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Characterizing microbial communities dedicated for conversion of coal to methane in situ and ex situ

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ABSTRACT

To enhance methane production in situ in bituminous coal seams, distribution of microorganisms in the formation water collected from a coalbed methane well was investigated. Based on next generation DNA sequencing, both bacteria (231 species) and archaea (33 species) were identified. Among the bacterial kingdom, polymer-degrading, benzoate, fatty acid and sugar utilizing bacteria were dominant. Among the archaea domain, the major methanogens (89.8%) belonged to the order of *Methanobacteriales* which are hydrogenotrophic. To develop a microbial consortium for ex situ coal bioconversion, the original microbial community was adapted to ground coals for five months in a laboratory environment. DNA sequencing revealed the presence of 185 bacteria species and nine archaea species which were dramatically different from those in the original formation water. In particular, the majority (90.4%) of methanogens were under the order of *Methanomicrobiales*. To increase methane production, two nutrient solutions were tested. Solution #2 which targeted methanogens provided a methane yield of 111 ft^3/ton in 20 days, which translated to a 5.6 ft^3/ton-day. In addition, the adapted consortium was found to be aerotolerant.

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1. Introduction

It is believed that an option for reducing CO_2 emissions is to replace coal-fired power plants with natural gas-fired ones. According to US Energy Information Administration's estimation, burning natural gas releases 1.21 pounds of CO_2 per kWh, which is approximately 60% of 2.07–2.17 pounds of CO_2 per kWh emitted from coal (bituminous to lignite). In addition, burning natural gas emits less sulfur, mercury and particulate matter than coal (Agyarko and Mansoori, 2013). Furthermore, recent development in high-temperature and more efficient natural gas combustion turbines by different manufacturers makes natural gas-fired power plants an even better option. Finally, combusting natural gas produces a clean stream of CO_2, ready for storage and/or utilization. Thus, during recent decades, searching for unconventional natural gas has been on the rise world-wide.

Unconventional gas mainly includes three categories: 1) shale gas, which is trapped in fine grained sedimentary rock called shale, requiring "hydraulic fracturing" technology to be produced; 2) tight gas, which is embedded in relatively impermeable hard rock, limestone or sandstone, sometimes with quantified limit of permeability; and 3) coalbed methane (CBM), which is contained in coal seams and adsorbed in the solid matrix of coal (McGlade et al., 2013). While production of shale gas increased ten-fold between 2006 and 2010, considering the extremely high uncertainty, it is still far from clear whether the expectations of huge and easily recoverable shale gas can be ultimately fulfilled (Berman, 2010; Berman and Pittinger, 2011). In addition, the shale gas extraction process, "fracking", has led to heated debates regarding its environmental impacts. For example, first, fracking necessitates high-pressure injection of water, chemicals and sand into shale formations to create and open fractures that enable hydrocarbons to flow. This practice leaves millions of gallons of water, mixed with additives, in the ground, which could result in pollution of underground aquifers; second, it is claimed that shale gas emissions of greenhouse gases are even higher than the conventional natural gas emissions, and may be equal or higher than the emissions caused by using coal or petroleum (Howarth et al., 2011).

Thus, considering the significant uncertainty, high cost and negative environmental impact associated with shale gas and difficulties in extracting tight gas, CBM is the best option among the three. CBM or microbially enhanced CBM (MECBM) through microbial processes appears to be favored in lower-rank coals, such as lignite or subbituminous coal, which have high permeability and highly branched compounds that may be accessible to microorganisms. Typical examples include, but are not limited to: Powder River Basin coals (Ayers, 2002; Flores et al., 2008; Ulrich and Bower, 2008), San Juan Basin coals (Scott et al., 1994) and south Sydney Basin coals (Faiz and Hendry, 2006). In the US, commercial production of CBM is mainly in states west of the Mississippi river, where 83.7% of coal is either subbituminous or lignite. For states that are east of the Mississippi river, where 96.4% of coal is

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bituminous (EIA), CBM has not been very successful. For example, State of Illinois has the largest overall as well as strippable bituminous coal reserves in the nation (Agyarko and Mansoori, 2013) but very limited CBM production due to low gas production rates. To solve this issue, the first objective of this study sought to understand the methane producing potential of an Illinois coal seam through elucidating structure of the microbial community in the formation water. Accomplishment of this objective would open doors for enhancing methane yield from this site.

Besides CBM, where methane is released in situ, methane can also be produced from mined out coals through surface mining and coal waste. Using coal waste as an example, about 55 million tons of waste coals are generated annually in US (Tillman and Harding, 2004). For the state of Illinois, around 5 million tons of mined coals are fine and/or ultrafine coal, and considered waste. This translates into an approximately $250 M loss to the Illinois coal industry (Chen et al., 2003). In addition to economic loss, coal wastes are either piled up at mine sites or dumped in ponds where the environmental risk is extremely high but difficult to assess.

In order to address the coal waste issue and utilize mined out coals for methane production instead of burning for electricity, the second and third objectives of this investigation aimed to develop/characterize an adapted microbial consortium that can be used for bioconversion of coal to methane ex situ and evaluate the effect of biostimulation on methane yield from bituminous coals collected from the Illinois basin, respectively. Keeping potential future large scale operation in mind, the adapted microbial consortium was intentionally exposed to air to select those that are aerotolerant. Therefore, this is the first study to report: 1) detailed population distribution of a microbial community originally in the Illinois basin; 2) microbial structure of an adapted consortium that tolerate air exposure and is still active in methane production from coal; and 3) methane yield from Illinois coals operated ex situ. The major advantage of using the adapted consortium is that the delicate, expensive, cumbersome and strict anaerobic environment for handling anaerobic cultures can be avoided. The developed microbial consortium can thus be used ex situ in industrial scale bioreactors or injected into coal seams where indigenous community capable of converting coal to methane is not available. Such coal seams have been reported for Sydney and Port Philip basins in Australia (Li et al., 2008).

2. Materials and methods

2.1. Coal samples

Chunks of coals were collected from a coal mining site (38.2461° N, 89.7528° W, 450–500 ft) in Washington County, Illinois (Fig. 1). This coal mine is part of the Herrin Seam, # 6 of the Illinois basin. We chose to study coals from this seam considering that the Herrin seam is one of the two regions in the Illinois Basin where most of the mining activity is currently taking place and a significant amount of methane extraction is currently underway. This seam is known to contain high volatile B bituminous coal (Korose and Elrick, 2010). Results from proximate and ultimate analyses of the coals used in this study also confirmed the same coal rank (Table 1). To simulate mined out coal or coal waste, the collected coal samples were not kept in a strict anaerobic chamber, but instead immersed in water in a bucket at room temperature. Prior to testing, the coal was ground and only the portion that passed through a 40 mesh (<0.42 mm) screen was kept in ziploc bags and maintained in a humidity chamber to avoid water loss.

2.2. Microbial community in the formation water

2.2.1. Genomic DNA extraction

Formation water was obtained from a CBM operation in southern Illinois. Water was retrieved from a depth of ~850 ft. It was sealed tight in five-gallon containers and brought to our laboratories, where it was immediately transferred to smaller containers and stored in a refrigerator. To select aerotolerant microorganisms, reducing agents, such as sodium sulfide was intentionally not added.

