rha-C10-C10 and Rha-Rha-C10-C10. The percentage of these compositions is more than 30%. Another abundant structure composition of rhamnolipids from P. aeruginosa is Rha-Rha-C10-C12 or Rha-Rha-C12-C10. They have same pseudomolecular ion and total amount is more than 10%.

Table 3. Structure and relative abundance of the rhamnolipids from various sources

Structure	Pseudomolecular Ion (m/z)	Relative abundance (%)			
		JBR425	PEER02/G	PEER02/S	TnERAB
Rha-Rha-C_8-C_{10}	621	2.06	2.53	3.77	ND
Rha-Rha-C_{10}-C_8	621	1.37	3.51	0.30	ND
Rha-C_8-C_{10}	475	1.41	4.73	ND	5.65
Rha-C_{10}-C_8	475	0.52	2.21	3.35	5.65
Rha-Rha-C_{10}-C_{10}	649	36.45	19.37	20.20	ND
Rha-Rha-C_{10}-$C_{12:1}$	675	4.08	9.70	13.24	ND
Rha-Rha- $C_{12:1}$-C_{10}	675	ND*	ND	ND	ND
Rha-C_{10}-C_{10}	503	28.13	15.33	12.07	57.08
Rha-Rha-C_{10}-C_{12}	677	8.13	10.37	11.59	ND
Rha-Rha-C_{12}-C_{10}	677	2.18	5.76	6.62	ND
Rha-C10- $C_{12:1}$	529	1.15	0.37	6.33	0.14
Rha- $C_{12:1}$-C_{10}	529	0.78	5.25	ND	9.05
Rha-Rha- $C_{12:1}$-C_{12}	703	ND	ND	ND	ND
Rha-Rha-C_{12}- $C_{12:1}$	703	ND	0.87	ND	ND
Rha-Rha-C_{10}-$C_{14:1}$	703	ND	0.16	1.07	ND
Rha-Rha-$C_{14:1}$-C_{10}	703	ND	0.44	0.68	ND
Rha-C_{10}-C_{12}	531	2.15	4.46	3.96	1.50
Rha-C_{12}-C_{10}	531	0.42	2.35	2.64	2.25
Rha-Rha-C_{12}-C_{12}	705	ND	2.15	2.14	ND

JBR425: commercial rhamnolipids; PEER02/G: rhamnolipids from *P. aeruginosa* PEER02 with glucose as substrate in MS media; PEER02/S: rhamnolipids from *P. aeruginosa* PEER02 with soybean oil as substrate in MS media; TnERAB: rhamnolipids from *E. coli* TnERAB with glucose as substrate in MS media and IPTG induction; * ND: none detected

Interfacial tension (IFT) analysis of Rhamnolipids

Like their chemical counterpart, biosurfactants also reduce interfacial tension between oil and water, thus decreasing the energy required to extract trapped oil in the porous matrix and displace this into the mobile liquid phase. But under what conditions can biosurfactants play a significant role in this process? How does one know when to expect biosurfactants to be a significant factor in some system under investigation? Here, rhamnolipids produced by *P. aeruginosa* PEER02 with soybean oil as carbon source was used to examine one of interfacial phenomena of biosurfactants - how rhamnolipids reduce interfacial tension and the effects of altering salt concentration, pH and temperature on rhamnolipids activity.

First, we investigated the effects of different concentrations of rhamnolipids on IFT to find a suitable concentration for analyzing IFT of rhamnolipid in various conditions. All of these tests used n-octane as the hydrocarbon phase. As shown in Fig. 6a, the IFT of rhamnolipid solution rapidly decreases with an increase of rhamnolipid concentration. When the concentration exceeds

the critical micelle concentration (CMC), the change of IFT becomes small. According to the results of Fig 6a, the CMC of our product is about 75mg/L. Hence, we selected 100ppm rhamnolipids for our subsequent IFT condition tests.

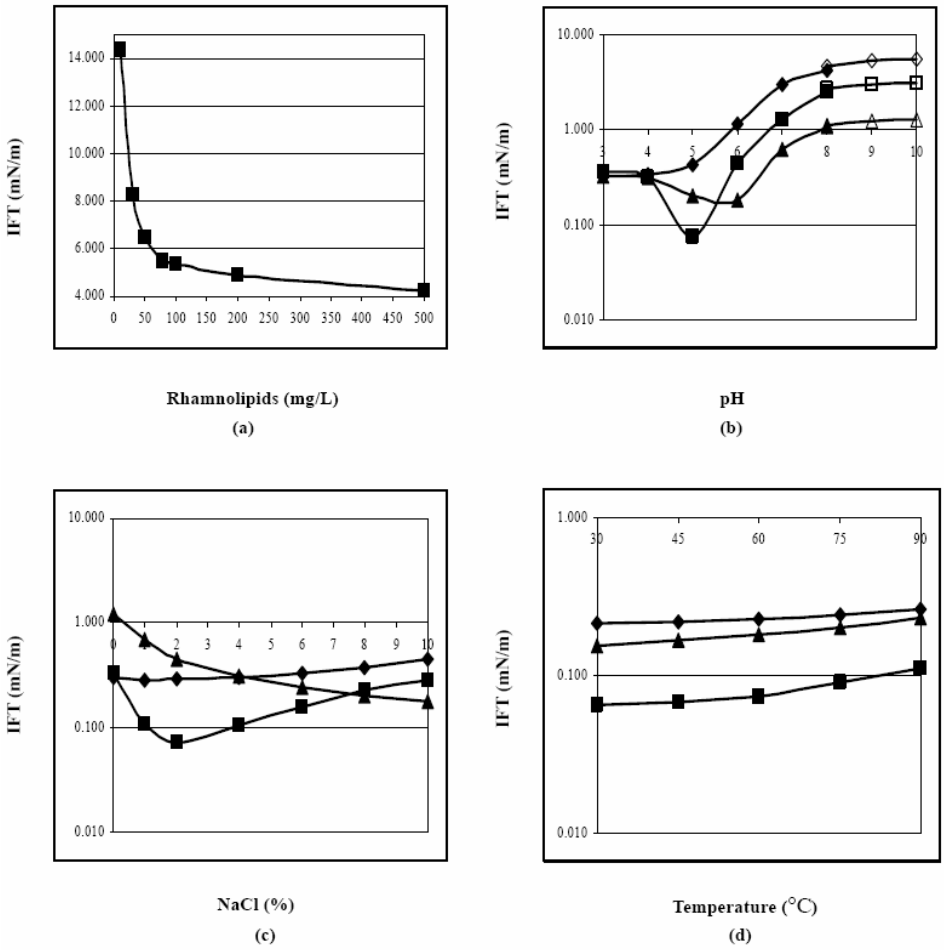


Fig. 6 IFT analysis of PEER02 rhamnolipids in various conditions. (a) Profile of IFT of different concentration of rhamnolipids in water; (b) Effects of Added Salinity, IFT of rhamnolipids, Diamonds - 1% NaCl, Squares – 2% NaCl and Triangles – 8% NaCl; (c) Effects of pH on IFT of rhamnolipids, Triangles – pH4, Squares – pH5 and Diamonds - pH6; (d) Effects of temperature on IFT of rhamnolipids, Diamonds – 25 C, Squares – 50 C, Triangles - 90 C.

From Fig 6b, we know that in the absence of additional salinity, rhamnolipids showed low IFT in the low pH buffer system, and in the range of pH 3-5, rhamnolipids showed the lowest IFT of about 0.3. If there is any additional salinity, the IFT profile is sensitive to pH. For example, when 2% NaCl was added into rhamnolipids with pH5, the IFT of this solution decrease dramatically, almost 5 fold. However, with the increase of salinity, not only the pH with lowest IFT will increase, but the

absolute value of IFT also increases. In addition, IFT profile of rhamnolipid solution with same pH but different salinity will be greatly different. For pH6, the IFT will decrease with the increase of salinity. But for pH4, the behavior is the reverse. Increasing salinity resulted in IFT increase in the range of 0~10% NaCl. However, in pH5, there is an optimal salinity. Overall, adding 2% NaCl to the pH5 buffer shows the lowest IFT (< 0.01 dyne/cm) for this rhamnolipid product. (Fig 6c).

Temperature is an important parameter for oil reservoir. Hence, temperature performance of rhamnolipid is indispensable when evaluating a product if you want to use it for enhancing oil recovery. Our experiments showed that IFT only changed slightly (increased slightly) in the range of 30-90°C (Fig 6d).

Core flooding test of PEER02 rhamnolipid for enhanced oil recovery

After an oil well loses its self flow (primary recovery by natural forces), and secondary flow (secondary recovery by pressurization using water flood and gas), approximately 2/3 of the original oil remains in the reservoir. Much of the remaining oil can be recovered by enhanced oil recovery techniques such as polymer flooding or surfactant flooding. Effective emulsifying surfactants and a better understanding of the formation of their emulsions with the crude oil are of paramount importance to economically recover the abundant residual oil in reservoirs. Entrapment of petroleum hydrocarbon by capillary forces is a major factor that limits oil recovery. Hydrocarbon displacement can occur if interfacial tension between the hydrocarbon and aqueous phases is reduced by several orders of magnitude. Microbial-produced biosurfactants may be an economical method to recover residual hydrocarbons since they are effective at low concentration. The IFT analysis of our engineered rhamnolipids showed that they can reduce remarkably interfacial tension of oil (n-octane)/water at the certain combination of higher salinity and acidic pH. Based on these data from IFT analysis, sand-packed core flooding tests of rhamnolipids from *P. aeruginosa* with soybean oil as carbon source were performed. .

Playground Sand purchased from Home Depot was packed into a 1” diameter by 2-foot long stainless steel tube to make a sand packed core. The sand pack was evacuated and saturated with brine. The brine composition and make-up brine for the rhamnolipid surfactant solution were the same as the pH5 buffer-2% NaCl brine – the conditions for minimum IFT. The pore volume and porosity of this core were calculated, and also brine permeability of this core (approximately 50 Darcies) was measured at different flow rates. Then, the brine was displaced with oil (n-Octane) in

the core until no water (brine) comes out. The oil-saturated core was aged for 1 day at room temperature. After 1day aging, brine was injected to displace oil, and the oil production was recorded. Water injection continues until no oil comes out. Next, 3 PV of 250mg/L rhamnolipids solution was injected and then the core with rhamnolipids was shut in overnight at room temperature. On the next day, an additional 3 PV of 250mg/L rhamnolipids solution was injected, followed then by injection of 6 PV of the brine. The recovered oil was measured and oil recovery change with injected pore volume of brine/rhamnolipids solution was calculated (Fig 7a and b).

Two-stage rhamnolipids flooding gave two peaks of oil recovery (Fig. 7a). The first 3 PV of rhamnolipids injection only gave a small amount of oil recovery, with a cumulative oil recovery of about 12% (Fig 7b). Then, another 3 PV of rhamnolipids was flooded following shut-in overnight. Then, a larger peak of oil recovery was noted (Fig 7a), and by the end of this stage, the cumulative oil recovery reached 37% (Fig7b). After rhamnolipids injection and displacement by a 6 PV brine flush, the final cumulative oil recovery reached 42% (Fig7b). The profile of IFT of the effluent shows IFT decreased with oil recovery. At the point of maximum oil cut, the lowest IFT was observed. Thereafter, with lower oil cut, IFT increased, and then maintained a relatively stable level. The subsequent brine flooding made IFT of the effluent increase yet further. This is because the concentration of rhamnolipids decreased with brine flooding. Our flooding technology showed that overnight incubation of core with rhamnolipids would increase oil recovery effectively and reduce the water cut.

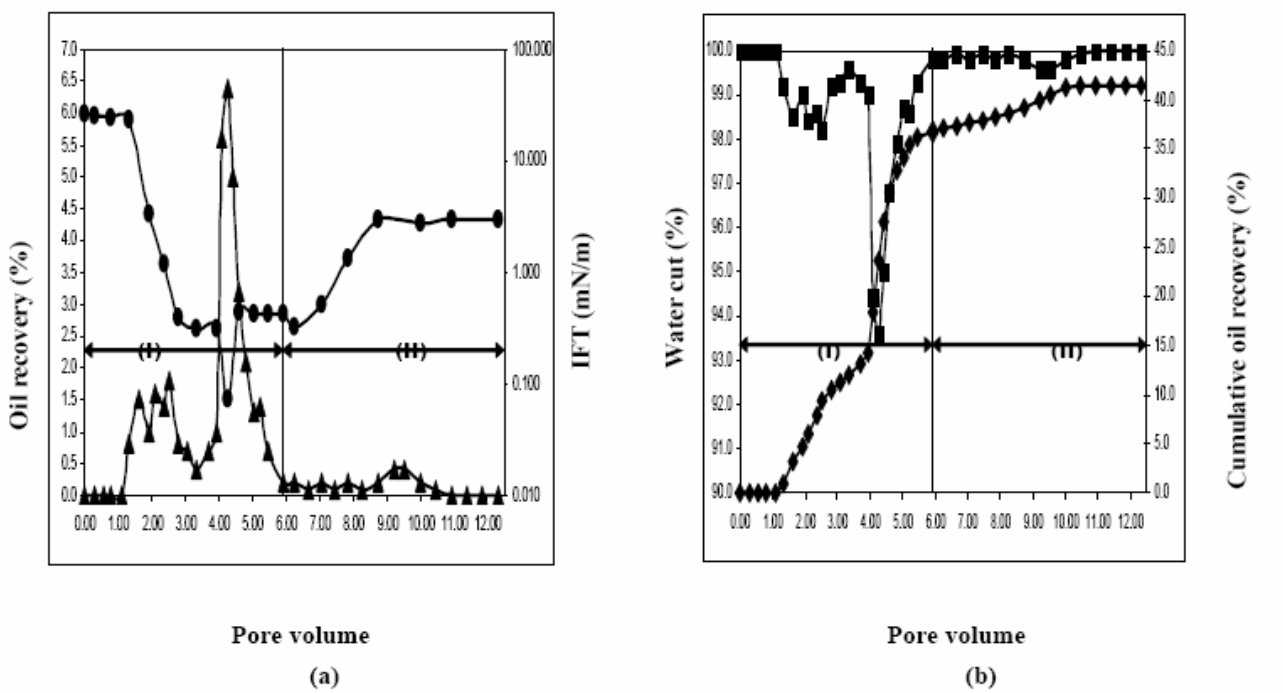


Fig. 7 Oil recovery test of sand-packed core by rhamnolipids flooding with 240 ppm of PEER02.. (a) Profile of oil recovery and IFT during flooding; (b) Water cut and cumulative oil recovery. (I) rhamnolipid flooding; (II) Brine flooding. Water cut: is the percent of water phase in the effluent.

Rhamnolipids produced by engineered strain PEER02 -- Effect of biosurfactant concentration

Concentrations of 80ppm, and 500ppm of PEER02 rhamnolipids solution also were injected in this series of sand pack oil displacement experiments. Summary results for the 240ppm case were presented above. Comparative results for different concentrations are shown in Fig 8. For the 80ppm of PEER02 rhamnolipid concentration, less than 12% of the remaining oil was recovered. At 240ppm, nearly 47% of the remaining oil was recovered. However, oil recovery was not increased further when 500ppm of rhamnolipid was used. Fig.9 shows the rhamnolipid adsorption and IFT data for the 240ppm sand rhamnolipid pack experiment.

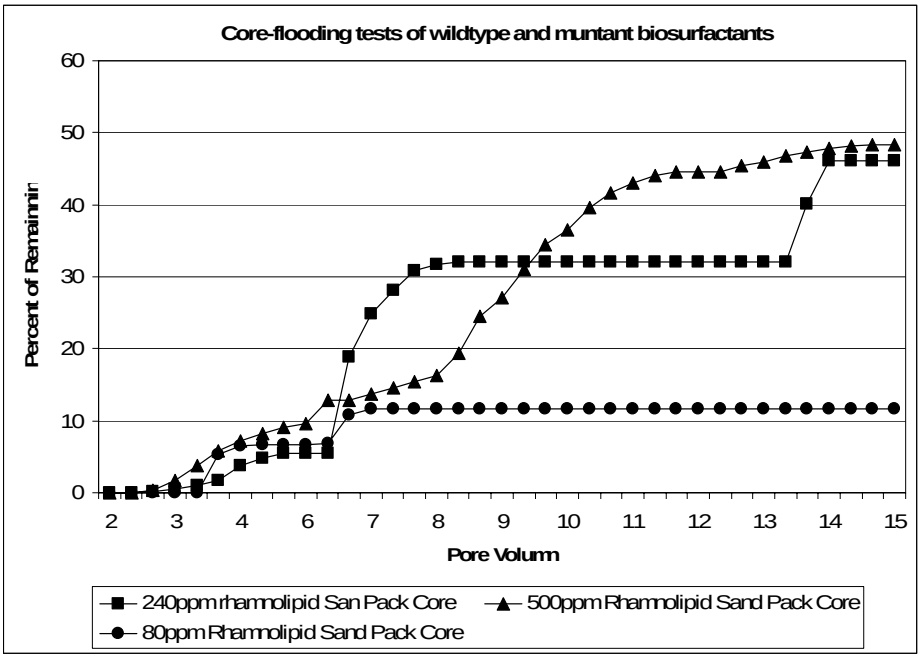


Fig 8. Sand-pack core flooding oil recovery tests of rhamnolipid biosurfactants produced by the PEER02 mutant strain.