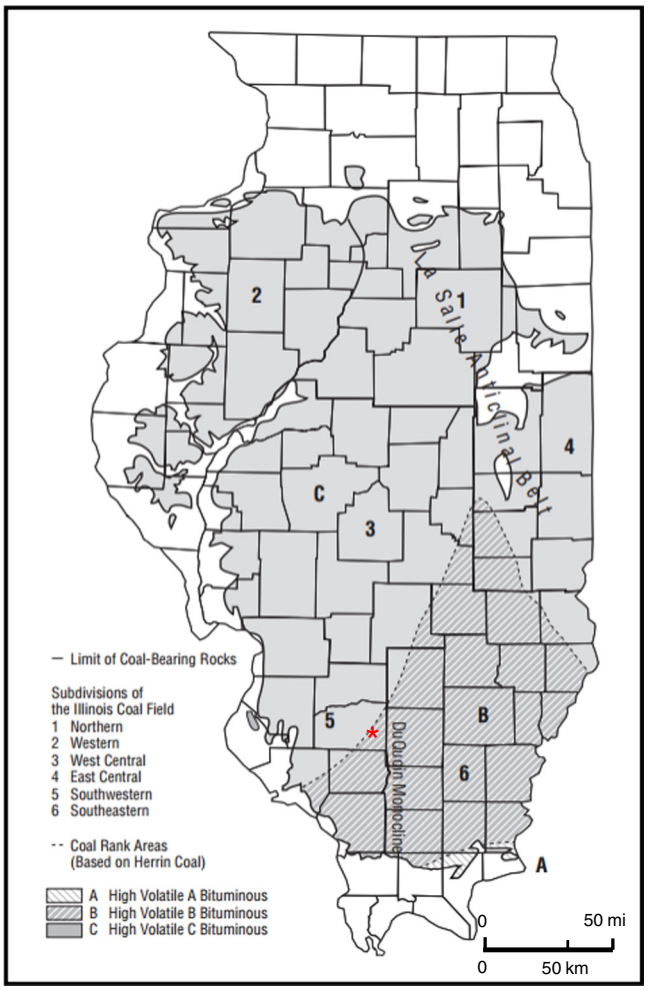

Fig. 1. Location of the site (red star) where coal samples were collected. Adapted from Korose and Elrick, 2010. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

To extract genomic DNA from the retrieved water, 1 L of water sample was passed through a membrane filter (90 mm, 0.22 μm). The formation water contained suspended coal particles. In order not to lose those microorganisms attached to coal particles, all that were retained on the filter were then processed for DNA extraction using Powerwater DNA extraction kit (Mo Bio, Carlsbad, CA, USA). Following extraction, DNA samples were quantified and evaluated through use of a Nanodrop

Table 1
Ultimate and proximate analysis of the coal samples used.

Parameter	Average ± STDEV (%)
Ultimate analysis (dry basis)	
Carbon	70.07 ± 0.36
Nitrogen	1.39 ± 0.01
Hydrogen	5.21 ± 0.03
Sulfur	0.63 ± 0.03
Oxygen	15.41 ± 0.20
Proximate analysis (dry basis)	
Ash	7.50 ± 0.05
Volatile matter	49.93 ± 0.18
Fixed carbon	42.58 ± 0.17
Heating value (BTU/lb)	12,547.50 ± 36.06

spectrophotometer. Three DNA samples with excellent quality (A_{260}/A_{280}: 1.8–2.0) and high concentrations (30–50 ng/μL) were subject to sequencing.

2.2.2. DNA sequencing

For each sample, three different assays were conducted. To understand the overall diversity of the microbial population, the 16S rRNA gene V4 variable region PCR primers 515/806 (Caporaso et al., 2011) were used. Single-step PCR using the HotStarTaq Plus Master Mix Kit (Qiagen, Valencia, CA, USA) was performed under these conditions: 94 °C for 3 min, 28 cycles of 94 °C for 30 s, 53 °C for 40 s and 72 °C for 1 min, and a final elongation step at 72 °C for 5 min. Sequencing was conducted at Molecular Research (Shallowater, TX, USA) on an Ion Torrent PGM following the manufacturer's guidelines. Sequence data (15–20,000 reads/assay) were processed using a proprietary analysis pipeline (Molecular Research, Shallowater, TX, USA). In summary, barcodes, primers, sequences < 150 bp, and sequences with ambiguous base calls and with homopolymer runs exceeding 6 bp were removed. Following the removal, sequences were denoised, chimeras were removed and operational taxonomic units (OTUs) were generated. OTUs were defined by clustering at 3% divergence (97% similarity) (Dowd et al., 2008; Edgar, 2010). Final OTUs were taxonomically classified using BLASTn against a curated GreenGenes database (DeSantis et al., 2006) and compiled into each taxonomic level into both "counts" and "percentage" files. Count files contain the actual number of sequences while the percent files contain the relative (proportion) percentage of sequences within each sample that map to the designated taxonomic classification.

To elucidate distribution of eubacterial species, the 16S eubacterial primers 27 F (AGRGTTTGATCMTGGCTCAG) and 519 R (GTNTTACNGCGGCKGCTG) were used for amplifying the 492 bp region of 16S rRNA genes through 454 pyrosequencing. To understand diversity within the archaea kingdom, in particular, methane-producing methanogens, a primer pair of 86 F (GCTCAG TAACACGTGG) and 448R (GCGGCGGCTGGCACC) was adopted for 454 pyrosequencing as well. Both 454 sequencing provided 3000 reads/assay. PCR conditions were the same as explained above. Following PCR, all amplicon products from different samples were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA, USA). Samples were sequenced by Molecular Research (Shallowater, TX, USA) utilizing Roche 454 FLX titanium instruments and reagents following the manufacturer's guidelines. Sequencing data were processed and analyzed in the same way as detailed above.

2.3. Converting coal to methane

Microorganisms that were initially in formation water and collected on membrane filters were added to 10 g of ground coal (<40 mesh) in three different media. The first medium was a standard Tanner medium dedicated for cultivating anaerobic bacterial strains (Tanner et al., 2007). The second medium was a MS medium for growing methanogens (Bonin and Boone, 2006). The third one was formation water depleted with microbes after filtration. All media were autoclaved at 121 °C for 30 min before use.

Once cells were added to a serum bottle (100 mL) containing 10 g of coal and 50 mL of solution, the bottle was closed with a butyl rubber stopper and sealed by an aluminum crimp. Each bottle was purged with N_2 for around 30 min to drive out air. After the entire content in each bottle appeared to be colorless, all bottles were kept in dark at 28 °C. To test effects from different medium, nutrient solution # 2 and # 3 were investigated at their original strength (100%) with two replicates. For medium # 1 and # 2, two other concentrations, 20% and 50% of the initial strength were also studied. Dilution to these lower concentrations was made by adding deionized and distilled water (DDW). At different time points, the headspace was sampled to obtain concentrations of methane and CO_2 by using a gas chromatograph (GC) as detailed below.

After 65 days of cultivation, cells in the microcosms with 100% medium #2 were used to inoculate 10 g of fresh coal samples. Briefly, 5 mL of inoculum was added to 10 g of coal with 45 mL of medium #2. After setting up four microcosms under the same conditions, two bottles were purged with N_2 while the other two were not. All microcosms were maintained at 28 °C in darkness. On day 10, 20 and 30, the headspace gas was tested for methane and CO_2 concentrations using GC as detailed below.

2.4. DNA sequencing of the adapted microbial consortium, coal, and coal residue

Following 30-day cultivation of the aforementioned microcosms, the entire content was allowed to settle. The liquid phase was referred to as the adapted consortium, the solid portion was named as coal residue. The liquid phase was withdrawn and centrifuged at 5000 *g* for 10 min to collect suspended cells. These cells, coal residue (2.56 g, wet weight) and coal (1.5 g) that was used in the above experiments were subjected to DNA extraction separately through use of the Powerwater DNA extraction kit with a slightly modified procedure to fit into our application. All extracted DNA samples were handled in the same way as described above for the original community in the formation water. Three sequencing assays were performed on the three sets of samples using the same procedures described above.

2.5. Analysis

A Shimadzu gas chromatograph (GC) 17A with a Flame Ionizing Detector (FID) was used to measure methane and CO_2 content in headspace of serum bottles. Briefly, a 50 μL aseptic syringe connected to a sterile 25 gauge needle was used to withdraw and inject a 25 μL of gas sample to a 60 m × 0.53 mm RT-Msieve 5A molecular sieve capillary column (Restek Corp., Bellefonte, PA, USA). The carrier gas (Argon) flow rate was set at 10.1 mL/min with a velocity of 55 cm/s. The isothermal zone temperatures for the injector and detector were set at 75 °C and 310 °C, respectively. The retention time for methane was 4.73 min and that for CO_2 was 6.71 min. Calibration curves for methane and CO_2 were established using standard gases (Air Liquide, Plumsteadville, PA, USA).

3. Results and discussions

3.1. DNA sequencing: the original microbial community vs. the adapted consortium

Producing methane from coal through microbial processes requires the collective actions of microorganisms comprising three major metabolic groups: 1) hydrolytic and fermentative bacteria; 2) acetogenic bacteria, and 3) methanogenic archaea. During the initial stage of gas production, complex organic compounds in coals are decomposed to simpler molecules, such as, acetate, long chain fatty acids, CO_2, H_2, CH_4, and HS^- by fermentative anaerobes. Fatty acids, alcohols, and some aromatic and amino acids are then converted to H_2, CO_2, and acetate by H_2-producing acetogens while H_2-using acetogenic bacteria consume H_2 and CO_2 to produce more acetate. Finally, simple molecules are transformed to CH_4 by methanogens belonging to the domain of Archaea (Faiz and Hendry, 2006).

For all samples sent for sequencing, the results among three replicates were basically the same. Thus, the DNA extraction process we adopted is highly reliable and repeatable. Through the diversity assay using the 515/806 primer pair, it was revealed that (Fig. 2): 1) the formation water contained 44% of bacterial strains and 56% of archaea; 2) DNA extracted from the coal samples that were used in our studies comprised 92.6% of bacteria, 2.3% of fungi, 0.7% of archaea and 4.4% of

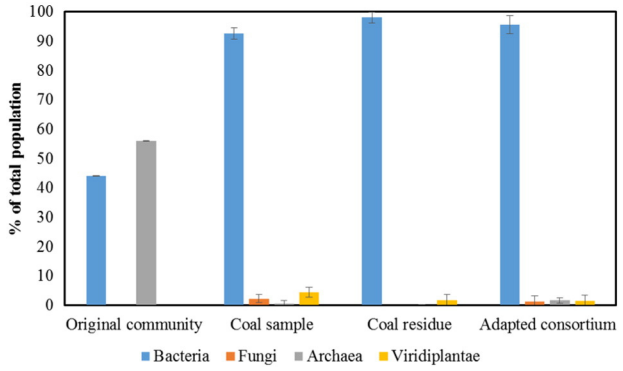

Fig. 2. Distribution of different kingdoms for DNA extracted from the original community, coal samples, coal residue and the adapted consortium.

viridiplantae (green plants); 3) DNA from coal residue after one-month of bioconversion consisted of bacteria, fungi, archaea and viridiplantae in the percentage of 98.0%, 0.04%, 0.09% and 1.83%, respectively; and 4) DNA from the adapted consortium cultivated on coal after one-month was composed of 95.6% of bacteria, 1.4% of fungi, 1.6% of archaea and 1.4% of viridiplantae.

For the formation water, the 515 F assay revealed 231 bacterial species and 33 archaea at the species level. The 16 F and 86 F assay recovered 147 bacteria and 25 methanogen species, respectively. The difference lies in the fact that the 515 F assay provided a much more in-depth sequencing compared to the other two. However, the dominant species disclosed between the 515 F and 16 F assays for bacterial species and those between the 515 F and 86 F analyses for archaea were very similar. Hence, only results from the 515 F assay were reported for both the bacteria and archaea kingdom.

Among all of the bacteria identified in the original microbial community, the major populations were: *Meniscus spp.* (11.1%), *Syntrophus sp.* (10.0%), *Ruminococcus spp.* (8.1%), *Dasania spp.* (4.7%), *Candidatus_solibacter spp.* (4.4%), *Crocinitomix spp.* (3.6%), *Desulfofustis spp.* (3.5%), *Thermovirga spp.* (3.5%), *Sulfurimonas spp.* (3.4%), *Desulfuromusa spp.* (3.4%), *Maritimimonas spp.* (3.3%), *Alkaliflexus spp.* (2.8%), *Desulfuromonas spp.* (2.8%), and *Desulfotignum spp.* (2.1%) (Fig. 3a). The genus of *Meniscus* is known to be aerotolerant and heterotrophic bacteria (Irgens, 1977). They can grow on a broad range of mono- and disaccharides provided the concentration of CO_2 is more than 1%. The *Syntrophus* genus includes anaerobic bacteria that are shown to catabolize benzoate and fatty acids in syntrophic association with hydrogen-using methanogens (Jackson et al., 1999; Mountfort et al., 1984). *Ruminococcus spp.* are generally found in rumen fluid and are featured by their capability to degrade cellulose and other plant-based polymers (Klieve et al., 2005; Koike and Kobayashi, 2001). Research on *Dasania spp.* is few. The only publication on a new species of *Dasania* described it as a nitrate reducer to nitrogen within the order of *Pseudomonadales* (Lee et al., 2007). *Candidatus_solibacter spp.* belongs to the phylum of *Acidobacteria* which are widespread in soils and sediments worldwide, and are abundant in many soils (Challacombe et al.,

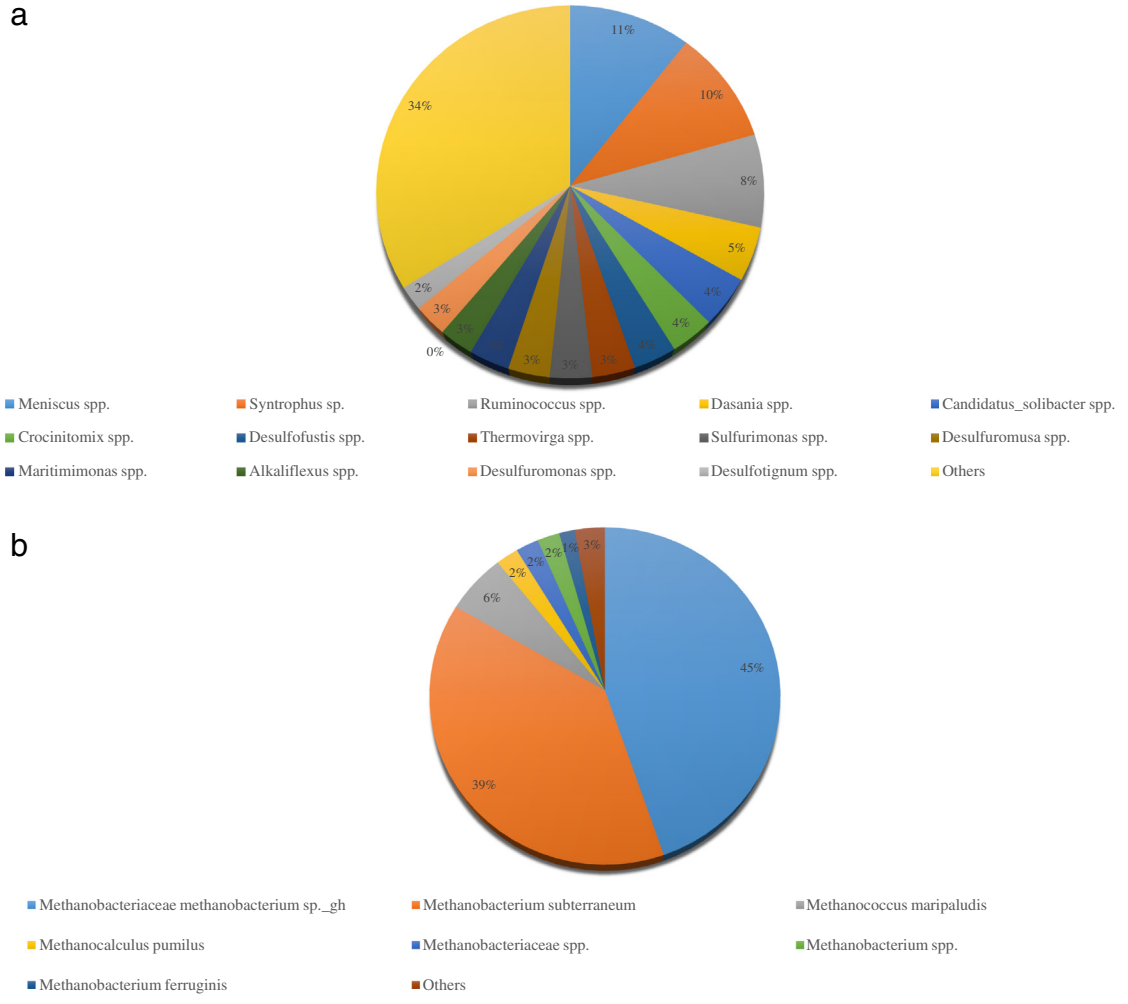

Fig. 3. Diversity of microbes in the formation water. a: bacterial species; b: archaea species.