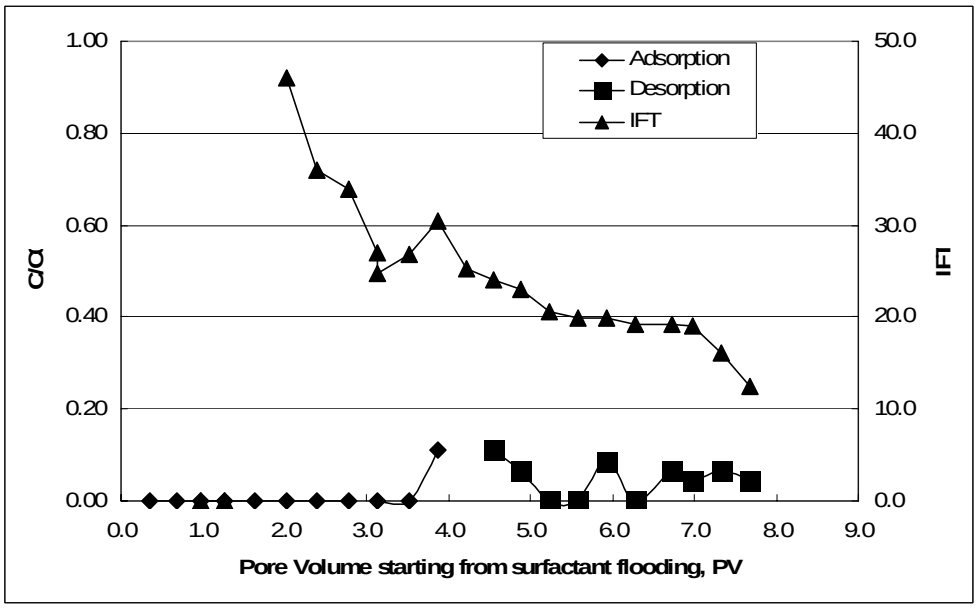


Fig. 9. Rhamnolipid adsorption in a sand-pack oil displacement test performed at 240ppm injection concentration of PEER02 rhamnolipid.

Discussion

Surface active compounds commonly used commercially usually are chemically synthesized. However, biosurfactants have received increasing attention to replace these synthetic surfactants

owing to their advantages such as biodegradability and low toxicity. Nowadays, the use of biosurfactant has been limited due to their high production cost. The main factor limiting commercialization of biosurfactants is associated with non-economical large scale production. To overcome the obstacle and to compete with synthetic surfactants, a combination of an inexpensive substrate and effective microorganism has to be developed for biosurfactant production. Agro-industrial wastes are perhaps the most promising source of substrate for biosurfactant production and can alleviate many processing industrial waste management problems. Nevertheless, biosurfactants can be produced with high yield by some microorganisms, especially *Pseudomonas* sp. These microorganisms can use various renewable resources, especially agro-industrial waters, as potential carbon sources. This would lead to the greater possibility for economical biosurfactant production and reduced pollution caused by those wasters (Maneerat, 2005). Here, with the novel biotechnique of transposome, we successfully integrated the key genes of rhamnolipids biosynthesis, RhlAB into the chromosome of *P. aeruginosa* and *E. coil* cells formerly unable to synthesize rhamnolipids and now produced rhamnolipids from these engineered strains.

The transposase is stably associated with the transposon but is inactive in the absence of Mg^{2+}. Magnesium ions present inside the bacteria activate the transposase following transformation, facilitating transposition into the bacteria chromosome. Thus, transposition is dependent simply upon activation of the enzyme and not the expression of a foreign transposase gene. The use of transposon-transposase complexes (transposome) will allow creating stable insertion mutation (Hoffman et al., 2000). Thus, stable mutants would be a great advantage for rhamnolipids production by fermentation as well as the future of microbial enhanced oil recovery.

LC/MS was very efficient for the analysis of complex rhamnolipids mixtures, in a relatively short and simple chromatographic run. If only using HPLC to characterize a rhamnolipid mixture, the sample required derivatisation with para-bromoacetophenone in order to detect the signal with UV (Schenk et al., 1995). Also HPLC could not provide structural information on the various compounds separated. Conversely, mass spectrometry gave useful structural information and allowed the quantification of chromatographically unresolved molecules, For example the very similar and co-eluting Rhal-C8-C10 and Rha-C10-C8 were easily distinguished and independently quantified by simple analysis of their mass spectra. In recent years, some publications have reported that Rha-C10-C10 and Rha-Rha-C10-C10 are in fact produced as part of a complex mixture of rhamnolipids. These compounds all contain one or two rhamnose groups linked to one or two 3-hydroxy fatty acids of different chain length, which may contain one double bond. The various

combinations of these groups generate a large number of possible rhamnolipids congeners. Liquid chromatography/mass spectrometry using elctrospray ionization was used to analyze rhamnolipids produced by engineered bacteria strains with soybean oil or glucose as carbon source. Identification and quantification of dozens of different rhamnolipids congeners was accomplished using a reverse-phase C18 column and a 30 min chromatographic run. Isomeric rhamnolipids that were not chromatographically resolved could be identified by interpretation of their mass spectra and their relative proportions estimated. The most abundant rhamnolipid produced by engineered P. aeruginosa PEER02 on soybean oil or glucose contained two rhamnoses and two 3-hydroxydecanoic acid groups or one 3-hydroxydecanoic acid group and one 3- hydroxydodecanoic acid group. The relative abundance of these two congeners all about 30%. However, in the commercial product of JBR245 from Jeneil Biosurfactant Company, the most abundant rhamnolipid was two rhamnoses and two 3-hydroxydecanoic acid groups. Its relative abundance is about 40%. Compared with commercial rhamnolipid, the percentage of longer carbon chain composition of engineered rhamnolipids from *P. aeruginosa* PEER02 increased. That's why our rhamnolipids showed better performance in IFT than that of commercial one (Jiang et al., 2004). In the engineered rhamnolipids from *E. coli*, the most abundant rhamnolipid contained one rhamnose and two 3-hydroxydecanoic acid groups. Its ratio is almost 50%. The rhamnolipids with the longest fatty acid side chain further from the rhamnose group tended to have slightly shorter retention time and were also produced in larger amounts than their counterparts. Table 3 clearly shows that the rhamnolipids produced by engineered bacteria differ both in quantity and in structure depending on the carbon source and species.

Substantial work has been carried out on the production and characterization of rhamnolipids. Here, we have systematically analyzed the interfacial activity of rhamnolipids from engineered *P. aeruginosa* with soybean oil as substrate. Supernatant containing rhamnolipids, produced from soybean oil was characterized under environmental parameters to better define appropriate conditions for its use as an agent for enhanced oil recovery (Patel and Desai, 1997). Owing to the amphiphilic nature of biosurfactants, not only hydrophobic but a range of interactions are involved in the possible adsorption of charged biosurfactants to interfaces.

Most natural interfaces have an overall negative or, rarely, positive charge. Thus, the ionic conditions and the pH are important parameters if interactions of ionic biosurfactants with interfaces are to be investigated. A fundamental characteristic of surfactants is their tendency to adsorb at interfaces in an oriented fashion. In comparing the performance of surfactants in an

interface, it is usually necessary to distinguish between the amount of surfactant required to produce a given amount of change in phenomenon under investigation and the maximum change in the phenomenon that the surfactant can produce, regardless of the amount used. The former parameter is the efficiency of the surfactant, the latter is effectiveness. These two parameters do not necessarily run parallel to each other- in fact, in many cases they run counter to each other (Rosen, 2004).

pH change usually causes marked changes in the adsorption of ionic surfactants onto charged solid substrates. As the pH of the aqueous phase is lowered; a solid surface will usually become more positive, or less negative, because of adsorption onto charged sites of proton from the solution, with consequent increase in the adsorption of anionic surfactants and decrease in the adsorption of cationics. The reverse is true when the pH of the aqueous phase is raised. In these cases, change in the pH may convert the surfactant from one containing an ionic group capable of strong adsorption onto oppositely charged sites on the adsorbent to a neutral molecule capable of adsorption only through hydrogen bonding or dispersion forces. Addition of neutral electrolyte, such as NaCl, causes a decrease in the adsorption of ionic surfactants onto an oppositely charged adsorbent and an increase in their adsorption onto a similarly charged adsorbent. These effects are presumably due to the decreased attraction between oppositely charges species and decreased repulsion between similarly charged species at higher ionic strength. Both efficiency and effectiveness of adsorption of ionic surfactants onto similarly charged substrates are increased by an increase in the ionic strength of the aqueous phase. Temperature increase generally causes a decrease in the efficiency and effectiveness of adsorption of ionic surfactants, the change being relatively small compared to that caused by pH change. Our results (Fig7d) showed similar performance at the range of 30-90°C.

Oil is an essential energy source that drives industrialization and continued economic growth. Current production technologies recover only about one-third to one-half of the oil contained in the reservoirs. New technologies that can recover even a part of the entrapped oil offer timely and cost-effective solutions. Thus, the long-term economic potential for enhanced oil recovery is great (Miller et al., 1991). Development of a simple biosurfactant based microbial enhanced oil recovery method can be beneficial for small independent operators. These operators operate low cost water floods and other recovery projects where low oil flow rates are accompanied by high water cut. A small improvement in oil production will increase profitability. Microbial enhanced oil recovery using biosurfactants can provide a cheap and simple tertiary oil recovery method. Biosurfactants can be generated within oil reservoirs by bacteria that grown in saline and anaerobic conditions.

This ability to generate biosurfactants within oil reservoirs might help overcome biosurfactant adsorption during flow through reservoir rocks and the resultant degradation that results in low biosurfactant concentrations. Our stable mutants might offer a marked improvement in microbial enhanced oil recovery.

3.3 Rhamnolipids production and characterization from various substrates by engineered *Pseudomona aeruginosa*

ABSTRACT

Rhamnolipids biosufractants that have been studied extensively. Mainly, they are biosynthezied from *P. aeruginosa* stains. With transoposome-mediated chromosome integration, a novel rhamnolipids producing strain, *P. aeruginosa* PEER02 was constructed. With different carbon sources as substrate, this enineered strain can produce rhamnolipids with different yield in the minimal media. Interfacial tension analysis (IFT) showed that different rhamnolipids from different substrates gave different performance. Those rhamnolipids with plant oil as substrate showed as low IFT as 0.05mN/m in the buffer solution with pH5.0 and 2% NaCl. Fed-batch fermentation with soybean oil as substrate greatly enhanced the production of rhamnolipids, and the yield reached about 25g/L.These results showed some potentials for producing high-performance rhamnolipids with high yield from low-cost renewable resources.

INTRODUCTION

Rhamnolipids, as one of main type of biosurfactants, have advantages over their chemicals counterparts because they are biodegradable, have low toxicity, are effective at extreme temperatures or pH values (Cameotra and Makkar, 1998; Liang et al., 1999) and show better environmental compatibility (Georgiou *et al.*, 1990). Nevertheless, from an economic standpoint, biosurfactants are not yet competitive with the synthetics. Rhamnolipids can only replace synthetic surfactants if the cost of the raw material and the process costs are reduced. So far, several renewable substrates from various sources, especially from industrial wastes have been intensively studied for microorganism cultivation and surfactant production at an experimental scale. Here we make efforts to use alternate low-cost substrates to facilitate industrial development of rhamnolipids production by engineered *Pseudomonas aeruginosa* which was constructed in our lab.

MATERIALS AND METHODS

Bacterial strain, media and chemicals

P. aeroginosa PEER02 (see Section 3.2) was used for rhamnolipids fermentation

Fermentation media and conditions

Nutrient broth from BD (Franklin Lakes, NJ) was used for seed culture of *P. aeruginosa*. The mineral salts medium and a 0.4% or 2% glucose or 2% soybean oil as carbon source was as rhamnolipd fermentation media. The basal mineral salts medium contained (g/L): $NaNO_3$, 15; KCl, 1.1; NaCl, 1.1; $FeSO_4.7H_2O$, 0.00028; KH_2PO_4, 3.4; K_2HPO_4, 4.4; $MgSO_4.7H_2O$, 0.5; yeast extract 0.5; and 5 ml of a trace elements solution containing (g/L): $ZnSO_4.7H_2O$, 0.29; $CaCl_2.4H_2O$, 0.24; $CuSO_4$. $5H_2O$, 0.25; $MnSO_4.H_2O$, 0.17 (Lindhardt et al, 1989). The trace element solution was filter-sterilized through a 0.22-µm membrane filter (Millipore, type GS) and then added to the medium, which had been autoclaved and allowed to cool. *P. aeruginosa* was first grown in nutrient broth for 24h at 30ºC with shaking and then diluted 1:10 into mineral salts medium and 2% different substrates and incubated for 4 days. Incubation was carried out in 250mL Erlenmeyer flasks with 25mL medium and at 30ºC with orbital shaking at 250rpm. For fed-batch experiments, mineral salts medium plus 2 or 4% soybean oil was as initial medium and different amounts of soybean oil was supplemented with fermentation time.

Analytical method for rhamnolipids

Rhamnolipids were quantified in triplicate by weight and by the colorimetric determination of sugars with orcinol (Candrasekaran and Bemiller 1980). Rhamnolipids were purified by first separating the cells from supernatant by centrifugation (10000 ×g). The supernatant was then extracted with chloroform and ethanol. 0.5mL rhamnolipid sample was extracted with 1mL chloroform:ethanol (2:1, v/v). The organic phase was evaporated to dryness and 0.5mL of H_2O was added. To 0.1mL of each sample with suitable dilution, 0.9mL of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added. After heating for 30min at 80ºC the samples were cooled at room temperature and the OD_{421} was measured. The rhamnolipid concentrations were calculated from standard curves prepared with L-rhamnose (0-50mg/L).

Rhamnolipids purification and LC-MS analysis

The rhamnolipids were precipitated by acidifying culture supernatant to pH 2 with concentrated HCl and kept at 4°C overnight, and recovered by centrifugation at 10000g for 1h and dissolved in deionized water. Two volumes of chloroform:ethanol (2:1, v/v) was added to rhamnolipids solution and shaken 30 min for extraction. The organic phase was evaporated to dryness, and the rhamnolipid residue was then dissolved to 500mg/l final concentration. The HPLC-MS analyses were performed with a Gemini C18 column (3 mm x 100 mm, 5μm particle) from Phenomenex and a ThermoFinnigan LCQ Classic ion trap mass spectrometer. The LC gradient was started at 8%B and holding for 1 minute, then ramp to 75%B in 20 minutes, hold at 75%B for 10 minutes, back to 8%B in 1 minute and hold at 8%B for 5 minutes. Solvent B is 10:90 water:acetonitrile with 0.1% acetic acid. Solvent A is 98:2 (v/v) water:acetonitrile with 0.1% acetic acid. The mass spectrometer was operated in the negative ion mode scanning 250-950 m/z range. Data dependent MS/MS was set to perform MS/MS of the more abundant ions in the mass range.

Interfacial tension analysis of Rhamnolipids

The IFT was determined by using a spinning drop tensiometer (Temco Inc.) as detailed by Cayais (1975). Aqueous rhamnolipid solution was loaded into glass tube (Inner diameter, 2mm and Outer diameter, 5mm), followed by injection of 1.5μL octane. The glass tube with solution was spun in the tensiometer, and IFT was determined from the octane drop geometry. After 10min-spinning, the data were collected for analysis. In these studies, we measured the "fresh" IFT values without pre-mixing rhamnolipid solution and octane together to reach phase equilibrium. To adjust pH of rhamnolipid solution, 50mM citrate-Na_2HPO_4 buffer was used.

RESULTS AND DISCUSSIONS

Current status of rhamnolipid production (Literature review – Table 1)

Table 1 Production of rhamnolipids by *Pseudomonas* strains using different substrates as reported in the literature

Strain	Carbon source (g/L)	Rhamnolipids (g/L)	$Y_{P/S}$	Fermentation time (h)
P. aeruginosa PTCC1637	Glucose (60)	12.5	0.208	150
P. aeruginosa DSM2659	Glucose(18.2)	1.5	0.082	144
P. aeruginosa BS2	Sucrose (20)	1.85	0.093	44
P. aeruginosa GL1	Glycerol (30)	5.8	0.193	150
P. aeruginosa PA1	Glycerol (30)	9.5	0.316	168
P. aeruginosa BS2	Whey(20)	1.78	0.089	44
P. aeruginosa GS3	Molasses (70)	0.72	0.011	96
Pseudomonas sp. BOP100	Ethanol (30)	3.0	0.100	120
P. aeruginosa IFO 3924	Ethanol (70)	32.0	0.457	168
P. aeruginosa DSM 7108	Soybean oil(125)	78.0	0.624	167
P. aeruginosa DSM 7101	Soybeanl oil (160)	112.0	0.687	264
P. aeruginosa DS10-129	Soybean oil (6)	4.3	0.716	288
P. aeruginosa UI29791	Corn oil (75)	46.0	0.613	192
P. aeruginosa 47T2	Frying oil (40)	8.1	0.203	80
P. aeruginosa 44T1	Olive oil(20)	10.0	0.500	110
Pseudomonas sp. JAMM	Olive oil mill (24)	1.4	0.058	150
P. aeruginosa UW-1	Canola oil(60)	24.3	0.405	216
P. aeruginosa LBI	Soapstock(35)	15.9	0.454	54
P. aeruginosa DSM2874	Rapwseed oil(90)	45.0	0.500	384
P. aeruginosa KY4025	n-paraffin (90)	2.0	0.067	144
Pseudomonas sp.	n-paraffin (50)	14.0	0.280	120
Pseudomonas sp. MUB	n-C14/15(20)	2.9	0.145	48
P. aeruginosa DSM2874	n-C14/15(80)	12.8	0.160	180

Rhamnolipids production from various carbon sources in minimal medium

Fermentation experiments show *P. aeruginosa* PEER02 can produce rhamnolipids in minimal salts media containing various carbon sources, including glucose, fructose, glycerol, ethanol, soybean oil, corn oil, frying oil, palm oil, peanut oil, coconut oil, olive oil, grape seed oil, etc. The results of preliminary carbon source test are shown below in Table 2.