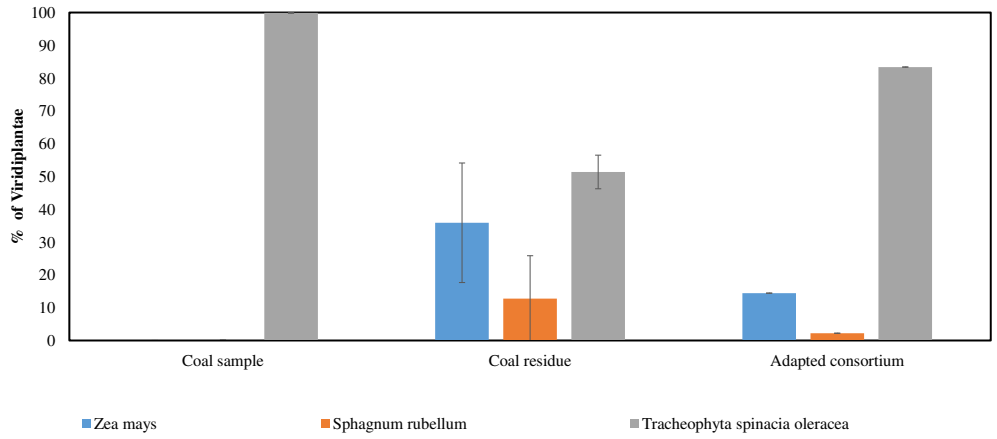

Fig. 4. Distribution of *Viridiplantae* (green plant DNA) for coal samples, coal residue and the adapted consortium.

2011). Exact functions of this genus is unclear except they may be acidophilic based on their lineage.

Among the top 13 Genera, *Desulfofustis spp.*, *Desulfuromusa spp.* and *Desulfotignum spp.* are related to dissimilatory sulfate reduction. For example, the Genus of *Desulfuromusa spp.* is categorized as obligately anaerobic, sulfate and Fe (III)-reducing bacteria (Liesack and Finster, 1994; Vandieken et al., 2006). They were picked by the next generation sequencing, but were not detected in clone libraries developed from samples collected from the same, although different part of the Illinois basin (Strąpoć et al., 2008). In addition, *Sulfurimonas spp.* which can oxidize sulfur to sulfate was also observed. Thus, in the coal mine where the formation water was sampled, sulfate reduction and oxidation are actively performed by different microbial strains.

Among all identified archaea species in the original community, the dominant ones at the species level were: *Methanobacteriaceae methanobacterium* sp. (44.7%); *Methanobacterium subterraneum* (38.7%); *Methanococcus maripaludis* (5.8%) *Methanobacteriaceae spp.* (2.2%), and *Methanocalculus pumilus* (2.1%) (Fig. 3b). Thus, the majority (89.8%) of archaea belonged to the order of *Methanobacteriales*. The *Methanobacteriales* are generally hydrogenotrophic, using hydrogen to reduce CO_2 to CH_4. Formate, CO and secondary alcohols can also be used as electron donors by some members of this order for CO_2 reduction (Bonin and Boone, 2006).

Besides *Methanobacteriales*, the order of *Methanococcales* and *Methanomicrobiales* were 5.8% and 3.6% of the total, respectively. Based on these results, enhancing methane production in situ may benefit from providing H_2 and/or other electron donors to stimulate methanogenic activities.

Regarding the coal samples that were used in this study, it is surprising to identify green plant DNA. As shown in Fig. 4, the majority or 99.9% of green plant DNA in the original coal belonged to *Tracheophyta spinacia oleracea* (Spinach). The *Sphagnum rubellum* (Red Peatmoss) DNA was 0.1%. But for coal residue and the adapted microbial consortium, DNA from *Zea mays* (corn) and Red Peatmoss was present and had a larger representation compared to that in the original coal samples. Here, we hypothesized that all of these green plant DNA originated from the biomass that was the initial material for coal formation. As coal degradation took place in the microcosms, more DNA in coal was mobilized and showed up in the extracted samples. Although proving this hypothesis requires more investigations, it is interesting to get a glimpse of the origin of Illinois coal.

In addition to DNA from green plants, fungal DNA was also detected in the coal samples that we have used (Fig. 5). For untreated coals, DNA from *Emericella variecolor* (61.1%), *Cryptomyces maximus* (20.4%), *Amylomyces rouxii* (10.7%), and *Zygozyma arxii* (7.8%) was identified. For coal residue and the adapted consortium, however, different fungal DNA from *Phoma herbarum* (a fungal plant pathogen), *Didymocrea sadasivanii* (a filamentous fungus), *Bimuria novae_zelandiae* (isolated from barley field) and *Byssochlamys nivea* (an extremely heat resistant mold) were observed. Since the formation water had no fungal strains, the fungal DNA revealed in the adapted consortium must be from coals added to the microcosms. The presence of fungal DNA in the original coal samples could be from: 1) fungal strains initially associated with coal. During coal formation, some fungal DNA was preserved in the process; 2) fungal strains in air and water. As described above, the coal samples were intentionally exposed to air and water during storage to simulate mined out coals and coal wastes. The exact source of these DNA deserves further investigation. To explain the difference between DNA detected in the original coal and that in the coal residue/adapted consortium, we hypothesized that: 1) during coal bioconversion, more DNA was mobilized as porosity of coal was increased; and 2) some DNA associated with the original coal was degraded by enzymes released from the mixed consortium. Again, proving this hypothesis demands further studies.

It is not surprising to find microorganisms that are associated with coal as they have been reported for subbituminous coals at Texas

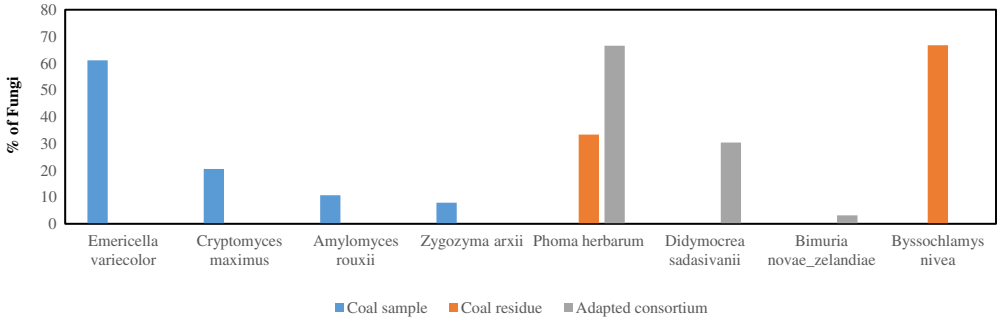

Fig. 5. Distribution of fungal DNA for coal samples, coal residue and the adapted consortium.