Table 2 Effect of various carbon sources on rhamnolipids production by *P. aeruginosa* PEER02 in minimal media

Substrate	Fermentation time (day)								
	2 days			4 days			6 days		
	Growth	RL (g/L)	Conversion	Growth	RL (g/L)	Conversion	Growth	RL (g/L)	Conversion
Glucose	3.86	3.277	0.164	2.32	2.532	0.127	1.73	2.379	0.119
Fructose	1.39	0.156	0.008	2.84	1.239	0.062	4.04	4.221	0.211
Xylose	0.49	0.162	0.008	0.62	0.264	0.013	0.47	0.345	0.017
Lactose	NG**	ND***	ND	NG	ND	ND	NG	ND	ND
Sucrose	NG	ND	ND	NG	ND	ND	NG	ND	ND
Maltose	NG	ND	ND	NG	ND	ND	NG	ND	ND
Soluble starch	NG	ND	ND	NG	ND	ND	NG	ND	ND
Ethanol	0.89	0.060	0.003	2.33	1.233	0.062	2.43	1.989	0.099
Glycerol	3.89	2.472	0.124	4.73	9.969	0.498	4.97	7.296	0.365
Soybean oil	4.88	5.049	0.252	4.09	7.095	0.355	1.70	3.648	0.182
Corn oil	5.21	5.112	0.256	4.22	8.166	0.408	2.10	4.797	0.240
Peanut oil	5.16	5.523	0.276	4.39	6.723	0.336	2.68	6.708	0.335
Palm oil	5.71	4.233	0.212	2.49	4.362	0.218	2.37	4.071	0.204
Coconut oil	5.71	6.099	0.305	3.82	5.448	0.272	2.23	6.363	0.318
Grapeseed oil	5.51	5.166	0.258	1.85	4.152	0.208	0.94	4.863	0.243
Olive oil	5.66	3.489	0.174	4.74	4.668	0.233	3.00	5.583	0.279
Frying oil	5.49	4.881	0.244	4.00	7.347	0.367	2.05	4.230	0.212
n-Octane	NG	ND	ND	NG	ND	ND	NG	ND	ND
n-Decane	NG	ND	ND	NG	ND	ND	NG	ND	ND
n-Dodecane	NG	ND	ND	NG	ND	ND	NG	ND	ND
n-Tetradecane	NG	ND	ND	NG	ND	ND	NG	ND	ND
n-Hexadecane	NG	ND	ND	NG	ND	ND	NG	ND	ND
Crude oil	NG	ND	ND	NG	ND	ND	NG	ND	ND
Mineral oil	NG	ND	ND	NG	ND	ND	NG	ND	ND

* For liquid substrate, the conversion is g/mL, and for solid substrate, g/g;

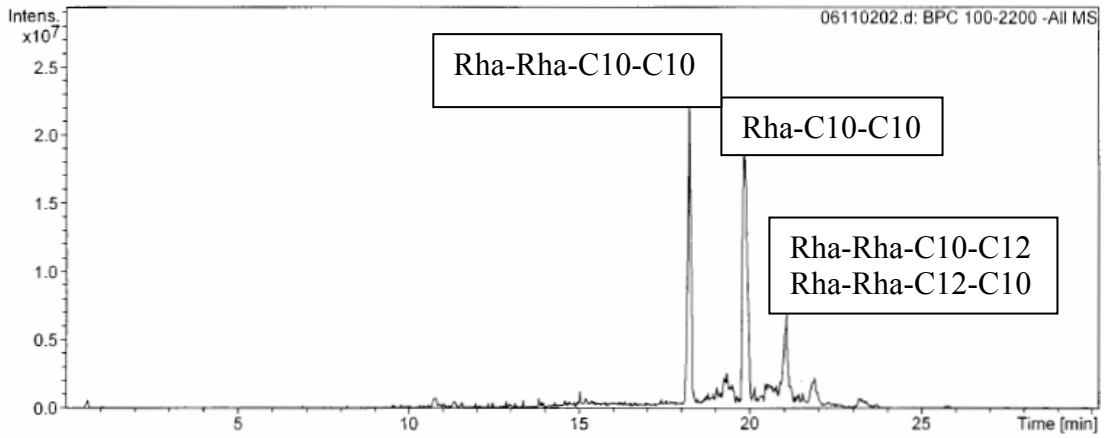
** NG: no growth; *** ND: none detected

According to Table 2, *P. aeruginosa* PEER02 can not grow in sucrose, lactose, maltose as well as all hydrocarbons tested. Thus, this engineered strain can not produce rhamnolipids with these substrates. Compared with the substrates of various plant oils, *P. aeruginosa* PEER02 produce less rhamnolipids with sugars as substrates. Of all plant oils, corn oil is the best substrate for

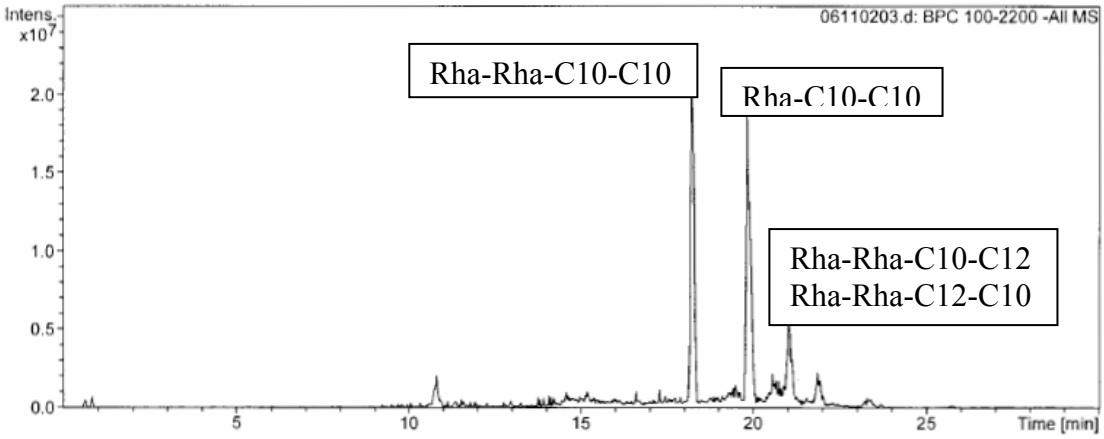
rhamnolipids production by *P. aeruginosa* PEER02. The yield is about 8g/L, and the productivity (conversion rate) is about 34%. However, in our investigation, glycerol will give the highest yield and productivity. Its yield and productivity is about 10g/L and 50%, respectively.

Rhamnolipids characterization from different substrates

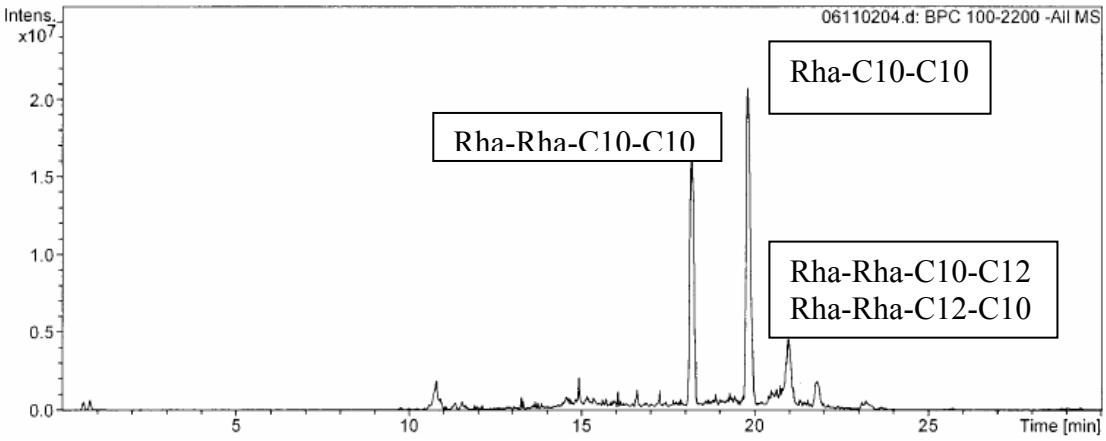
Rhamnolipids were produced with *P. aeruginosa* PEER02 from 12 different substrates, including 8 plant oil, 2 sugars, two others, glycerol and ethanol. Rhamnolipids from different substrates were purified for HPLC/MS analysis (Fig 1a-l). From the base peak ion intensity chromatograms of rhamnolipids from various sources, the main compositions of each samples and their percentage are summarized in Table 3 The purified samples were analyzed also for interfacial tension agains n-Octane (Table 4). Generally, the higher the percentage of di-rhamnolipids with longer carbon chain lengths, the lower is its IFT.



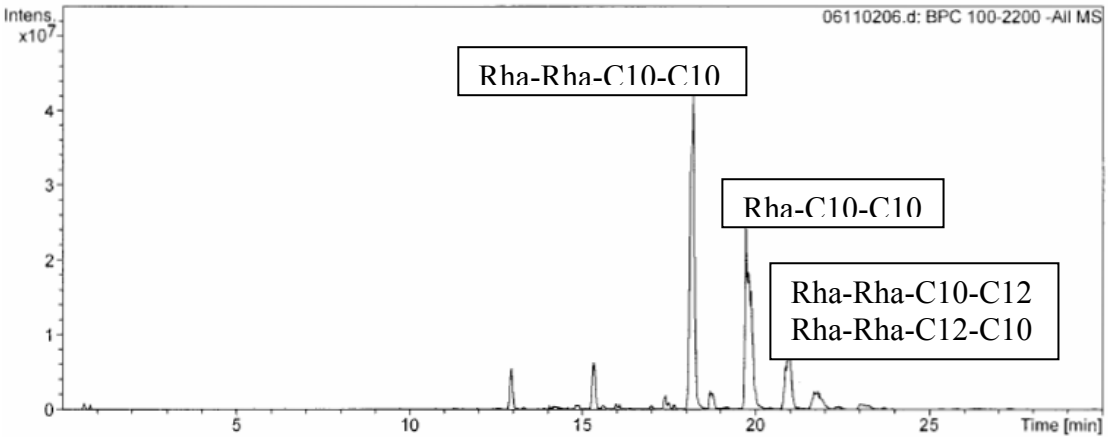
(a) Soybean oil



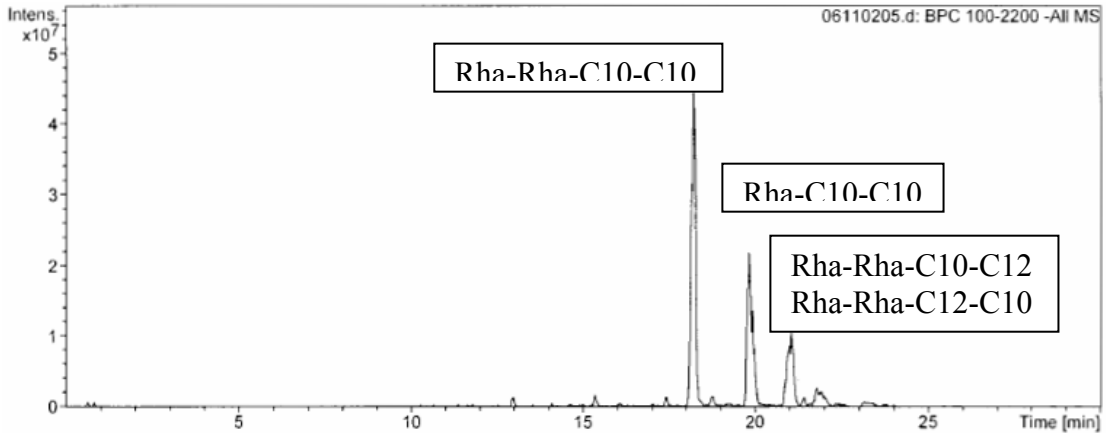
(b) Corn oil



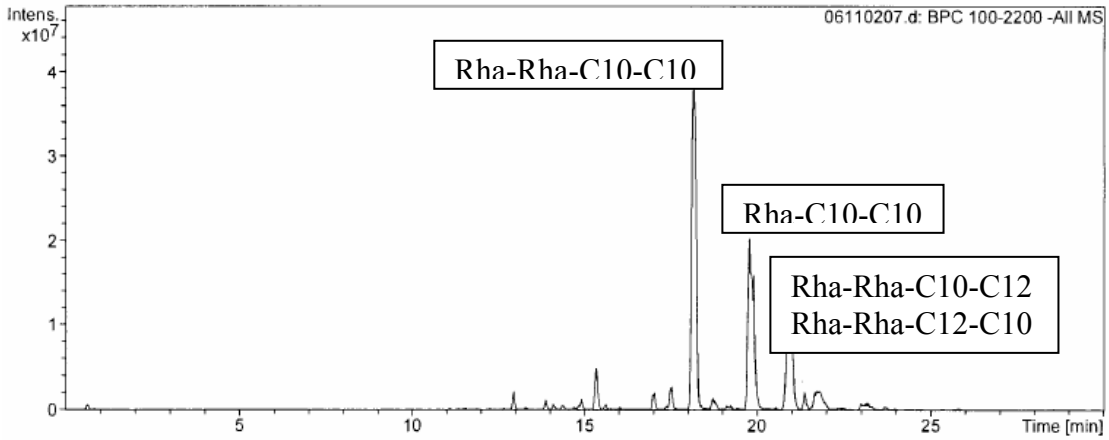
(c) Frying oil



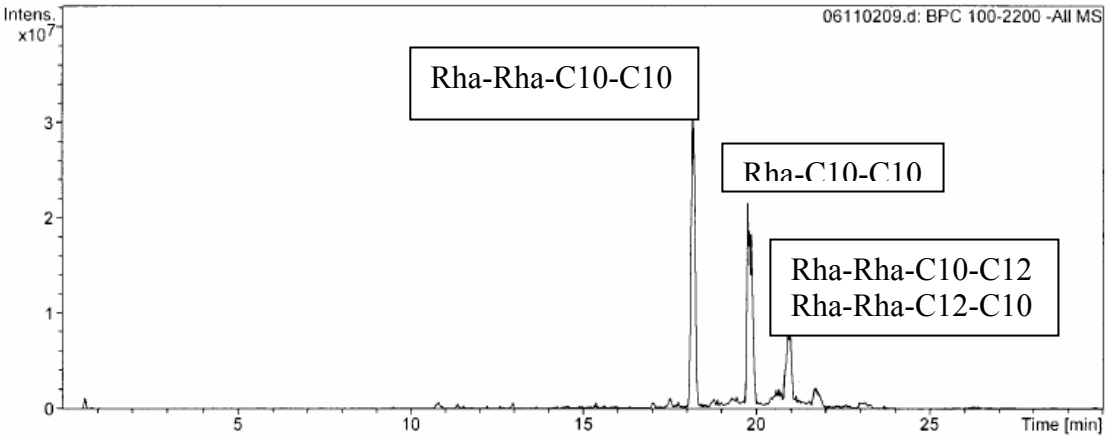
(d) Palm oil



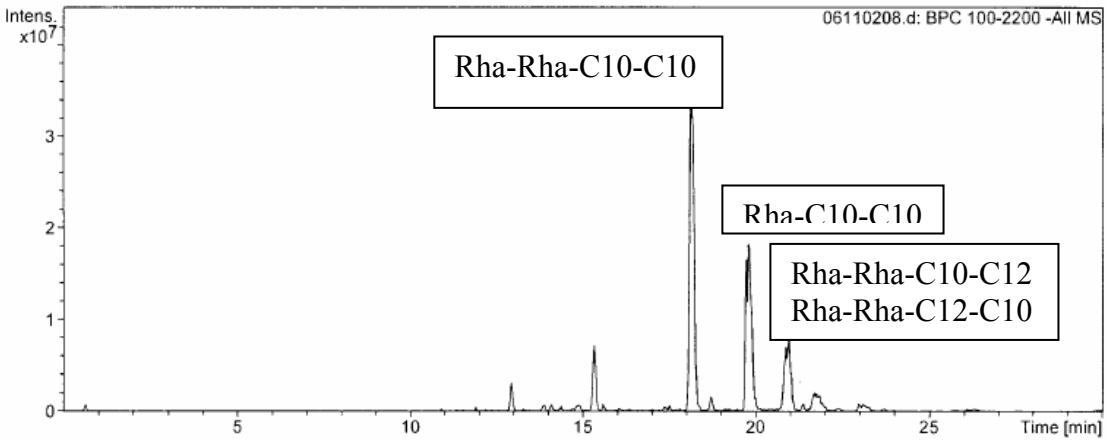
(e) Coconut oil



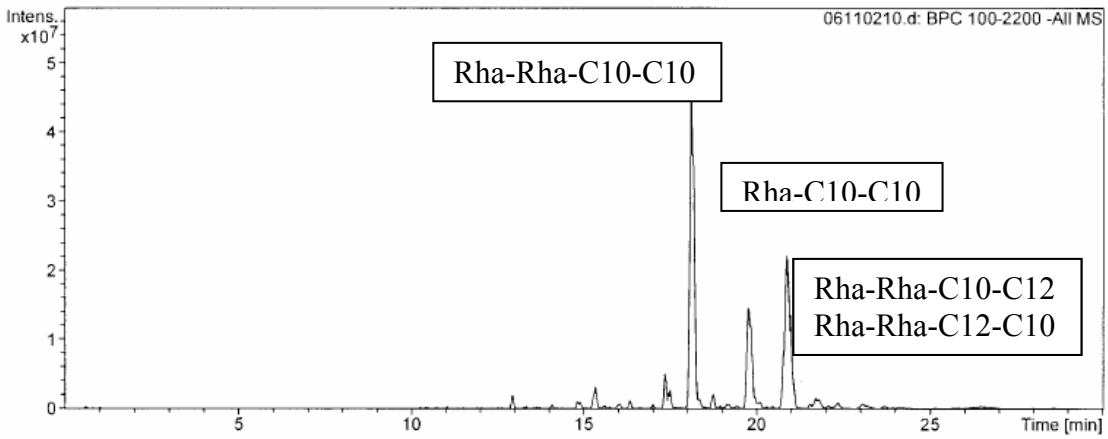
(f) Peanut oil



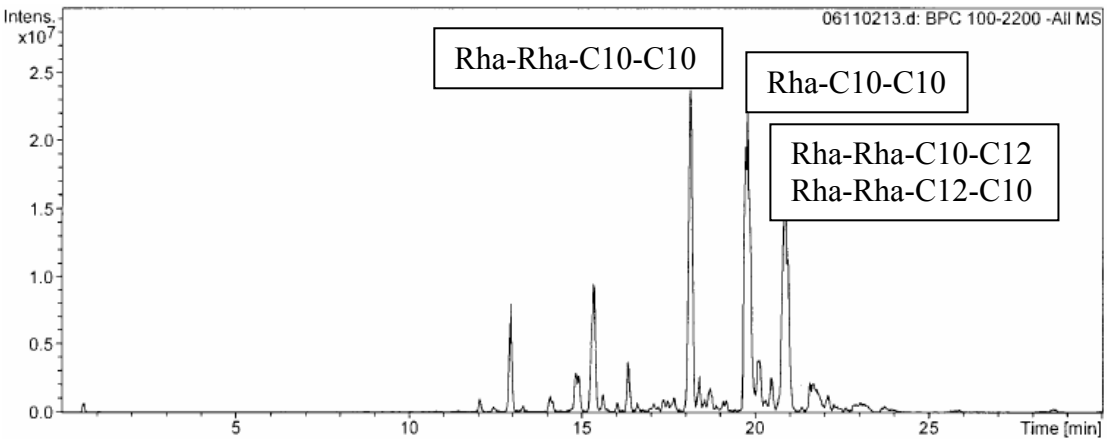
(g) Olive oil



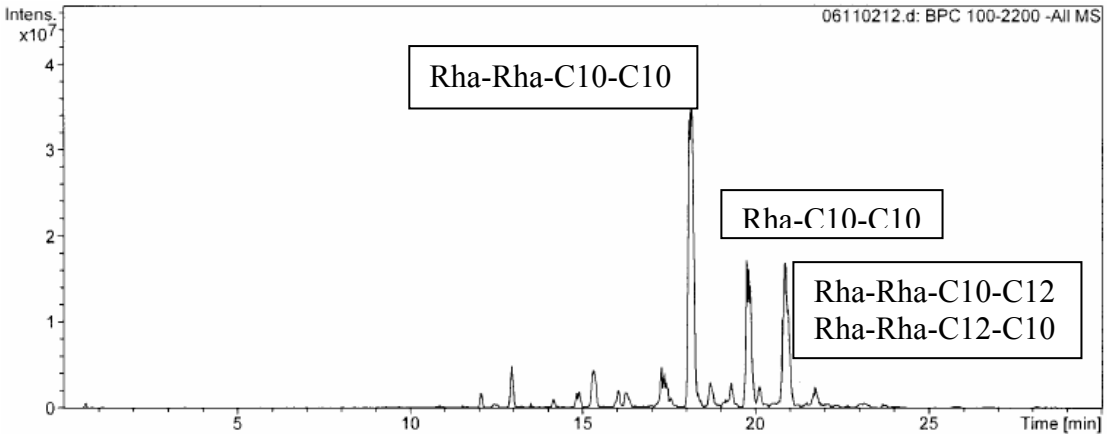
(h) Grapeseed oil



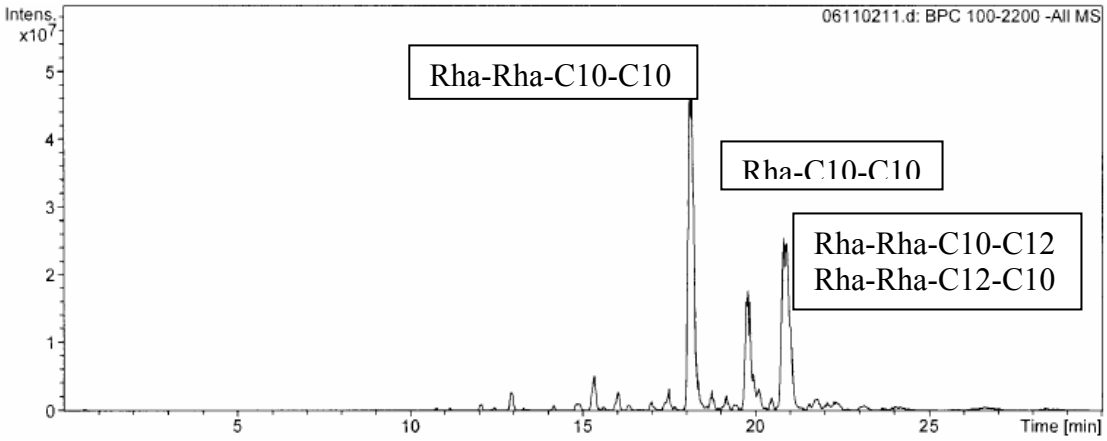
(i) Glucose



(j) Glycerol



(k) Ethanol



(l) Fructose

Fig. 1. Base peak ion intensity chromatograms of rhamnolipids from various sources. (a) soybean oil; (b) corn oil; (c) frying oil; (d) palm oil; (e) coconut oil; (f) peanut oil; (g) olive oil; (h) grape seed oil; (i) glucose; (j) glycerol; (k) ethanol; (l) fructose. The main compositions are labeled in each chromatogram.

Table 3. Structure and relative abundance of the rhamnolipids from PEER02 from various sources

No.	Substrate	Rha-Rha-C10-C10 m/z 649	Rha-C10-C10 m/z 503	Rha-Rha-C10-C12 (or C12-C10) m/z 677
1	Soybean oil	37.4	47.7	14.9
2	Corn oil	43.9	43.5	12.6
3	Frying oil	36.8	48.8	14.4
4	Palm oil	51.3	31.3	17.4
5	Coconut oil	47.7	33.6	12.4
6	Peanut oil	49.6	32.6	17.8
7	Olive oil	47.6	31.5	14.6
8	Grapeseed oil	44.3	38.5	17.2
9	Glucose	49.5	18.4	32.1
10	Glycerol	52.5	22.2	34.6
11	Ethanol	23.3	33.2	25.2
12	Fructose	47.9	17.5	24.4

Table 4 IFT analysis of Rhamnolipids produced from PEER02 from various substrates

No.	Substrate	(a) 6000rpm/30°C		(b) 3000rpm/30°C		
		mN/m @5min	mN/m @10min	mN/m @1min	mN/m @5min	mN/m @10min
1	Soybean oil	5.486	4.989	0.280	0.149	0.077
2	Corn oil	6.321	6.052	0.412	0.232	0.174
3	Frying oil	6.367	6.190	0.520	0.331	0.253
4	Palm oil	5.947	5.928	0.078	0.064	0.055
5	Coconut oil	5.292	5.233	0.033	0.130	0.012
6	Peanut oil	4.244	3.341	0.036	0.014	0.015
7	Olive oil	5.021	5.007	0.088	0.053	0.037
8	Grapeseed oil	5.050	5.012	0.181	0.111	0.061
9	Glucose	5.737	5.955	0.138	0.140	0.128
10	Glycerol	5.049	4.981	0.188	0.168	0.151
11	Ethanol	2.498	2.439	0.040	0.022	0.011
12	Fructose	5.942	4.976	0.430	0.418	0.413

(a) dilute supernatant of fermentation to 100 ppm rhamnolipid with distilled water
(b) dilute supernatant of fermentation to 100 ppm rhamnolipid in buffer pH5 and 2 wt% added NaCl

Fed-batch fermentation of Rhamnolipids production by *P. aeruginosa* PEER02

Fed-batch fermentations of rhamnolipids production starting with 2% (S2) and 4% (S4) soybean oil in the fermentative media, respectively, were investigated. For S2, an additional 2% soybean oil was added into the media every day of the first 4 days. For S4, an additional 3% soybean oil was added as a supplemental substrate on the second and fourth day, respectively. The final concentration of soybean oil was 10% for both fed-batch fermentations.

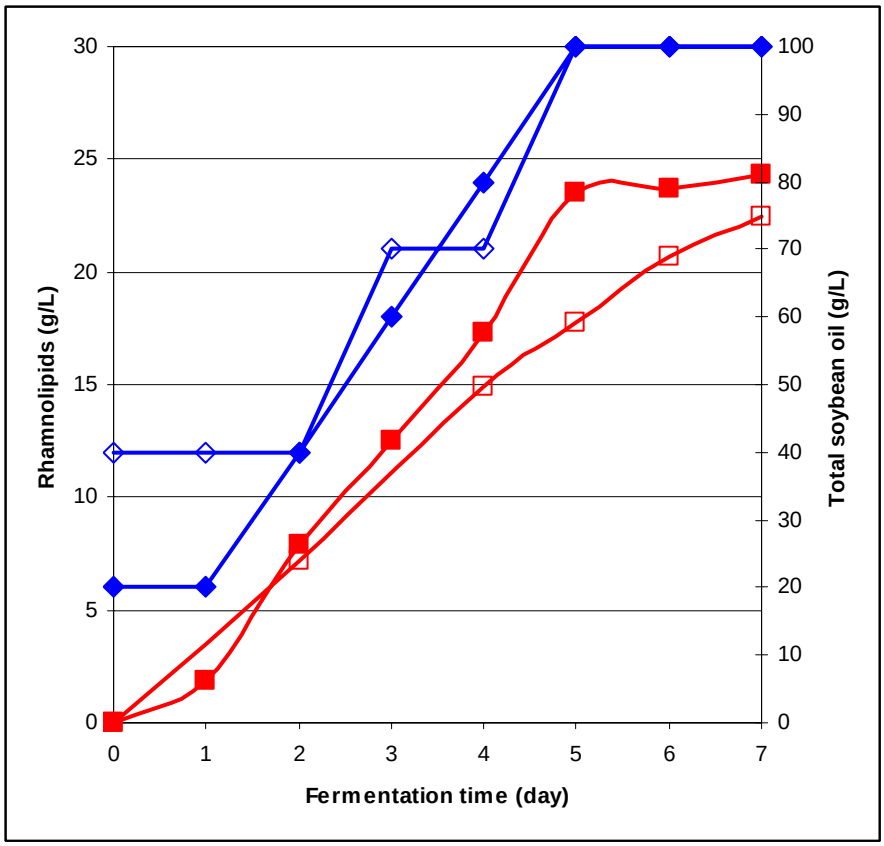


Fig. 2. Fed-batch fermentation of Rhamnolipids production by *P.aeruginosa* PEER02. Red line with solid square: rhamnolipids production curve of S2; Red line with unfilled square: rhamnolipids production curve of S4; Blue line with solid diamond: fed-batch soybean added concentration for S2; Blue line with unfilled diamond: fed-batch line for S4.

From Fig.2, fed-batch fermentation greatly enhanced the production of rhamnolipids, with the yield reaching 23~25g/L. Generally speaking it is more efficient to add the substrate step wise, rather than all at the start of the fermentation process. It could be expected that the surfactant production and percent conversion could be improved from this result when optimized as a commercial process.

3.4 Rhamnolipid production and divergent evolution in *Escherichia coli*

ABSTRACT

Rhamnolipids, one of the most extensively studied biosurfactants, are a subclass of glycolipids and mainly produced from *Pseudomonas aerugina.* Besides the comprehensive application in the field

of envionment, rhamnolipids also have potential application in medicine, such as for their antimicrobial activity and anti-adhesive acitivity. Rhamnolipids production in Pseudomonads involves the process of quorum sensing with complex regulation and is quite difficult to optimize. In addition, many *P. aeruginosa* strains are pathogenic strains that are limited or not suitable for industrial development. Here, through the methodology of synthetic biology and metabolic engineering, *E. coli* was designed to produce rhamnolipids. *E. coli* strains harbor various gene combinations from *P. aeruginosa* to produce either mono-rhamnolipids or dir-rhamnolipids. Engineered rhamnolipids showed different performance in interfacial tension and antimicrobial activity. HPLC/MS analysis confirmed they mainly contained C_{10}-C_{10} and C_{10}-C_8 carbon chain as well as carbon chains with other lengths at low abundance. Theses results indicate that the key enzyme for rhamnolipids biosynthesis, rhamnosyltransferase 1, would function promiscuously. Generally, it is believed that protein with promiscuous function divergently evolved to acquire higher specificity or activity. A mutant with different product selectivity was found via directed evolution and subsequent high-throughput screening. Compared with the wild-type strain, the mutant produced rhamnolipids with almost only C_{10}-C_{10} carbon chain. Creation of this mutant enzyme demonstrates the feasibility of exploiting the underlying ability to engineer the result, and provides evidence for the feasibility of new enzyme design.

INTRODUCTION

Rhamnolipids are mainly produced from *Pseudomonas aerugina* or other Pseudomonads. Rhamnolipids production in Pseudomonads is a part of quorom sensing process, which has complex regulation. Thus, optimizing production of rhamnolipids in Pseudomonads is quite difficult. In addition, many *P. aeruginosa* strains are pathogenic strains that are limited or not suitable for industrial applicatiion. *E. coli*, as generally recoginzed as safe (GRAS) microorgainsm, has been applied comprehensively as an engineered host strains for many bioproducts production, including proteins, enzymes, metabolites and other pharmaceuticals.

The type of rhamnolipid produced depends on the bacterial strain, the carbon source used, and the process strategy (Mulligan *et al*., 1993). The lengths of the fatty acid chains of rhamnolipids can vary significantly, resulting in a multitude of different rhamnolipd compositions. Fatty acyl chains composed of 8, 10, 12, and 14 carbons in length, as well as 12- or 14-carbon chains with double bonds. The different structures of rhamnolipids have different properties with respect to interfacial

tension, solubility, and charge (Zhang and Miller, 1995). For example, the interfacial tension between hexadecane and water was decreased to <0.1 mN/m by the dR-Me (a methyl ester form of rhamnolipids) but was only decreased to 5 mN/m by the dR-A (the acid form of rhamnolipids). Solubilization and biodegradation of two alkanes in different physical states, liquid and solid, were determined at dirhamnolipid concentrations ranging from 0.01 to 0.1 mM (7 to 70 mg/liter). The low product selectivity of rhamnolipids biosynthesis in bacteria showed that the key biosynthetic enzyme, here RhlAB, would function promiscuously (Khersonsky et al., 2006; James and Tawfik 2001). Many promiscuous functions of enzymes were proven to be susceptible to directed evolution. Here, in to order to improve the performance of rhamnolipids from *E coli* directed evolution of RhlAB was investigated.

MATERIALS AND METHODS

Bacterial strain, media and chemicals

E. coli BL21(DE3) (*F^- ompT gal dcm lon hsdS$_B$(r_B^- m_B^-) λ(DE3)*) which can not produce rhamnolipids were the parental strain for engineering rhamnolipids-producing strain. *E. coli* DH5α (F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–) was as the host strain for constructing various recombinant plasmids. The *E. coli* strains were commercially available. Except for rhamnolipids fermentation, all these bacteria were grown on LB media supplementing suitable antibiotics at 37 ºC (Sambrook and Russell, 2001). Unless noted otherwise, antibiotics were used at the following concentration: chloramphenicol 25μg/mL; ampicillin, 50μg/mL; Kanamycin, 40μg/mL. All enzymes used for DNA manipulation were purchased from New England Biolabs (Beverly, MA). Pfx DNA polymerase and dNTP mixture for PCR amplification were purchased from Invitrogen (Carlsbad, CA). EZ::TN™ Transposase was purchase from EPICENTRE Biotechnologies (Madison, WI). Genomic DNA of *Pseudomonas aeruginosa* PAO1 was purchased from ATCC.

Plasmids and strains construction

The encoding region of rhamnolipids biosynthetic gene RhlA was amplified with primer RhlA-11 (5'- AGTTGGTACCATGCGGCGCGAAAGTCTGTTGG -3') and RhlA-12 (5' - AATTGAATTCAGGCGTAGCCGATGGCCATCTC - 3') and *P. aeruginosa* PA01genomic DNA as template; the PCR product of RhlA was digested with KpnI and EcoRI and cloned into

pET30a(+) (Fig 2, Novagen) to produce the recombinant plasmid pETRA (Fig2). The encoding region of rhamnolipids biosynthetic gene RhlAB was amplified with primer RhlAB-11 (same as RhlA-11) and RhlAB-12 (5' -AACCAAGCTTTCAGGACGCAGCCTTCAGCCATCG - 3') and *P. aeruginosa* PA01genomic DNA as template; the PCR product of RhlAB was digested with KpnI and HindIII and cloned into pET30a(+) to produce the recombinant plasmid pETRAB (Fig2). The encoding region of rhamnolipids biosynthetic gene cluster RhlABRI was amplified with primer Rhl-11 (5'- AGTTGAGCTCATGCGGCGCGAAAGTCTGTTGG-3') and Rhl-12 (5'-CAGCCGAAGCTTGAAGCGGAAAAAAGTGCGCGAAACG- 3') and *P. aeruginosa* PA01genomic DNA as template; the PCR product of RhlABRI was digested with SacII and HindIII and cloned into pET30a(+) to produce the recombinant plasmid pETRhl (Fig. 2). The encoding region of rhamnolipids biosynthetic gene RhlC was amplified with primer RhlC-11 (5'-TCAGAAGCTTAAG***AAGGAG***ATATACATATGGACCGGATAGACATGGGCGTG-3') and RhlC-12 (5' - TTCAGCGGCCGCATTCGTTCTACTTCCTCGACCC - 3') and using *P. aeruginosa* PA01genomic DNA as template. The open reading frame of RhlC was followed by a Shine-Dalgarno ribosomal binding sequence (boldface) and a spacer (***AAGGAG***ATATACAT). The PCR product of RhlC was digested with HindIII and NotI and cloned into pETRA, pETRAB, and pETRhl to produce the recombinant plasmids pETRAC, pETRABC and pETRhl-RC, respectively (Fig. 2). Then, these six recombinant plasmids pETRA, pETRAB, pETRhl, pETRAC, pETRABC and pETRhl-RC were transformed into *E. coli* BL21(DE3) to obtain engineered strains *E. coli* ETRA, ETRAB, ETRhl, ETRAC, ETRABC and ETRhl-RC, respectively.

To facilitate exchange of RhlA or RhlB in the recombinant plamsid pETRAB, the genes RhlA and RhlB were redesigned by introducing another restriction site (SacI at the 3'-end of RhlA, at the 5'end of RhlB). In addition, the open reading frame of RhlB was followed a Shine-Dalgarno ribosomal binding sequence (boldface) and a spacer (***AAGGAG***ATATACAT). So, the new primer for RhlA and RhlB cloning were RhlA-21 (same as RhlA-11) and RhlA-22 (5' TCAGGAGCTCTTATGCAACCGCAAAGCCCG-3'), RhlB21 (5'-GACGGAGCTCAAG***AAGGAG***ATATACATATGCACGCCATCCTCATCGCCATC-3') and RhlB-22 (same as RhlB-12). The new PCR products of RhlA-SacI and RhlB-SacI were digested with KpnI and SacI, and SacI and HindIII, then cloned into pET30a(+) to produce new recombinant plasmid pETRAB-SacI (Fig2). The engineered strains harboring pETRAB or pETRAB-SacI have no difference for rhamnolipids production.