(Jones et al., 2010) and anthracite coal in a coal mine in Hubei, China (Wei et al., 2014). For the former, only bacteria species was identified. Cloning methanogens and sulfate-reducing bacteria was proven to be unsuccessful. For the latter, both bacteria and archaea were detected through pyrosequencing and quantified by real time PCR. The total population for each kingdom was estimated to be 10^5 and 10^4 cells/g coal, respectively. Methanogens were calculated as 10^4 cells/g coal and 10^5 cells/mL of mine water.

In terms of coals used in this study, it contained 113 different bacterial species (Fig. 6a). The top species were *Pseudomonas putida* (35.1%), *Staphylococcus pasteuri* (11.2%), *Acinetobacter septicus* (4.3%), *Staphylococcus epidermidis* (2.7%), *Luteipulveratus mongoliensis* (2.6%) and *Ruminococcus torques* (2.6%). For the coal residue left after bioconversion, 109 species were detected (Fig. 6b). The major ones were: *Pseudomonas putida* (26.7%), *Erwinia pantoea dispersa* (7.7%), *Pantoea agglomerans* (6.0%), *Candidatus_chloracidobacterium spp.* (3.5%), *Rubellimicrobium spp.* (3.2%), *Methylobacterium adhaesivum* (3.2%), *Pantoea spp.* (3.1%), *Methylobacterium aquaticum* (3.0%), *Staphylococcus pasteuri* (2.9%), *Clostridium bifermentans* (2.6%), *Pseudomonas oryzihabitans* (2.3%), and *Massilia spp.* (1.8%). With regard to the adapted consortium, a total of 185

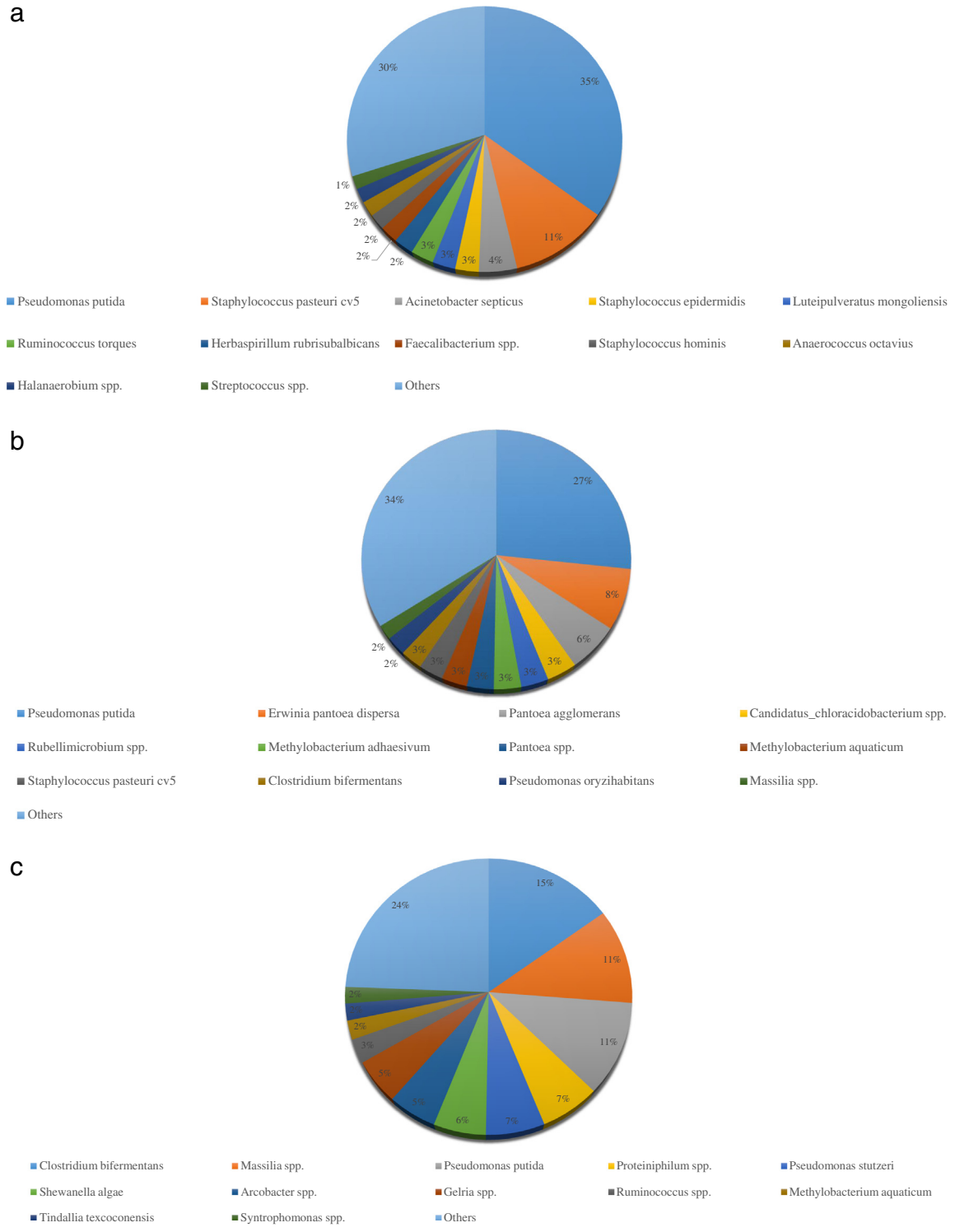

Fig. 6. Diversity of bacterial species in different samples. a: coal samples used for this study; b: coal residue after bioconversion for 30-days; c: adapted consortium.

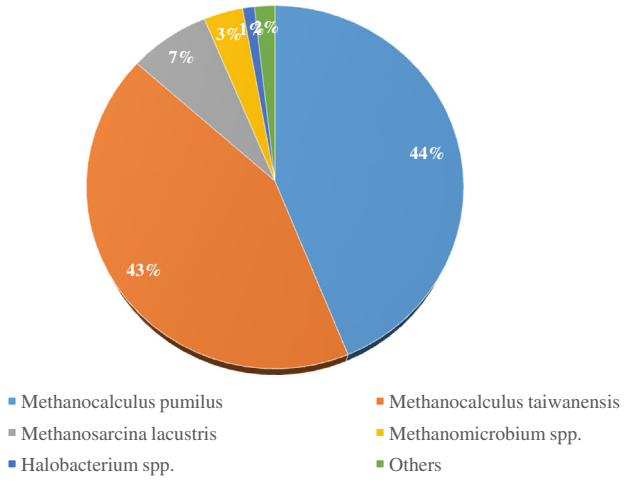

Fig. 7. Diversity of archaea species in the adapted consortium.

species were identified (Fig. 6c). The abundant ones were: *Clostridium bifermentans* (15.1%), *Massilia spp.* (11.1%), *Pseudomonas putida* (11.1%), *Proteiniphilum spp.* (6.5%), *Pseudomonas stutzeri* (6.4%), *Shewanella algae* (5.7%), *Arcobacter spp.* (5.3%), *Gelria spp.* (2.8%), *Ruminococcus spp.* (2.2%), *Methylobacterium aquaticum* (2.0%), *Tindallia texcoconensis* (2.0%), and *Syntrophomonas spp.* (1.9%).

Comparing bacterial species in the original formation water and those in the adapted consortium, a dramatic difference was observed. Among the top ten species in the formation water, except *Rumicoccus spp.* whose percentage decreased from 8.1% to 2.8% in the adapted consortium, the other top nine bacterial species originally in the formation water became minor ones. In contrast, some less prevalent species became dominant in the adapted consortium. However, since they were present in both the formation water and in the coal samples, it is not clear at this point where exactly they came from. Undoubtedly, these species, under the experimental conditions adopted for this study, might be more competitive than those predominant ones initially in the formation water.

The class of *Clostridia* was found to dominate in an enrichment culture derived from formation water collected from subbituminous coal beds in western Canada (Penner et al., 2010). It was also reported to prevail in yeast extract amended CBM well water samples (Green et al., 2008; Li et al., 2008). *Clostridium bifermentans* was identified as hydrogen producers when grown on wastewater sludge (Wang et al., 2003). The genus *Massilia* belongs to the family of *Oxalobacteraceae* and class of *Betaproteobacteria*. Research on this genus is not much. But, novel species, such as *Massilia tieshanensis* isolated from mining soil (Du et al., 2012) and *Massilia kyonggiensis* isolated from forest soil in Korea (Kim, 2014) were demonstrated to have no growth under anaerobic conditions (Du et al., 2012). Apparently, the strains in the adapted consortium were different from those isolated and studied. Similarly, identification of *P. putida* in the adapted consortium contradicts the common recognition that this species includes strictly aerobic bacteria (Sohn et al., 2010). Therefore, the adapted consortium may contain some novel strains that have not been investigated and reported so far. Apart from these controversies, *Proteiniphilum spp.* are reported as obligately anaerobic strains. For two *Proteiniphilum acetatigenes*, the major fermentation products from yeast extract and peptone are acetic acid and propionic acid (Chen and Dong, 2005). *P. stutzeri*, also detected in a Canadian coal mine, was identified as a denitrifier (Penner et al., 2010).

In terms of archaea identification, the coal samples contained only one species: *Methanobrevibacter spp.* For the coal residues, two species were detected: *Methanobrevibacter arboriphilus* (51.7%) and

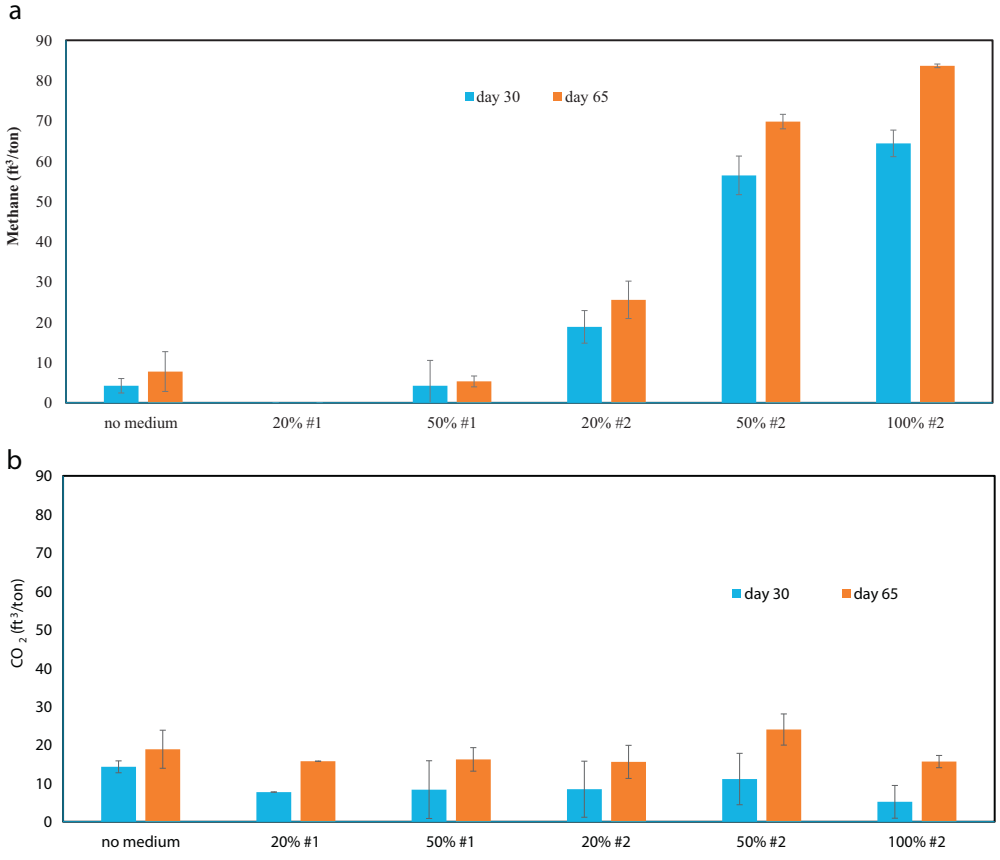

Fig. 8. Gas production from microcosms containing different nutrient supplementation. a: methane; b: CO_2.

Methanobacterium bryantii (48.3%). For the adapted consortium, a total of nine species were observed (Fig. 7): *Methanocalculus pumilus* (43.9%), *Methanocalculus taiwanensis* (42.7%), *Methanosarcina lacustris* (7.1%), *Methanomicrobium spp.* (3.5%), *Halobacterium spp.* (1.0%), *Methanosaeta spp.* (0.9%), *Thermoplasma spp.* (0.5%), *Methanocalculus halotolerans* (0.4%), and *Methanobacterium ferruginis* (0.01%). Thus, in the adapted consortium, the majority of archaea belonged to the *Methanocalculus* genus and the *Methanomicrobiales* order. Again, comparing with archaea strains initially in the formation water, the minor order of *Methanomicrobiales* became the dominant one (90.4%) while the major order of *Methanobacteriales* turned to be the minor one (0.01%).

Both pure strains of *Methanocalculus pumilus* and *Methanocalculus taiwanensis* have been isolated and studied. The former was isolated from a waste-disposal site containing high concentrations of metals (Mori et al., 2000) and the latter was isolated from an estuarine environment in Taiwan (Lai et al., 2002). Belonging to the same genus of *Methanocalculus*, both strains can perform methanogenesis from formate and CO_2/H_2. Acetate was required for cell growth, but this substrate cannot be converted to methane. Knowing the presence of either these two methanogens or other strains that are similar to these two in the adapted consortium will assist us in designing strategies to maximize methane production from coal.

3.2. Methane yield from coal

To evaluate whether microorganisms in the formation water can convert coal to methane ex situ, we first tested methane yield with the addition of two different media. As shown in Fig. 8a, compared with the control (no medium) which contained only the formation water and concentrated microbial cells, medium #1 which was the Tanner medium specifically for strict anaerobes, did not have much effect at two concentrations studied: 20% and 50%. Medium #2, however, demonstrated increased methane production with increased medium concentration, from 20%, 50% to 100%. For the microcosms with 100% medium #2, the methane yield was 84 ft^3/ton of coal, which translated to a methane production rate of 1.3 ft^3/ton/day in 65 days. For this study, we stopped the experiment at day 65 since methane production rate (ft^3/ton-day) between day 30 and 65 was lower than that during the first 30 days. This could be due to many reasons, such as: inhibitory gas and liquid compounds in the reactors, nutrient depletion, etc. Currently, we are conducting comprehensive experiments to understand the coal bioconversion process and increase the methane production rate. Although methane yield was all different under different conditions, there was no statistically significant difference in terms of CO_2 concentration in all of the microcosms (Fig. 8b). The presence of CO_2 indicated that the microbial community was active even though for some, no or little methane was detected.