Fermentation media and conditions

LB for seed culture of *E. coli*. The mineral salts medium with 0.4% glucose or LB with 0.4% glucose as carbon source was as rhamnolipd fermentation media. The mineral salts medium contained (g/L): $NaNO_3$, 15; KCl, 1.1; NaCl, 1.1; $FeSO_4.7H_2O$, 0.00028; KH_2PO_4, 3.4; K_2HPO_4, 4.4; $MgSO_4.7H_2O$, 0.5; yeast extract 0.5; and 5 ml of a trace elements solution containing (g/L): $ZnSO_4.7H_2O$, 0.29; $CaCl_2.4H_2O$, 0.24; $CuSO_4$. $5H_2O$, 0.25; $MnSO_4.H_2O$, 0.17 (Lindhardt et al, 1989). The trace element solution was filter-sterilized through a 0.22-µm membrane filter (Millipore, type GS) and then added to the medium, which had been autoclaved and allowed to cool.

E. coli wild-type and mutant were first grown in LB for 24h at 30ºC with shaking and then diluted 1:10 into mineral salts medium or LB with 0.4% glucose supplementing 50µM IPTG (isopropyl-beta-D-thiogalactopyranoside) as inducer and incubated for 24h. Incubation was carried out in 250mL Erlenmeyer flaks with 25mL medium and at 30ºC with orbital shaking at 250rpm.

Analytical method for rhamnolipids

Rhamnolipids were quantified in triplicate by weight and by the colorimetric determination of sugars with orcinol (Candrasekaran and Bemiller 1980). Rhamnolipids were purified by first separating the cells from supernatant by centrifugation (10000 ×g). The supernatant was then extracted with chloroform and ethanol. 0.5mL rhamnolipid sample was extracted with 1mL chloroform:ethanol (2:1, v/v). The organic phase was evaporated to dryness and 0.5mL of H_2O was added. To 0.1mL of each sample with suitable dilution, 0.9mL of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added. After heating for 30min at 80ºC the samples were cooled at room temperature and the OD_{421} was measured. The rhamnolipid concentrations were calculated from standard curves prepared with L-rhamnose (0-50mg/L).

Rhamnolipids purification Thin-layer chromatography and LC-MS analysis

The rhamnolipids were precipitated by acidifying culture supernatant to pH 2 with concentrated HCl and kept at 4°C overnight, and recovered by centrifugation at 10000g for 1h and dissolved in deionized water. 2 volumes of chloroform:ethanol (2:1, v/v) was added to rhamnolipids solution

and shaken 30 min for extraction. The organic phase was evaporated to dryness, and the rhamnolipid residue was then dissolved to 500mg/l final concentration. The purified rhamnolipids were separated, visualized, and compared to known rhamnolipids samples (JBR425, Jeneil BIosurfactant Co., LLC) using thin layer chromoatography (TLC; silica gel 60 plates). Simply, a 10μL sample was loaded into silica gel 60 plates. After being dried at room temperature, the silica gel was developed with a solution of chloroform-methanol-water (65:15:2 by volume), then visualized using a 50:1:0.05 mixture of the solution glacial acetic acid-sulfuric acid-anisaldehyde at 90°C for 30min. The HPLC-MS analyses were performed with a Gemini C18 column (2 mm x 50 mm, 5μm particle) from Phenomenex and a ThermoFinnigan LCQ Classic ion trap mass spectrometer. The LC gradient was: starting at 8%B and holding for 1 minute, then ramp to 75%B in 20 minutes, hold at 75%B for 10 minutes, back to 8%B in 1 minute and hold at 8%B for 5 minutes. Solvent B is 10:90 water:acetonitrile with 0.1% acetic acid. Solvent A is 98:2 (v/v) water:acetonitrile with 0.1% acetic acid. The mass spectrometer was operated in the negative ion mode scanning 250-950 m/z range. Data dependent MS/MS was set to perform MS/MS of the more abundant ions in the mass range.

Interfacial tension analysis of Rhamnolipids

The IFT was determined by using a spinning drop tensiometer (Temco Inc.) as detailed by Cayais (1975). Aqueous rhamnolipid solution was loaded into glass tube (Inner diameter, 2mm and Outer diameter, 5mm), followed by injection of 1.5μL octane. The glass tube with solution was spun in the tensiometer, and IFT was determined from the octane drop geometry. After 10min-spinning, the data were collected for analysis. In these studies, we measured the "fresh" IFT values without pre-mixing rhamnolipid solution and octane together to reach phase equilibrium. To adjust pH of rhamnolipid solution, 50mM citrate-Na_2HPO_4 buffer was used.

RESULTS AND DISCUSSIONS

Rhamnolipids production and characterization from *E. Coli*

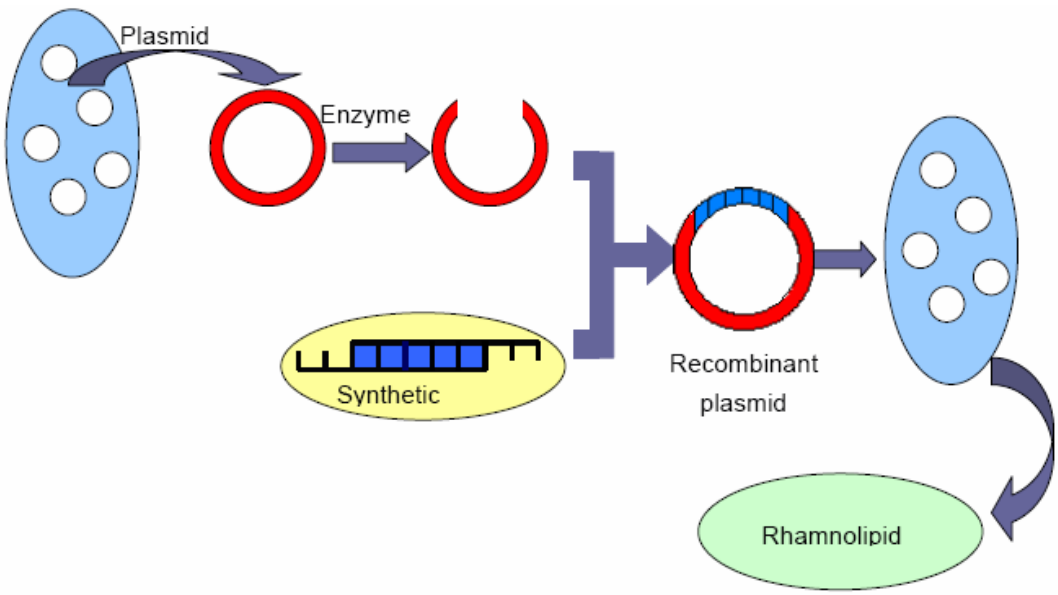
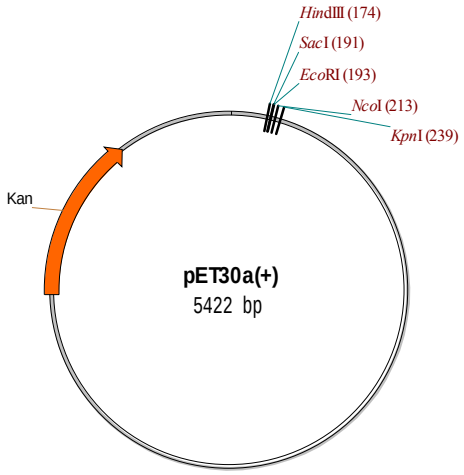


Fig 1 Diagram for Metabolic Engineering Methodology for Engineering Rhamnolipid-producing *E. coli*

From the biosynthetic pathway, the synthesis of rhamnolipids proceeds by sequential glycosyl transfer reactions, each catalyzed by a specific rhamnosyltransferase (rhamnosyltransferase 1 or 2) with TDP-rhamnose acting as a rhamnosyl donor and 3-hydroxyalkanoyl-3-hydroxyalkanoate acting as acceptor. L-rhamnosyl-3- hydroxyalkanoyl-3-hydroxyalkanoate (Rha-C_m-C_n, m and n: 8, 10, 12, or 14) and L-Rhamnosyl-L- rhamnosyl-3-hydroxyalkanoyl-3-hydroxyalkanoate (Rha-Rha-C_m-C_n) were referred to as rhamnolipids 1 (mono-rhamnolipids) and 2 (di-rhamnolipds), respectively. Rhamnosyltransferase 1 is encoded by the RhlA and B (or RhlAB) genes, which are organized in an operon and responsible for biosynthesis of mono-rhamnolipds. The active enzyme complex is located in the cytoplasmic membrane, with the RhlA protein being localized in the periplasm and the catalytically active RhlB component crossing the membrane. Rhamnosyltransferase 2 is encoded by the RhlC gene that is located in an operon with an upstream gene (PA1131) of unknown in *P. aeruginosa* PAO1 and not organized with RhlAB.

According to the metabolic engineering methodology for engineering rhamnolipid-producing *E. coli* (Fig.1), various gene combinations were constructed (Fig.2) and transformed into *E. coli* BL21(DE3) to obtain the corresponding engineered strains which can produce either mono-rhamnolipids or dirhamnolipids. The corresponding engineered strains and control strain were designated *E. coli* ETRA, ETRAC, ETRAB, ETRABC, ETRhl, ETRhl-RC and ET30, respectively.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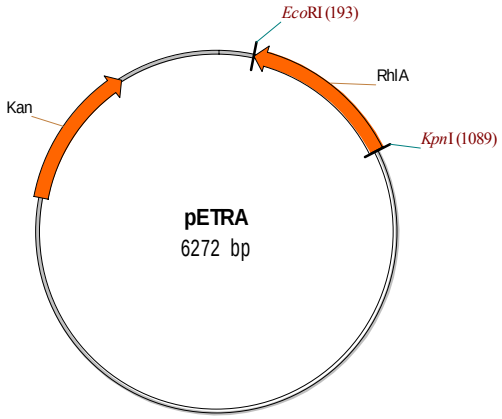
SacI (191)
EcoRI (193)
NcoI (213)
KpnI (239)
Kan
pET30a(+)
5422 bp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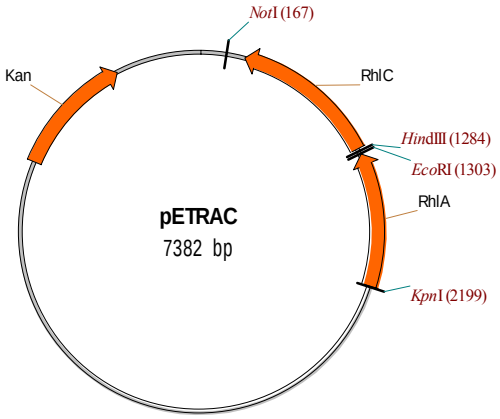
RhlA
Kan
KpnI (1089)
pETRA
6272 bp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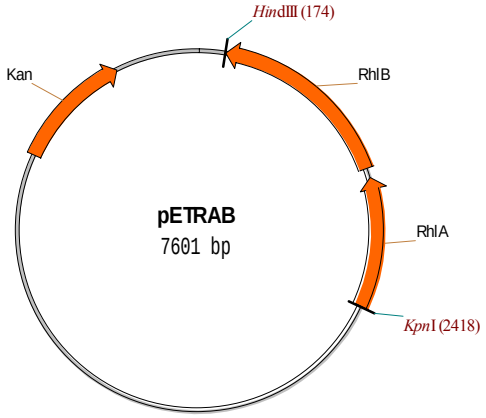
Kan
RhlC
HindIII (1284)
EcoRI (1303)
RhlA
pETRAC
7382 bp
KpnI (2199)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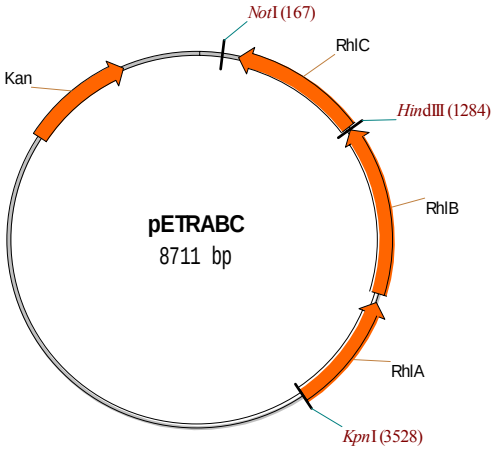
Kan
RhlB
pETRAB
7601 bp
RhlA
KpnI (2418)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RhlC
Kan
HindIII (1284)
RhlB
pETRABC
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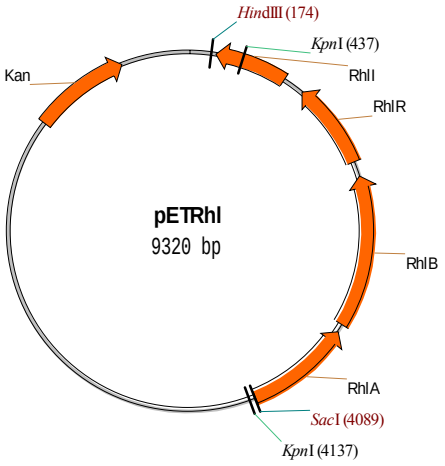
RhlA
KpnI (3528)


HindIII (174)
KpnI (437)
RhlI
RhlR
Kan
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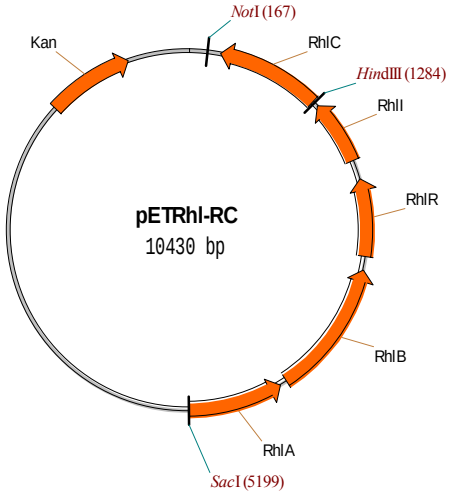
RhlB
RhlA
SacI (4089)
KpnI (4137)


NotI (167)
Kan
RhlC
HindIII (1284)
RhlI
RhlR
pETRhl-RC
10430 bp
RhlB
RhlA
SacI (5199)

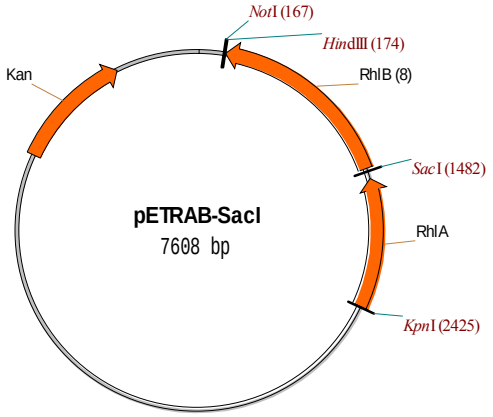


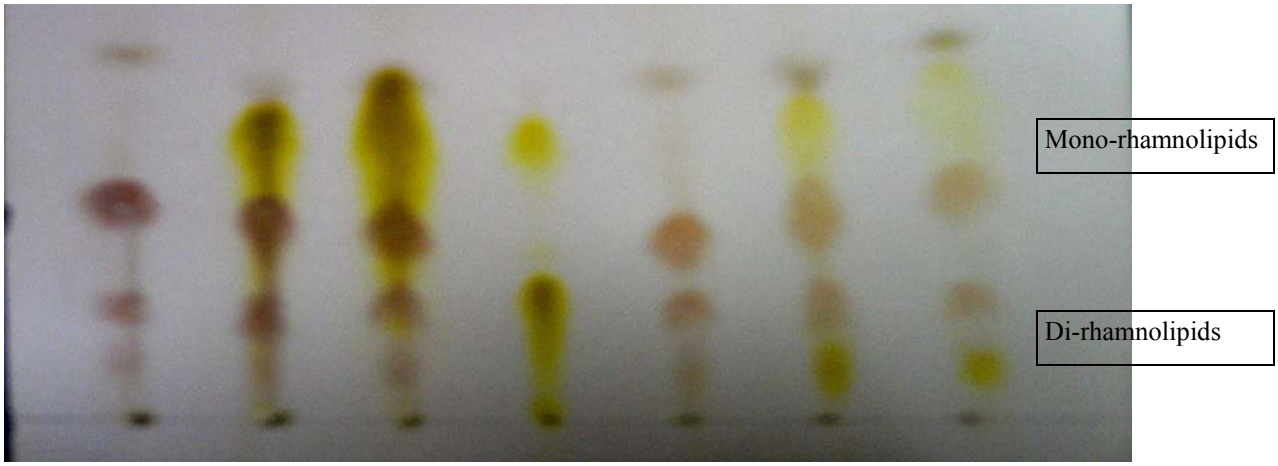
Fig 2 Plasmid map for engineering rhamnolipid-producing *E. coli.* Parental plasmids pET30a(+) and Recombinant expression plasmids

Preliminary fermentation of rhamnolids by engineered *E. coli* in rich (LB) or minimal (MS) media plus 0.4% glucose was carried out. The rhamnolipids yield and the IFT of their fermentative broth were shown in Table 1.