To test whether purging with N_2 was necessary after the microcosms were just established, we used 100% medium #2 and acclimated cells from our first experiment to start the second round of testing. For two microcosms, purging was conducted for 30 min. For another two, no purging was performed. This time, we observed must faster generation of CH_4 (Fig. 9a) and CO_2 (Fig. 9b). With regard to methane production rate, the acclimated cells gave 5.6 ft^3/ton/day during the first 20 days. In terms of CO_2, the release rate in the first 10 days was 6.14 ft^3/ton/day, which was much higher than 0.24 ft^3/ton/day observed during

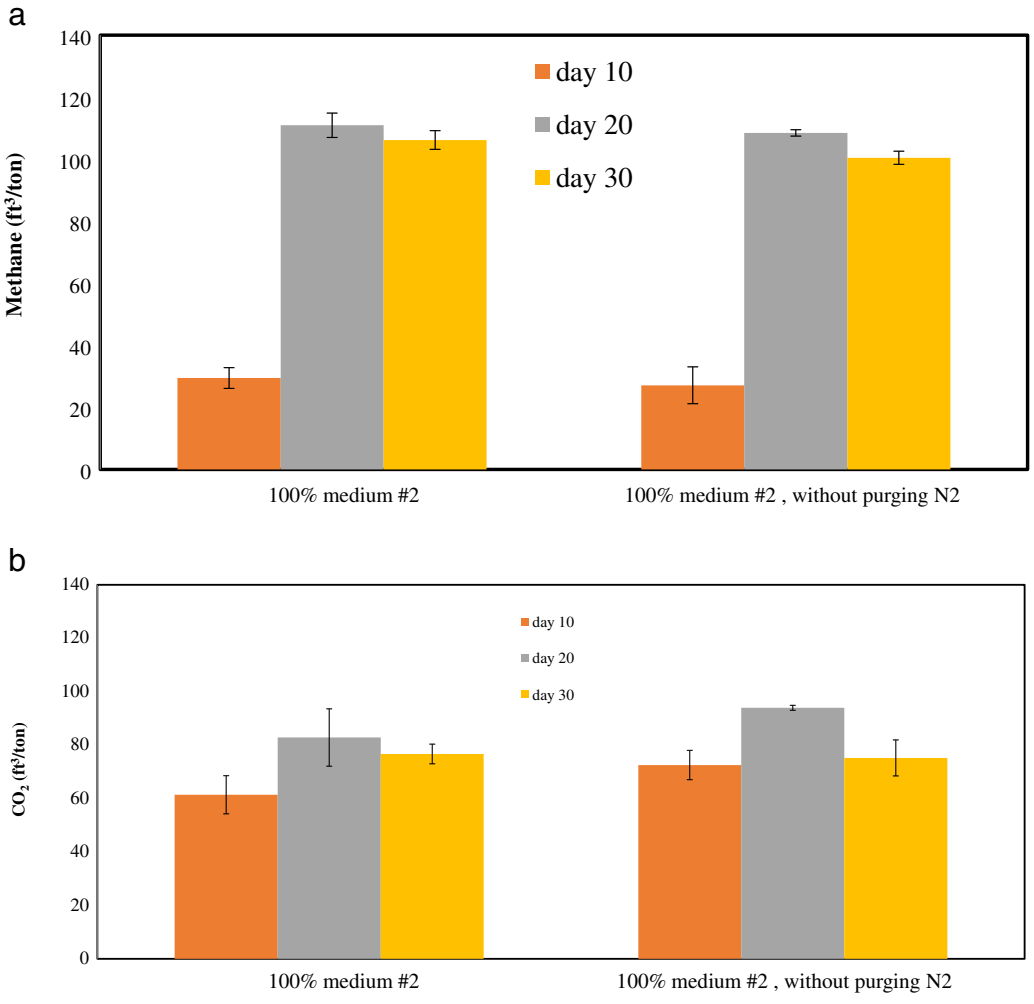

Fig. 9. Effect of purging on gas production from microcosms. a: methane; b: CO_2.

the first experiment. Regarding with or without nitrogen purging, between these two setups, there was no statistically significant difference in terms of yield of CO_2 and CH_4, which suggests that the microcosms can be initiated under a relaxed, and not strictly anaerobic condition. In other words, the microbial consortium is highly robust and nitrogen purging is not needed. Since CO_2 can be reduced to CH_4 if hydrogen is present, the high concentration of this gas indicated that the cultures might be hydrogen-limited.

The approach of converting coal to methane through biological processes has been explored by several research groups. The majority of studies on this aspect have been conducted in a strictly anaerobic environment with coal and the microbial community handled in a glove box (Green et al., 2008; Harding et al., 1993; Jones et al., 2010; Papendick et al., 2011). The only exception is the one reported by Opara et al. (2012) where both coal and the microbial inoculum were exposed to air during sample collection, transfer and handling. But even with air exposure, the methane production rate of 73.1 ft^3/ton/day was the highest among bituminous coals. This could be due to the smallest particle size (<74 μm) among all studies reported or owing to the robustness of the microbial community collected from a waste coal environment.

For this study, we took a similar strategy of evaluating methane production from coal under a relaxed anoxic condition. We strongly agree with the statement given by Opara et al. (2012) that an aerotolerant microbial consortium is a crucial element for large scale application of bioconversion of coal to methane. The statement is true for two scenarios: 1) injection of a microbial consortium to coal mines where indigenous microbial community is either unable or has limited capability to produce methane from coal; and 2) conversion of mined out coal or coal waste to methane ex situ.

As discussed above, in this study, we obtained a methane production rate of 5.6 ft^3/ton/day and a methane yield of 111 ft^3/ton in 20 days. This production rate is higher than 2.1 ft^3/ton/day from low volatile bituminous (Fallgren et al., 2013), 2.7 ft^3/ton/day from subbituminous B coals (Green et al., 2008), and 0.8 ft^3/ton/day from subbituminous coals (Jones et al., 2010). However, it is lower than 30 ft^3/ton/day reported for subbituminous coals from Australia (Papendick et al., 2011). This high methane production rate was obtained at 37 °C and with mixing at 50 rpm. Overall, we have proven that the approach of converting coal to methane ex situ is feasible. Further increasing methane yield may benefit from evaluating other parameters, such as, temperature, pH, coal particle size, mixing and the presence of solvent and/or surfactant, etc.