Table 1 `Preliminary fermentation of rhamnolids by engineered *E. coli* in rich (LB) or minimal (MS) media

Strain	MS media		LB media	
	IFT(mN/m)	Rhamnolipid (mg/L)	IFT(mN/m)	Rhamnolipid (mg/L)
E. coli ET30	40.1	<2	35.0	<5
E. coli ETRA	5.8	75.0	0.015	190.0
E. coli ETRAB	0.49	115.0	0.24	199.5
E. coli ETRhl	0.76	125.0	0.30	231.5
E. coli ETRAC	5.2005	83.3	0.013	253.8
E. coli ETRABC	0.35	213.3	0.22	230.0
E. coli ETRhl-RC	0.39	185.0	0.32105	235.0

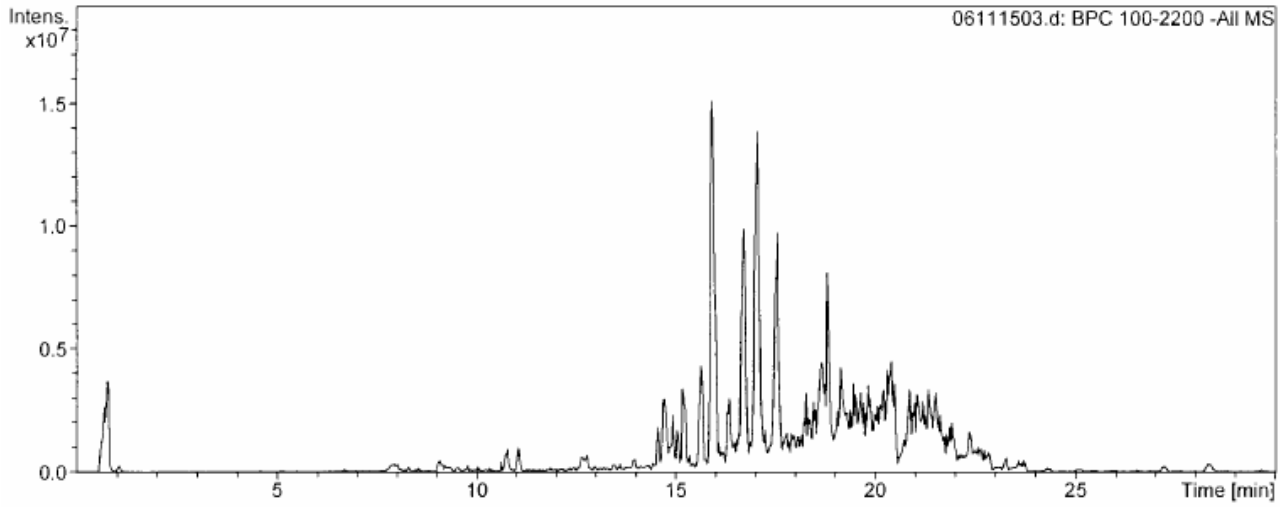
The IFT analysis and oil-spreading test indicate *E. coli* ETRA and *E. coli* ETRAC produced some kind of surface active material, but TLC analysis showed (Fig 3) that they did not produce mono-rhamnolipids and/or di-rhamnolipids (yellow dots). However, they do produce other kinds of compounds (purple dots) whose composition needs to be confirmed, e.g., LC-MS. *E. coli* ETRAB and ETRhl produced mono-rhamnolipids, and *E. coli* ETRABC and ETRhl-RC produced mono-rhamnolipids and di-rhamnolipids. The ratio of di/mono-rhamnolipids were approximately 2:1~3:1.



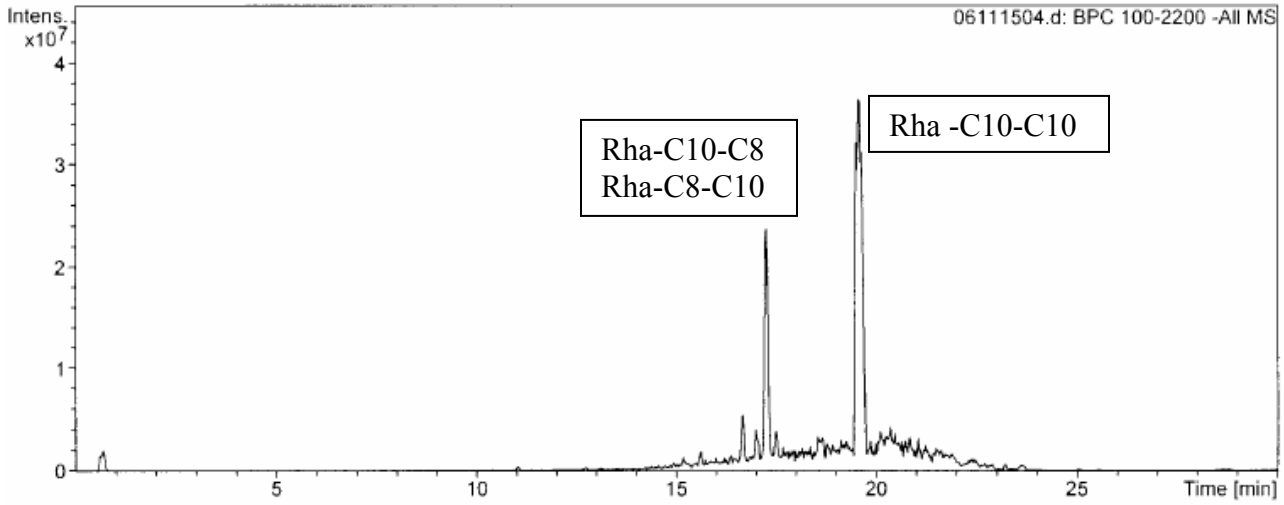
(a) (b) (c) (d) (e) (f) (g)

Fig 3 Thin layer chromatography (TLC) of rhamnolipids from engineered *E coli*.
(a) ETRA; (b) ETRAB; (c) ETRhl; (d) Commercial rhamnolipids (as standard); (e) ETRAC; (f) ETRABC; (g) ETRhl-RC.

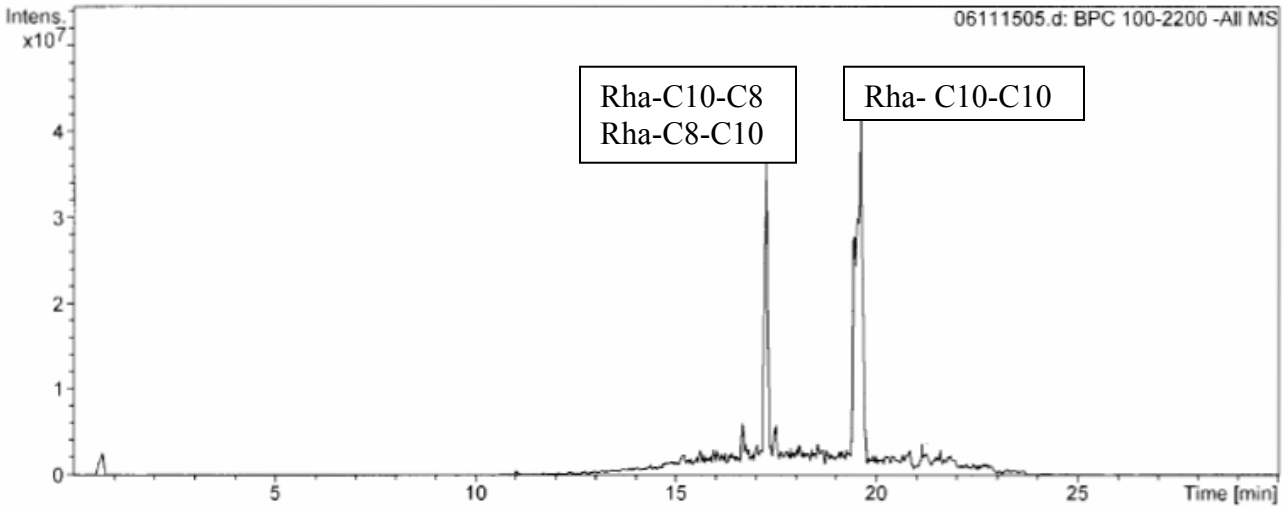
HPLC-MS analysis of these rhamnolipids further confirmed the structures of various surfactants from engineered strains. Note for the ETRA and the ETRAC that the peaks are much lower intensity than the other 4 samples, plus their elution times are shorter. This further confirms that these 2 strains did not make rhamnolipid surfactants.


Intens.
x10^7
06111503.d: BPC 100-2200 -All MS
1.5
1.0
0.5
0.0
5
10
15
20
25
Time [min]

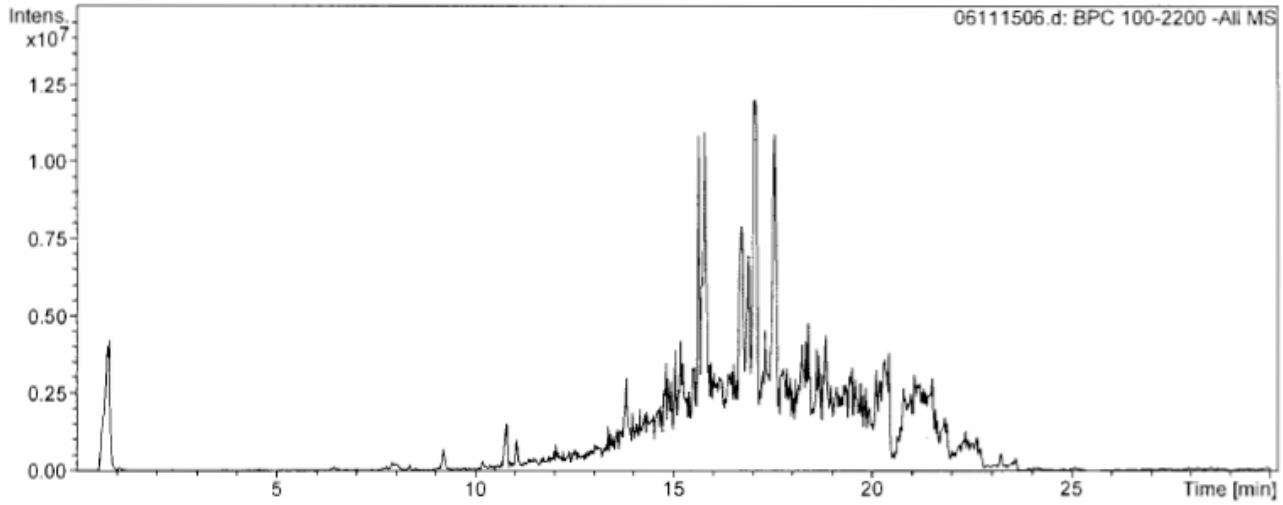
(a)


Intens.
x10^7
06111504.d: BPC 100-2200 -All MS
4
3
2
1
0
Rha-C10-C8
Rha-C8-C10
Rha -C10-C10
5
10
15
20
25
Time [min]

(b)


Intens.
x10^7
06111505.d: BPC 100-2200 -All MS
Rha-C10-C8
Rha-C8-C10
Rha- C10-C10
4
3
2
1
0
5
10
15
20
25
Time [min]

(c)


Intens.
x10^7
06111506.d: BPC 100-2200 -All MS
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0.75
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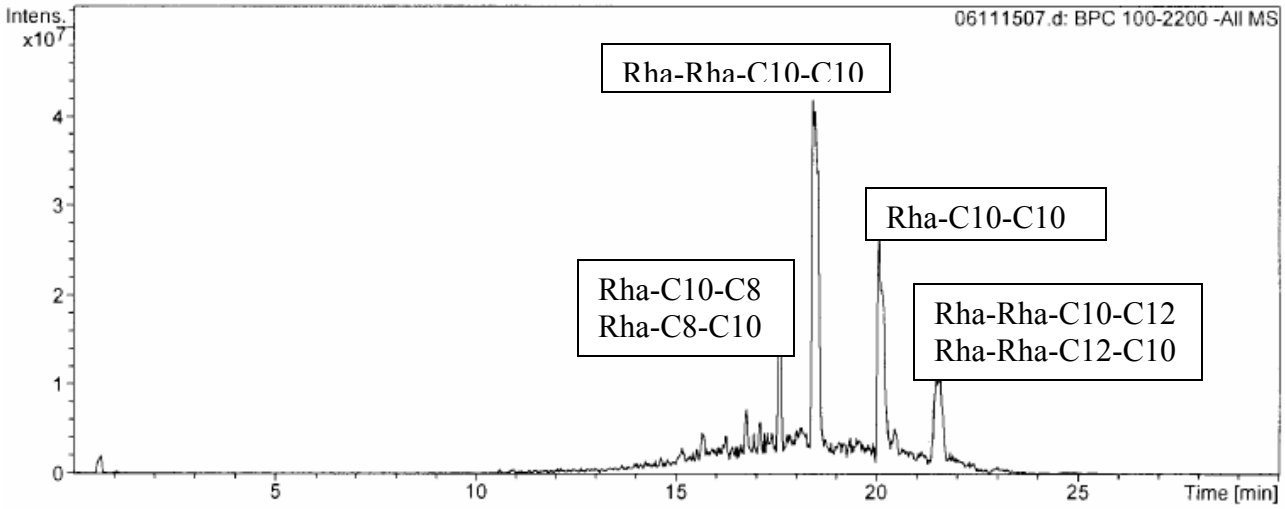
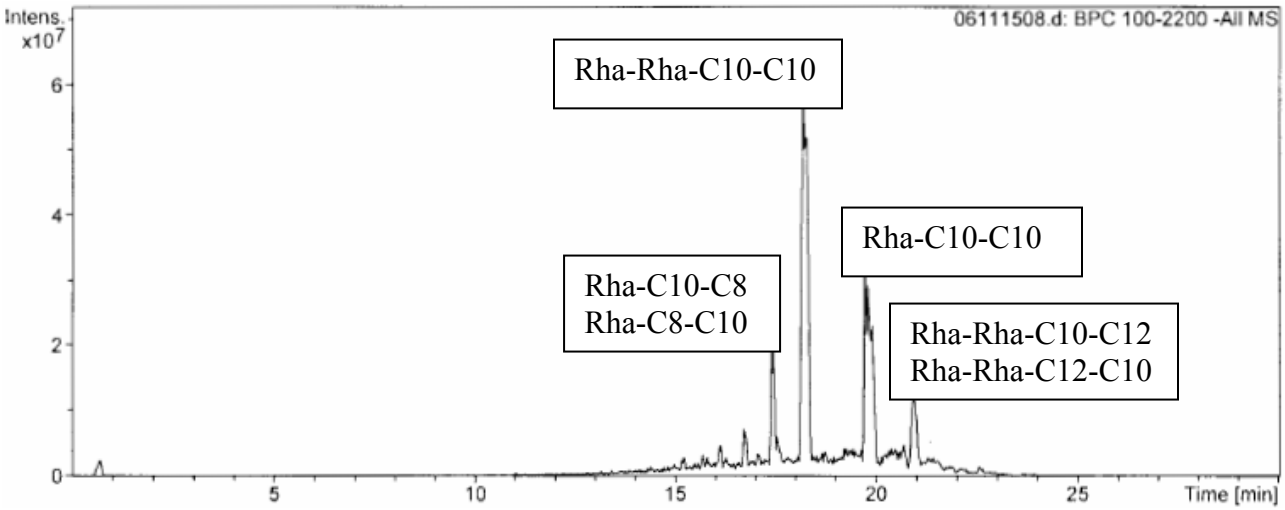
0.25
0.00
5
10
15
20
25
Time [min]

(d)



(e)



(f)

Fig. 4. Base peak ion intensity chromatograms of rhamnolipids from different engineered *E. coli* strains. (a) ETRA; (b) ETRAB; (c) ETRhl; (d) ETRAC; (e) ETRABC; (f) ETRhl-RC. The main compositions are labeled in each chromatogram.

Table 2. Structure and relative abundance of the rhamnolipids from various engineered *E. coli strains*

Strains	Rha-Rha-C10-C10 m/z 649	Rha-C10-C10 m/z 503	Rha-Rha-C10-C12 (or C12-C10) m/z 677	Rha-C8-C10 (or C10-C8) m/z 475
E. coli ETRA	-	-	-	-
E. coli ETRAB	-	73.1	-	26.9
E. coli ETRhl	-	69.7	-	30.3
E. coli ETRAC	-	-	-	-
E. coli ETRABC	45.2	28.7	15.0	6.0
E. coli ETRhl-RC	45.8	30.2	8.9	8.5

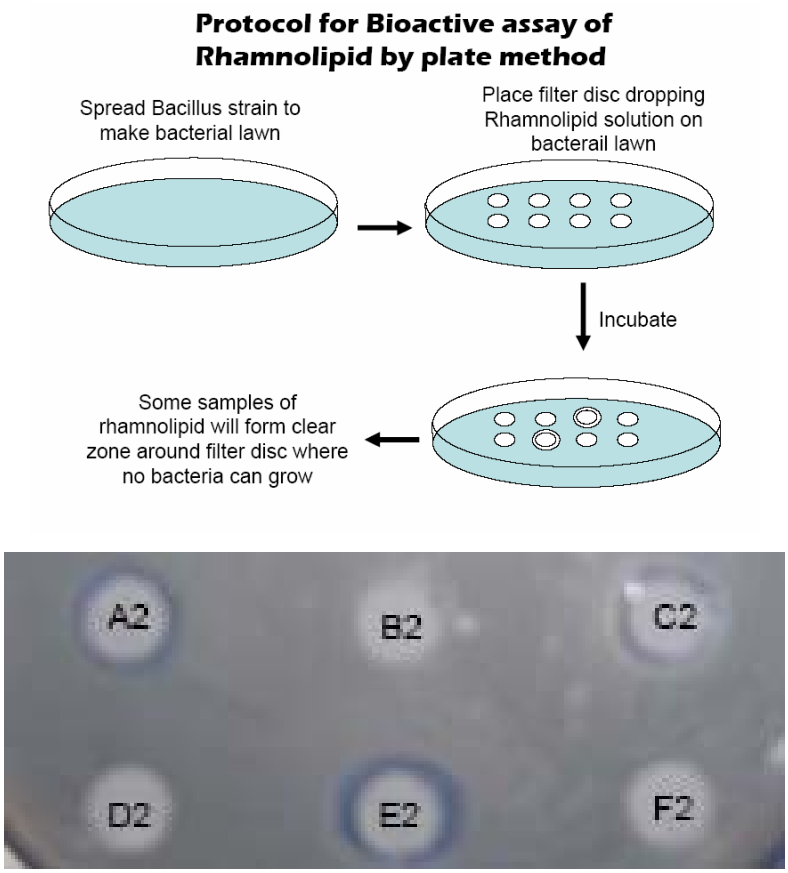
- denotes that it has no evidence of containing that chemical structure

Directed evolution of RhlAB to improve rhamnolipids production in *E. coli*

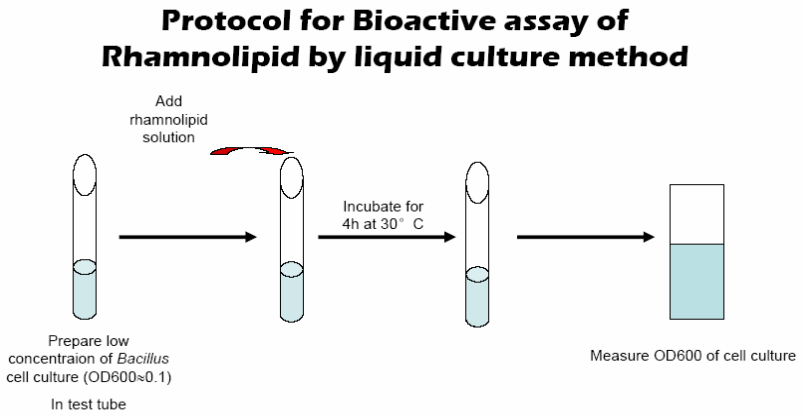
Mutant RhlA and RhlB genes were produced by error-prone PCR with RhlA-21 (5'-AGTTGGTACCATGCGGCGCGAAAGTCTGTTGG -3') and RhlA-22 (5' TCAGGAGCTCTTATGCAACCGCAAAGCCCG-3'), and RhlB21 (5'-GACGGAGCTCAAG***AAGGAG***ATATACATATGCACGCCATCCTCATCGCCATC-3') and RhlB-22 (5' -AACCAAGCTTTCAGGACGCAGCCTTCAGCCATCG - 3'). Muant RhlA or RhlB PCR products were digested with the corresponding restrictione enzymes (KpnI and SacI for RhlA; SacI and HindIII for RhlB) and cloned into pETRAB-SacI to replace wild-type genes of RhlA or RhlB. The mutants plamids were transformed into *E. coli* BL21(DE3) to produce a mutant library.

To screen the mutant strains and identify those with desirable properties, a robust high throughput screening method should be developed. Here, we focused on panning the mutants which have high performance of interfacial tension from *E. coli* mutant library. First, rhamnolipids from *E. coli* showed bioactivity according to the results of plate methods (Fig 5a). Filter discs containing rhamnolipids could inhibit effectively the growth of *Bacillus subtilis* cells and the rhamnolipids with low interfacial tension create a larger clearing zone (Fig 5a, E2). Then, using a liquid culture method (Fig. 5b adding

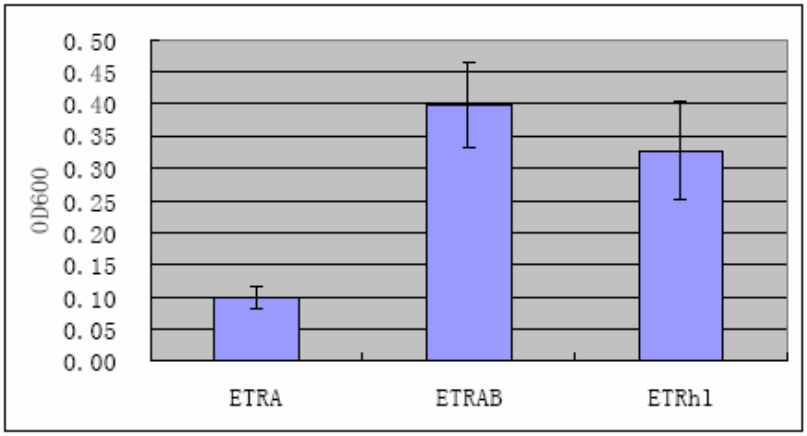
different rhamnolipids to diluted *Bacillus subtilis* cell culture), different rhamnolipids showed a variation of ability for growth inhibition (Fig. 5c).



(a)



(b)



IFT(mN/m)	0.01482±0.00552	0.24253 ±0.03686	0.302976 ±0.04883

(c)

Fig.5. Bioactive assay of rhamnolipids from *E. coli*. (a) Plate method; (b) Liquid culture method, and (c) results. This suggests a correlation of the plate results and IFT. Cultures creating lower IFT inhibit cell growth to make a clear area of lower OD.

Based on the results from the plate method and liquid culture method of bioactive assay of rhamnolipids, a high throughput screening method for primary screening mutants which can produce rhamnolipids with high performance of interfacial tension was developed (Fig 6).

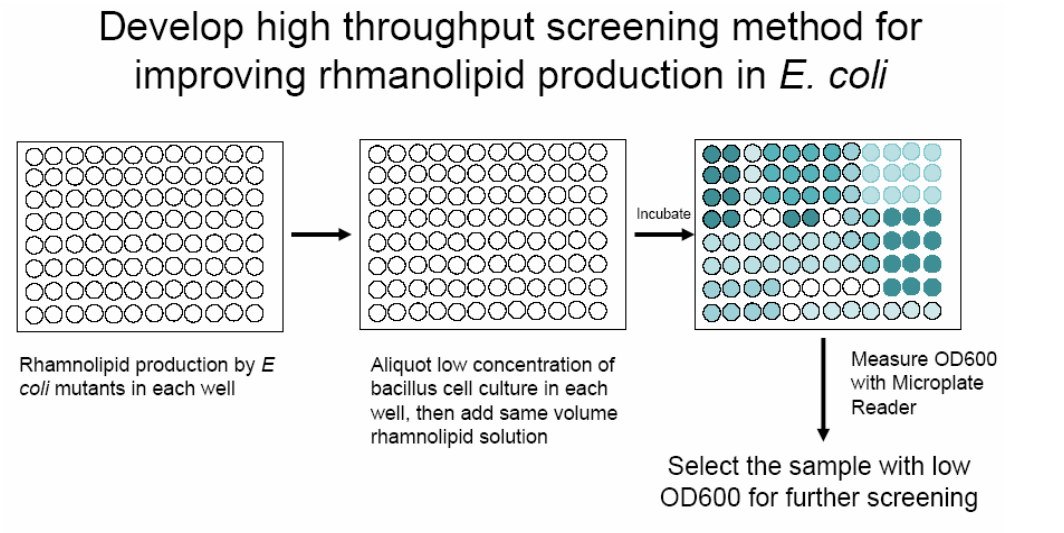


Fig.6. Developing high throughput screening method to identify candidates with improved rhamnolipids production in *E coli*.