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References

Agyarko, L.B., Mansoori, G.A., 2013. A review of non-renewable energy options in Illinois. Int. J. Oil Gas Coal Technol. 6, 288–347.
Ayers, W.B., 2002. Coalbed gas systems, resources, and production and a review of contrasting cases from the San Juan and powder river basins. Am. Assoc. Pet. Geol. Bull. 86, 1853–1890.
Berman, A.E., 2010. Shale gas—abundance or mirage? Why the Marcellus Shale will disappoint expectations. Energy Bull.
Berman, A.E., Pittinger, L., 2011. US shale gas: less abundance, higher cost. The Oil Drum. http://www. theoildrum. com/node/8212.
Bonin, A.S., Boone, D.R., 2006. The order methanobacteriales. The Prokaryotes. Springer, pp. 231–243.
Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Lozupone, C.A., Turnbaugh, P.J., Fierer, N., Knight, R., 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc. Natl. Acad. Sci. 108, 4516–4522.
Challacombe, J.F., Eichorst, S.A., Hauser, L., Land, M., Xie, G., Kuske, C.R., 2011. Biological consequences of ancient gene acquisition and duplication in the large genome of *Candidatus Solibacter usitatus* Ellin6076. PLoS One 6, e24882.
Chen, S., Dong, X., 2005. *Proteiniphilum acetatigenes* gen. nov., sp. nov., from a UASB reactor treating brewery wastewater. Int. J. Syst. Evol. Microbiol. 55, 2257–2261.
Chen, S.S., Khan, L., Rostam-Abadi, M., 2003. A novel fine coal dewatering process with integrated biomass utilization. ICCI Final Technical Report.
DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L., 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbiol. 72, 5069–5072.
Dowd, S.E., Callaway, T.R., Wolcott, R.D., Sun, Y., McKeehan, T., Hagevoort, R.G., Edrington, T.S., 2008. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). BMC Microbiol. 8, 125.
Du, Y., Yu, X., Wang, G., 2012. *Massilia tieshanensis* sp. nov., isolated from mining soil. Int. J. Syst. Evol. Microbiol. 62, 2356–2362.
Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460–2461.
Faiz, M., Hendry, P., 2006. Significance of microbial activity in Australian coal bed methane reservoirs—a review. Bull. Can. Petrol. Geol. 54, 261–272.
Fallgren, P.H., Jin, S., Zeng, C., Ren, Z., Lu, A., Colberg, P.J.S., 2013. Comparison of coal rank for enhanced biogenic natural gas production. Int. J. Coal Geol. 115, 92–96.
Flores, R.M., Rice, C.A., Stricker, G.D., Warden, A., Ellis, M.S., 2008. Methanogenic pathways of coal-bed gas in the Powder River Basin, United States: the geologic factor. Int. J. Coal Geol. 76, 52–75.
Green, M.S., Flanegan, K.C., Gilcrease, P.C., 2008. Characterization of a methanogenic consortium enriched from a coalbed methane well in the Powder River Basin, USA. Int. J. Coal Geol. 76, 34–45.
Harding, R., Czarnecki, S., Isbister, J., Barik, S., 1993. Biogasification of low-Rank Coal. Electric Power Research Inst., Palo Alto, CA (United States). ARCTECH, Inc., Chantilly, VA (United States).
Howarth, R.W., Santoro, R., Ingraffea, A., 2011. Methane and the greenhouse-gas footprint of natural gas from shale formations. Clim. Chang. 106, 679–690.
Irgens, R.L., 1977. *Meniscus*, a new genus of aerotolerant, gas-vacuolated bacteria. Int. J. Syst. Bacteriol. 27, 38–43.
Jackson, B.E., Bhupathiraju, V.K., Tanner, R.S., Woese, C.R., McInerney, M.J., 1999. *Syntrophus aciditrophicus* sp. nov., a new anaerobic bacterium that degrades fatty acids and benzoate in syntrophic association with hydrogen-using microorganisms. Arch. Microbiol. 171, 107–114.
Jones, E.J., Voytek, M.A., Corum, M.D., Orem, W.H., 2010. Stimulation of methane generation from nonproductive coal by addition of nutrients or a microbial consortium. Appl. Environ. Microbiol. 76, 7013–7022.
Kim, J., 2014. *Massilia kyonggiensis* sp. nov., isolated from forest soil in Korea. J. Microbiol. 52, 378–383.
Klieve, A.V., Yokoyama, M.T., Forster, R.J., Ouwerkerk, D., Bain, P.A., Mawhinney, E.L., 2005. Naturally occurring DNA transfer system associated with membrane vesicles in cellulolytic *Ruminococcus* spp. of ruminal origin. Appl. Environ. Microbiol. 71, 4248–4253.
Koike, S., Kobayashi, Y., 2001. Development and use of competitive PCR assays for the rumen cellulolytic bacteria: *Fibrobacter succinogenes*, *Ruminococcus albus* and *Ruminococcus flavefaciens*. FEMS Microbiol. Lett. 204, 361–366.
Korose, C.P., Elrick, S.D., 2010. Coal geology of Illinois. Keyst. Coal Ind. Man. 456–467.
Lai, M.-C., Chen, S.-C., Shu, C.-M., Chiou, M.-S., Wang, C.-C., Chuang, M.-J., Hong, T.-Y., Liu, C.-C., Lai, L.-J., Hua, J.J., 2002. *Methanocalculus taiwanensis* sp. nov., isolated from an estuarine environment. Int. J. Syst. Evol. Microbiol. 52, 1799–1806.
Lee, Y.K., Hong, S.G., Cho, H.H., Cho, K.H., Lee, H.K., 2007. *Dasania marina* gen. nov., sp. nov., of the order *Pseudomonadales*, isolated from Arctic marine sediment. J. Microbiol. 45, 505.
Li, D., Hendry, P., Faiz, M., 2008. A survey of the microbial populations in some Australian coalbed methane reservoirs. Int. J. Coal Geol. 76, 14–24.
Liesack, W., Finster, K., 1994. Phylogenetic analysis of five strains of gram-negative, obligately anaerobic, sulfur-reducing bacteria and description of *Desulfuromusa* gen. nov., including *Desulfuromusa kysingii* sp. nov., *Desulfuromusa bakii* sp. nov., and *Desulfuromusa succinoxidans* sp. nov. Int. J. Syst. Bacteriol. 44, 753–758.
McGlade, C., Speirs, J., Sorrell, S., 2013. Unconventional gas—a review of regional and global resource estimates. Energy 55, 571–584.
Mori, K., Yamamoto, H., Kamagata, Y., Hatsu, M., Takamizawa, K., 2000. *Methanocalculus pumilus* sp. nov., a heavy-metal-tolerant methanogen isolated from a waste-disposal site. Int. J. Syst. Evol. Microbiol. 50, 1723–1729.
Mountfort, D., Brulla, W., Krumholz, L.R., Bryant, M., 1984. Syntrophus buswellii gen. nov., sp. nov.: a benzoate catabolizer from methanogenic ecosystems. Int. J. Syst. Bacteriol. 34, 216–217.

Opara, A., Adams, D., Free, M., McLennan, J., Hamilton, J., 2012. Microbial production of methane and carbon dioxide from lignite, bituminous coal, and coal waste materials. Int. J. Coal Geol. 96, 1–8.
Papendick, S.L., Downs, K.R., Vo, K.D., Hamilton, S.K., Dawson, G.K., Golding, S.D., Gilcrease, P.C., 2011. Biogenic methane potential for Surat Basin, Queensland coal seams. Int. J. Coal Geol. 88, 123–134.
Penner, T.J., Foght, J.M., Budwill, K., 2010. Microbial diversity of western Canadian subsurface coal beds and methanogenic coal enrichment cultures. Int. J. Coal Geol. 82, 81–93.
Scott, A.R., Kaiser, W.R., Ayers Jr., W.B., 1994. Thermogenic and secondary biogenic gases, San Juan Basin, Colorado and New Mexico—implications for coalbed gas producibility. AAPG Bull. Am. Assoc. Pet. Geol. 78, 1186–1209.
Sohn, S.B., Kim, T.Y., Park, J.M., Lee, S.Y., 2010. In silico genome-scale metabolic analysis of *Pseudomonas putida* KT2440 for polyhydroxyalkanoate synthesis, degradation of aromatics and anaerobic survival. Biotechnol. J. 5, 739–750.
Strąpoć, D., Picardal, F.W., Turich, C., Schaperdoth, I., Macalady, J.L., Lipp, J.S., Lin, Y.-S., Ertefai, T.F., Schubotz, F., Hinrichs, K.-U., 2008. Methane-producing microbial community in a coal bed of the Illinois Basin. Appl. Environ. Microbiol. 74, 2424–2432.
Tanner, R.S., Hurst, C., Crawford, R., Garland, J., Lipson, D., Mills, A., Stetzenbach, L., 2007. Cultivation of bacteria and fungi. Man. Environ. Microbiol. 69–78.
Tillman, D.A., Harding, N.S., 2004. Fuels of opportunity. Characteristics and Uses in Combustion Systems. Elsevier, Kidlington, U.K, pp. 89–128.
Ulrich, G., Bower, S., 2008. Active methanogenesis and acetate utilization in Powder River Basin coals, United States. Int. J. Coal Geol. 76, 25–33.
Vandieken, V., Mußmann, M., Niemann, H., Jørgensen, B.B., 2006. *Desulfuromonas svalbardensis* sp. nov. and *Desulfuromusa ferrireducens* sp. nov., psychrophilic, Fe (III)-reducing bacteria isolated from Arctic sediments, Svalbard. Int. J. Syst. Evol. Microbiol. 56, 1133–1139.
Wang, C., Chang, C., Chu, C., Lee, D., Chang, B.-V., Liao, C., 2003. Producing hydrogen from wastewater sludge by *Clostridium bifermentans*. J. Biotechnol. 102, 83–92.
Wei, M., Yu, Z., Jiang, Z., Zhang, H., 2014. Microbial diversity and biogenic methane potential of a thermogenic-gas coal mine. Int. J. Coal Geol. 134–135, 96–107.