Mutant rhamnolipids characterization

With this screening method, those mutants with the greatest inhibition for *Bacillus subtilis* cell growth were selected. Then, mutant rhamnolipids were further screened through IFT measurement and structure analysis by HPLC/MS. Finally, one mutant which has mutation in RhlB (L168P, Fig 7) was confirmed to have different properties in IFT behavior and structure components (Fig. 8 and Table 3)

```
RHLB-WT      MHAILIAIGSAGDVFPFIGLARTLKLRGHRVSLCTIPVFRDAVEQHGIAF    50
RHLB-MUTANT  MHAILIAIGSAGDVFPFIGLARTLKLRGHRVSLCTIPVFRDAVEQHGIAF    50
Consensus    mhailiaigsagdvfpfiglartlklrghrvslctipvfrdaveqhgiaf
RHLB-WT      VPLSDELTYRRTMGDPRLWDPKTSFGVLWQAIAGMIEPVYEYVSAQRHDD   100
RHLB-MUTANT  VPLSDELTYRRTMGDPRLWDPKTSFGVLWQAIAGMIEPVYEYVSAQRHDD   100
Consensus    vplsdeltyrrtmgdprlwdpktsfgvlwqaiagmiepvyeyvsaqrhdd
RHLB-WT      IVVVGSLWALGARIAHEKYGIPYLSAQVSPSTLLSAHLPPVHPKFNVPEQ   150
RHLB-MUTANT  IVVVGSLWALGARIAHEKYGIPYLSAQVSPSTLLSAHLPPVHPKFNVPEQ   150
Consensus    ivvvgslwalgariahekygipylsaqvspstllsahlppvhpkfnvpeq
RHLB-WT      MPLAMRKLLWRCIERFKLDRTCAPEINAVRRKVGLETPVKRIFTQWMHSP   200
RHLB-MUTANT  MPLAMRKLLWRCIERFKPDRTCAPEINAVRRKVGLETPVKRIFTQWMHSP   200
Consensus    mplamrkllwrcierfk drtcapeinavrrkvgletpvkriftqwmhsp
RHLB-WT      QGVVCLFPAWFAPPQQDWPQPLHMTGFPLFDGSIPGTPLDDELQRFLDQG   250
RHLB-MUTANT  QGVVCLFPAWFAPPQQDWPQPLHMTGFPLFDGSIPGTPLDDELQRFLDQG   250
Consensus    qgvvclfpawfappqqdwpqplhmtgfplfdgsipgtplddelqrfldqg
RHLB-WT      SRPLVFTQGSTEHLQGDFYAMALRALERLGARGIFLTGAGQEPLRGLPNH   300
RHLB-MUTANT  SRPLVFTQGSTEHLQGDFYAMALRALERLGARGIFLTGAGQEPLRGLPNH   300
Consensus    srplvftqgstehlqgdfyamalralerlgargifltgagqeplrglpnh
RHLB-WT      VLQRAYAPLGALLPSCAGLVHPGGIGAMSLALAAGVPQVLLPCAHDQFDN   350
RHLB-MUTANT  VLQRAYAPLGALLPSCAGLVHPGGIGAMSLALAAGVPQVLLPCAHDQFDN   350
Consensus    vlqrayaplgallpscaglvhpggigamslalaagvpqvllpcahdqfdn
RHLB-WT      AERLVRLGCGMRLGVPLREQELRGALWRLLEDPAMAAACRRFMELSQPHS   400
RHLB-MUTANT  AERLVRLGCGMRLGVPLREQELRGALWRLLEDPAMAAACRRFMELSQPHS   400
Consensus    aerlvrlgcgmrlgvplreqelrgalwrlledpamaaacrrfmelsqphs
RHLB-WT      IACGKAAQVVERCHREGDARWLKAAS                           426
RHLB-MUTANT  IACGKAAQVVERCHREGDARWLKAAS                           426
Consensus    iacgkaaqvverchregdarwlkaas
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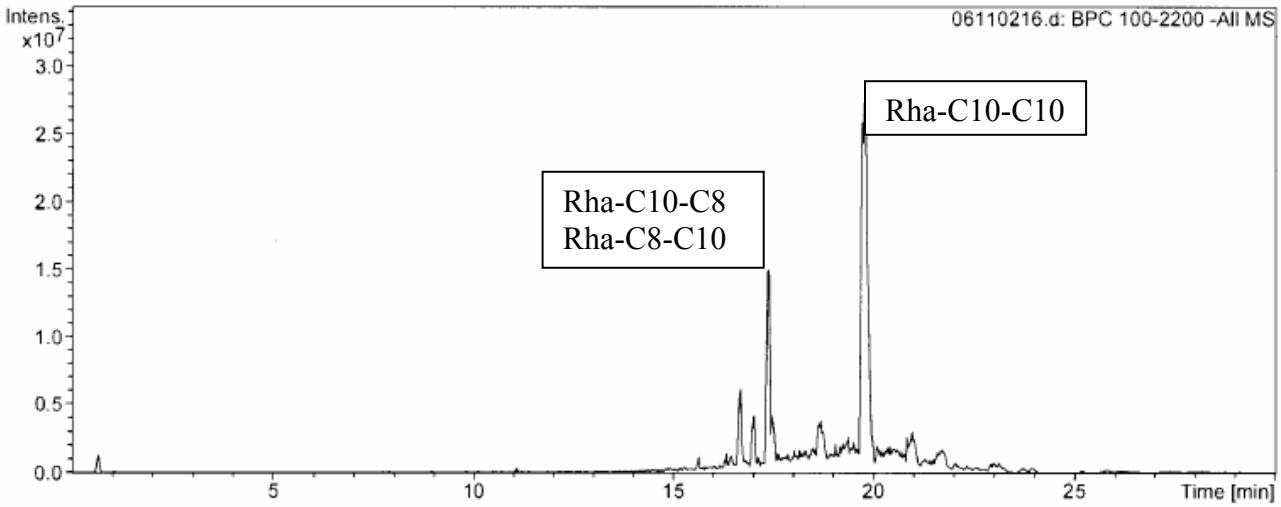
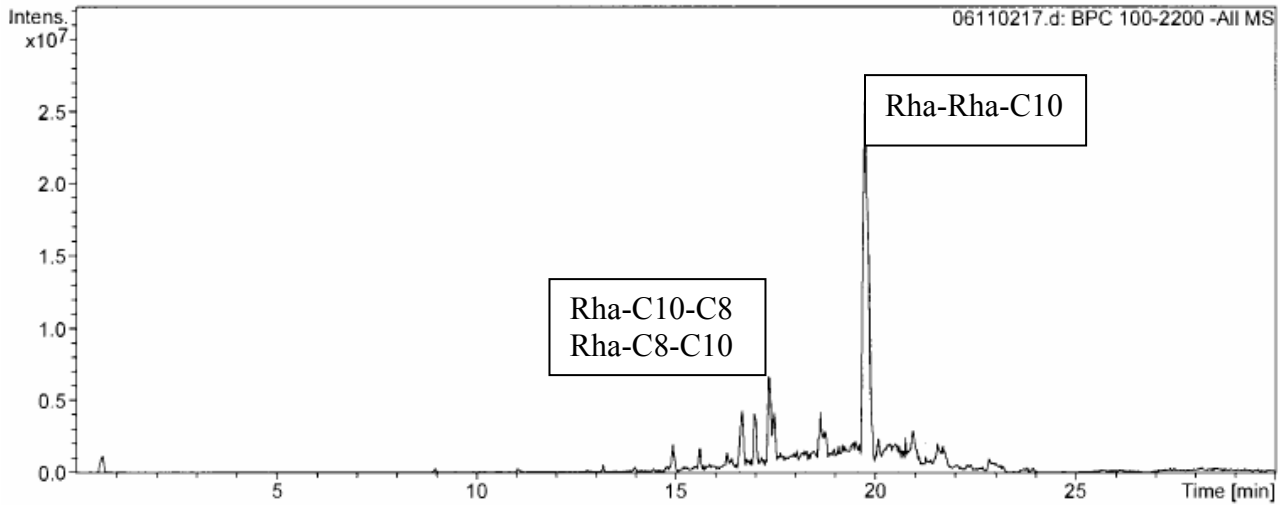
Fig. 7. Alignment of amno acid sequence of RhlB WT and Mutant H. Mutant H has a single mutant, as indicated, at position L168P.



(a)



(b)

Fig. 8. Base peak ion intensity chromatograms of rhamnolipids from engineered *E. coli* ETRAB-SacI WT and Mutant strains. (a) WT; (b) Mutant H. The main compositions are labeled in each chromatogram. Note for practical purposes, the ETRAB-SacI WT microbe is the same as ETRAB.

Table 3 IFT, structure and relative abundance of the rhamnolipids from *E. coli* ETRAB-SacI WT and Mutant strains

Strains	IFT (mN/m)*		Rha-C10-C10**	Rha-C10-C8	Rha-C8-C10
	pH7	pH8	m/z 503	m/z 475	m/z 475
WT	0.019	0.23	100	8.0	16.5
Mutant H	0.22	0.018	100	11.5	3.7

* rhamnolipids concentration is 100mg/L

** Given the relative amount of the Rha-C10-C10 100

3.5 Evaluation of Engineered *E. Coli* Rhamnolipids by IFT and Core Flooding

After an oil well loses its self flow (primary recovery by natural forces), and secondary flow (secondary recovery by pressurization using water flood and gas), approximately 65% of the original oil remains in the reservoir. It is estimated that 377 Billion Barrels of oil from the known US oil reserve still remain unrecoverable. Much of the remaining oil can be recovered by enhanced oil recovery techniques such as polymer flooding or surfactant flooding. Effective emulsifying surfactants and a better understanding of the formation of their emulsions with the crude oil are of paramount importance to economically recover the abundant residual oil in reservoirs. Entrapment of petroleum hydrocarbon by capillary forces is a major factor that limits oil recovery. Hydrocarbon displacement can occur if interfacial tension between the hydrocarbon and aqueous phases is reduced by several orders of magnitude. Microbial-produced biosurfactants may be an economical method to recover residual hydrocarbons since they are effective at low concentration. The IFT analysis of our engineered *E. Coli* rhamnolipids show that they can remarkably reduce interfacial tension of oil (octane)/water at the certain combination of salinity and pH. Based on these data from IFT analysis, sand-packed core flooding tests of rhamnolipids from *P. aeruginosa* with soybean oil as carbon source were performed.

MATERIALS AND METHODS

Materials:

1. Sand: Play Ground Sand From Home Depot, manufactured by Paragon Building Products Inc, Norco, CA 92860. Dried it at 90 C for 12hr.
2. Pack Tube: Diameter is 1 inch, wall thickness 0.083 inch and length is 2ft.
3. Brine: Varies – use the salinity of the surfactant solution with lowest IFT
4. Oil: n-octane was used as simulation oil.
5. Fermentation product from the 6 engineered E. Coli strains.

Interfacial Tension Analysis of Rhamnolipids

The IFT was determined by using a spinning drop tensiometer (Temco Inc.) as detailed by Cayais (1975). Aqueous rhamnolipid solution was loaded into glass tube (Inner diameter, 2mm and Outer diameter, 5mm), followed by injection of 1.5μL octane. The glass tube with solution was spun in the tensiometer, and IFT was determined from the octane drop geometry. After 10min-spinning, the data were collected for analysis. In these studies, we measured the "fresh" IFT values without pre-mixing rhamnolipid solution and octane together to reach phase equilibrium.

Sand-pack core flooding test

Dried playground sand manufactured by Paragon Building Products Inc (Norco, CA) was packed into a stainless iron tube (2×60cm) as a flooding core. The sand-packed core was evacuated and saturated with brine. Pore volume (PV), porosity, as well as brine permeability (approximately 50 Darcies) at three flow rates were calculated. Then n-octane (oil) displaced brine until no more water came out. The oil-saturated core was aged for 24h. Then, brine was injected into aged oil-saturated core to displace the oil which was not trapped strongly by sand and recovered with brine until no oil came out. At this point, the core was ready for biosurfactant (rhamnolipids) flooding. 3 PV of 250ppm rhamnolipid solution (in its IFT optimum pH buffer/salinity) was injected, and

oil recovery and water cut (percentage of non-oil phase in each elution fraction) were recorded. Next, the pack with rhamnolipids was incubated overnight at room temperature. Another 3 PV of rhamnolipid solution was injected during the next day. Similarly, oil recovery and water cut were recorded. After rhamnolipids flooding, 6 PV of that same brine was injected, and the oil and water cut in this stage were also recorded.

Rhamnolipids produced by Six Engineered strains Displayed Different Optimal IFT Values in Response to Different pH Conditions

Rhamnolipid biosurfactants were fermented and adjusted to final concentration of 250ppm by oil spreading assay. Then the pH values were adjusted from 5 to 12 using two different buffer systems – citric acid wide range buffer system and boric acid universal buffer system. IFT values were then measured under each different pH conditions and plotted in Figures 1A and 1B. In the citric acid based buffer system, the lowest IFT were obtained at pH 8 with the products of strain ETRA and ETRAC, while the lowest IFT of the products of other 4 strains were obtained at pH 7. But in the boric acid buffer system, the pH values of the lowest IFT were shifted downwards, with products of strain ETRA and ETRAC obtaining lowest IFT at pH 7; and products of strain ETRhl, ETRABC and ETRhl-RC obtaining lowest IFT at pH 7. The only exception was the product of strain ETRAB, which obtaining lowest IFT at pH 7 in both buffer systems. Figure 1C show the effects of the salinity on IFT at pH 7. The lowest IFT values for all six strains were obtained when 2% NaCl was added to the buffer. What surprised us was that even though there were no detectable rhamnolipids produced by strain ETRA and ETRAC (as shown in Table 2 in section 3.4), even though their products created very low IFT values.

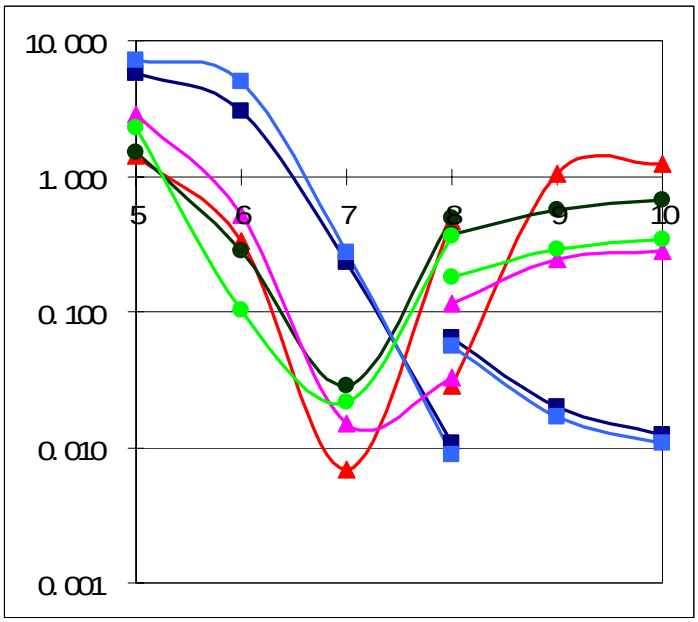


Fig. 1A IFT versus the solution pH. IFT of 250ppm rhamnolipids produced by engineered E.coli strains in the citric acid buffer system. Dark blue squares: ETRA; Red triangles: ETRAB; Black circles: ETRhl; Light blue squares: ETRAC; Pink triangles: ETRABC; Light green circles: ETRhl-RC;

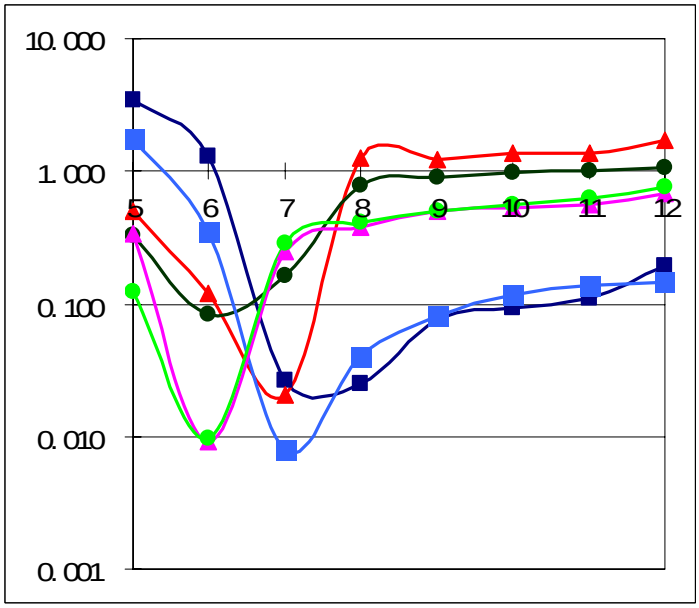


Fig. 1B IFT versus the solution pH. IFT of 250ppm rhamnolipids produced by engineered E.coli strains in the boric acid buffer system. Dark blue squares: ETRA; Red triangles: ETRAB; Black circles: ETRhl; Light Blue squares: ETRAC; Pink triangles: ETRABC; Light green circles: ETRhl-RC.

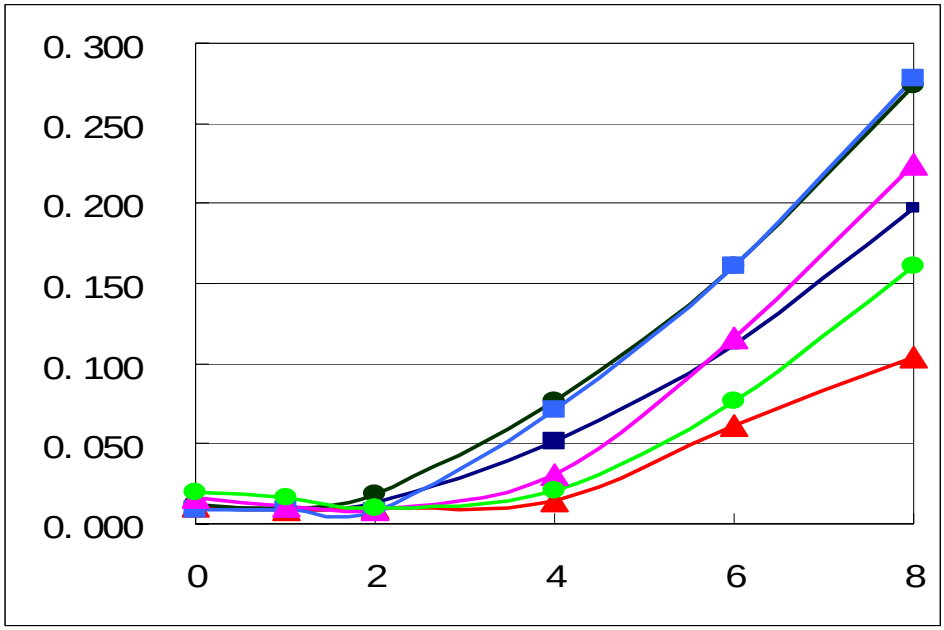


Fig 1C. IFT of the rhamnolipids produced by six E.coli strains with different added NaCl. Dark blue squares: ETRA in pH8 40mM Citric acid-Na2HPO4 and 0-8% NaCl buffer; Red triangles: ETRAB in pH7 40mM Citric acid-Na2HPO4 and 0-8% NaCl buffer; Dark green circles: ETRhl in pH7 40mM Citric acid-Na2HPO4 and 0-8% NaCl buffer Light blue squares: ETRAC in pH8 40mM Citric acid-Na2HPO4 and 0-8% NaCl buffer; Pink triangles: ETRABC in pH7 40mM Citric acid-Na2HPO4 and 0-8% NaCl buffer; Light green circles: ETRhl-RC in pH7 40mM Citric acid-Na2HPO4 and 0-8% NaCl buffer

Sand-pack Core Flooding Tests Show that Only the Strains Producing Rhamnolipids that were Detected by LC-MS can Recover Oil Effectively

The fermentation products of the each engineered E.coli strains were adjusted to 250ppm based on oil spreading assay and their optimal pH which gives the lowest IFT value. Each of those solutions was then tested for its oil recovery capability by the sand-pack core flooding experiments. The results are summarized in Figure 2A to 2C. The strains ETRhl, ETRABC and ETRhl-RC (demonstrated to produce rhamnolipids according to LC-MS results in Table 2 in Section 3.4), could recover 24 - 45% of the remaining oil after water flooding. On the other hand, the products of the strains ETRA and ETRAC (which did not produce LC-MS detectable rhamnolipids) could not recover oil effectively. Thus, although those two strains produced some surface-active products that

gave low IFT values, they could not recover oil nearly as well as the strains that produced either mono or di- rhamnolipid biosurfactants.

Apparently, the oil recovery efficiency is correlated to the IFT values (Fig. 2B). Interestingly, the strain ETRAB could produce rhamnolipids in good quantity, but it recovered least amount of oil among the six E.coli strains. It seemed that the high adsorption of the rhamnolipids produced by ETRAB could be the contributing factor of its low oil recovery (Fig. 2C). Further experiments need to be conducted to investigate the difference between ETRAB and other stains that contain the rhamnosyltransferase-B gene.

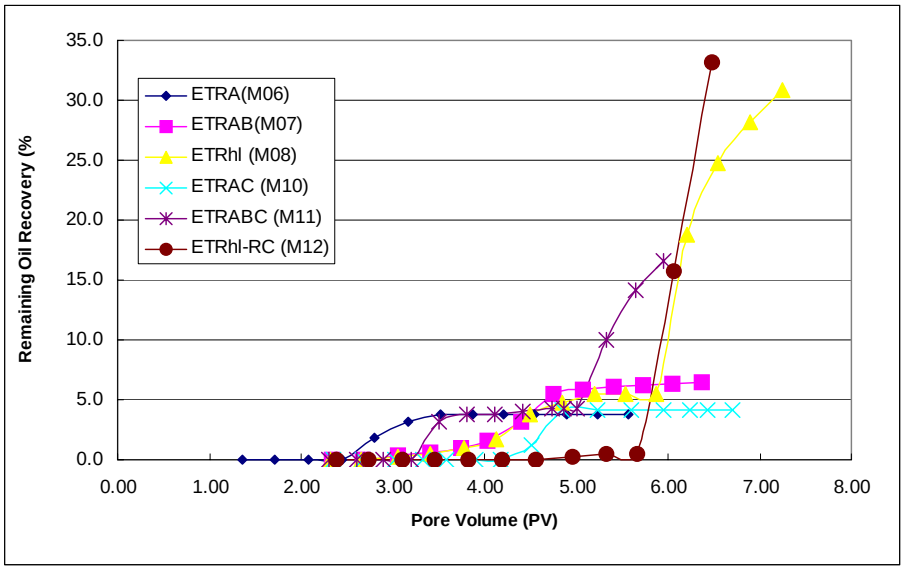


Fig 2A. Sand-pack core flooding tests of the rhamnolipids produced by six engineered E.coli strains. Rhamnolipids were adjusted to 250ppm and optimal pH values based on the IFT tests. Blue diamonds – ETRA; Pink Squares- ETRAB; Yellow triangles – ETRhl; Light blue crosses – ETRAC; Brown stars – ETRABC and Brown circles – ETRhl-RC.

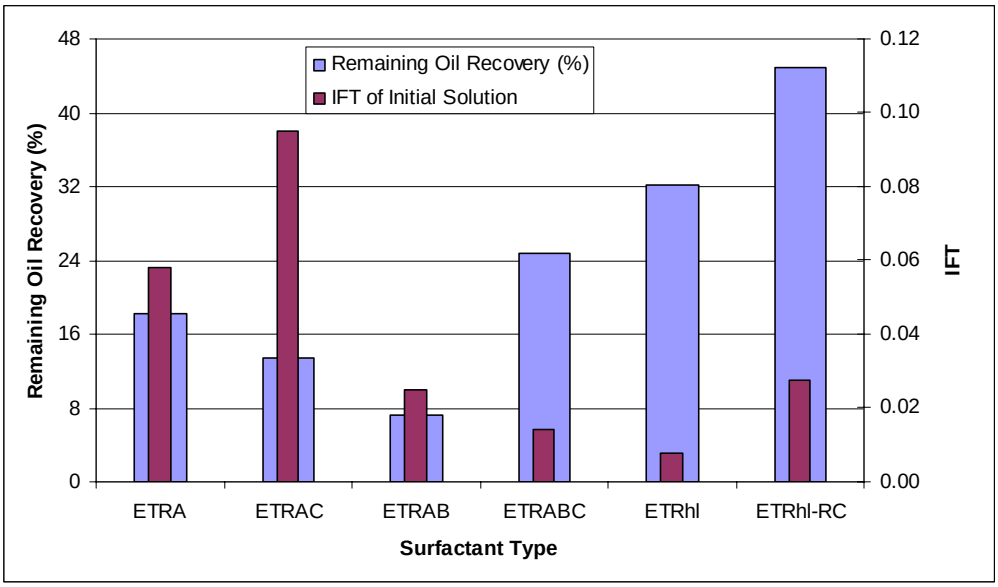


Fig 2B. Summary of the remaining oil recovery and IFT of the initial rhamnolipids injected of the fermentation products of the six E.coli strains.

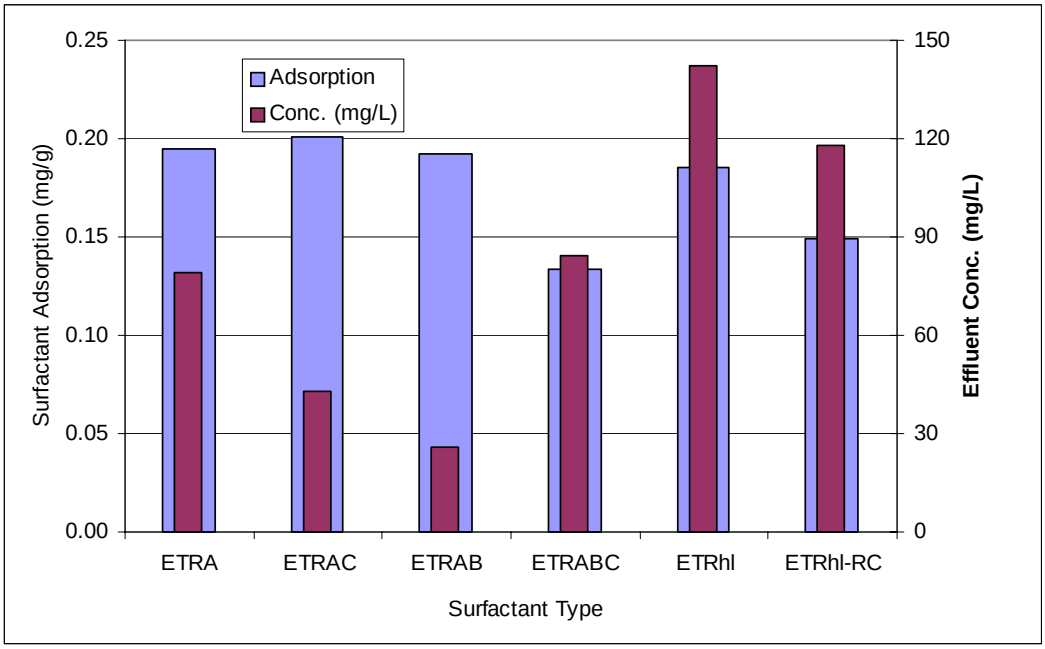


Fig 2C. Summary of the surfactant adsorption of the fermentation products of the six E.coli strains.

3.6 References

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4. Schedule and Milestone Status

During the second year of study, we have met the following milestones for the project:

1. We have completed the comprehensive evaluation studies of rhamnolipid as a surfactant product for conventional chemical EOR applications. (month 21)
2. We are continuing to improve lab methods to clone a bio-surfactant mutant into candidate thermophilic oil field microbes. (month 20)
3. We have successfully engineered *P. aeruginosa* PEER02 strain and several E. Coli strains and mutants to produce rhamnolipid biosurfactants effectively. (Month 24)

5. Summary of Significant Accomplishments

1. We successfully engineered the new mutant strains *P. aeruginosa* PEER02 and *E. coli* TnERAB that can produce rhamnolipids.
2. Rhamnolipid biosurfactants produced by our engineered bacteria have been evaluated by core flooding tests and are proven to be effective EOR agent
3. We have successfully produced rhamnolipids from many different plant oils and other agriculture products and waste products.
4. We have successfully developed bioactive assay based high throughput screening method for screening our mutants and improving rhamnolipids production in *E coli*.
5. A complete LC-MS analytical methods have been documented for structural analysis of the rhamnolipids.

6. Future Work / Potential Problems

The main focus for the next year of project period will be:

1. Further improve the methods to evaluate rhamnolipid and surfactin for their EOR characteristics.
2. Improve our mutant strains and optimize fermentation process to increase the yield of

rhamnolipid production using agriculture products and waste streams as substrate.

3. Continue our directed evolution studies, screen and improve our mutant E.Coli strains to produce rhamnolipids with different structural verities.
4. Continue to search for better oilfield microbe strains that could be a carrier of our improved mutant genes for potential in-situ oilfield EOR applications.

7. Technology Transfer Activities

We have discussed our research work and potential technology value with several major oil companies, such as Chevron, Exxon-Mobil and Shell. We also presented some of our research results as a poster at the 28th Symposium on Biotechnology for fuels and Chemicals in Nashville, TN in May 2006, and a SPE workshop in Daqing, China in October 2006 (the traveling expenses were funded by another non-DOE project). We will also present our results at the SPE Oilfield Chemistry Symposium in Feb, 2007.

8. Acknowledgements

We would like to thank DOE for their financial support and guidance throughout this project.

9. Budget

MEOR-- (anticipating $227,088 in additional funding)

Budget Period: 10/1/04 - 9/30/06

Approved Budget: $674,663

DOE share: $539,731

Non-Fed share: $134,932

Funding Received from DOE: $539,697

Actual Costs Incurred for period 10/1/04 - 9/30/06: $769,493

Cost Sharing for period: $192,373