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A formation water-based nutrient recipe for potentially increasing methane release from coal in situ

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ABSTRACT

Biogasifying coal to methane represents an environmentally benign way to utilize the abundant and inexpensive coal resource. To increase methane yield from coal, numerous studies have investigated the approach of biostimulation through finding the best nutrient solutions to enhance microbial activities. Toward this end, however, almost all studies have adopted laboratory made medium that is tap water- or deionized and distilled water-based. As a matter of fact, this water is dramatically different from formation water in coal basins. Thus, in order to enhance methane release from coal in situ, this study aimed to design a formation water-based recipe. To accomplish this objective, the chemical and microbial compositions of the formation water collected from the San Juan basin were analyzed first. Equipped with this fundamental knowledge, a screening test was conducted to evaluate nine parameters to identify statistically significant ones affecting methane yield from coal. For those critical parameters, the optimal value for each was determined through response surface methodology. Finally, the predicted results by the models were verified by an experimental study adopting all optimum conditions. This study demonstrated that microbes capable of converting coal to methane were present at the San Juan basin and the developed recipe increased methane yield 24.3-fold compared to those without.

1. Introduction

It has been suggested that up to 20% of the world's natural gas is microbial in origin [1]. Specific to coal bed methane (CBM), biogenic methane production has been observed as a significant source in nearly every shallow coal seam at temperatures less than 80 °C [2]. In some basins, like the Illinois basin except the southeastern part in western Kentucky, methane gas is formed primarily through biogenic rather than thermogenic process [3]. In the US, the coal resources are estimated at 6 trillion tons, and 90% of it is currently unmineable due to seam thickness, depth, and structural integrity [4]. To convert these unmineable coals to methane through the biogenic pathways, four potential techniques, such as physically increasing microbial access to coal and distribution of amendments, increasing the bioavailability of coal organics, microbial augmentation, and microbial stimulation, can be applied [5,6].

The first two approaches can be achieved by hydraulic fracturing, a technique commonly used for releasing natural gas from shales [7,8]. The latter two deal with the microorganisms that initiate the coal conversion process. Regarding microbial augmentation, the purpose is to supplement a coal basin where coal-degrading microbes are not present. This could be needed for non-productive CBM wells as reported [9]. But the majority of recent studies have shown that indigenous microbes capable of gasifying coal to methane are present in coal seams. And this observation has been reported for coal basins across the globe. Representative examples include: the Powder River basin [10–12], the San Juan basin [13], the Illinois basin [14], the Indio formation [9], the Alberta coalbeds in western Canada [15], the Jiuligang formation in the Jingmen-Danyang basin in Hubei, China [16], the south Sydney basin [17] and the others listed in the review [18]. Since microbes co-exist with coal and/or inhabit the formation water, the last approach of biostimulation is the most reasonable one.

Methane yields in different basins are disclosed at different levels. It is 67 ft^3/ton for the Illinois basin [19], 50–70 ft^3/ton for the Powder river basin [20], 70–106 ft^3/ton for the Springfield (Indiana) [21], and 115–263 ft^3/ton for the Paleocene Fort Union coals in south-central Wyoming [22]. To further increase methane production from coal, different studies have evaluated effects of different recipes/chemicals on coal conversion to methane. These recipe/chemicals include, but not limited to: trypticase soy broth [23], a MS medium for methanogens

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[24], a commonly used anaerobic medium [25], non-ionic surfactants (Zonyl FSN, Triton X-100, and Brij 35) [26], and solvents (ethanol, methanol, pyridine, and N,N-dimethylformamide (DMF) [20] [27]. Besides these studied in the academic labs, other recipes have been tested at pilot scales by different companies [28]. However, except our previous study targeting developing a nutrient solution for biogasification of Illinois coal ex situ [24], none of the reported studies focused on finding the most suitablenutrient recipes for a specific coal basin. To fill this gap, this study was designed to identify the optimal nutrient recipe for the San Juan basin. Specifically, this recipe is aimed for in situ application. For this purpose, the formation water collected from the coal seam was used as the basis for developing the nutrient solution. The basin specific recipe was developed through a systematic approach considering the chemical and microbial composition of the formation water and the in situ temperature. To determine the optimal nutrient solution, a three-step methodology: screening, optimization and verification was adopted as detailed below.

2. Materials and methods

2.1. Coal and formation water sample collection and preparation

Chunks of coals were collected from a coal mining site at southwest of the San Juan basin in the United States (US). This seam is well known as the oldest natural gas production area in the US from both conventional and unconventional tight sand, CBM, and shale formations. The collected coal samples were immersed in water in a bucket at room temperature in darkness. Prior to use, the surface layer of the coal chunk was peeled off. The remaining 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. Prior to use, the coal samples were subject to elemental and proximate analysis as reported before [29]. From the former, the percentage of carbon, nitrogen, hydrogen, sulfur, and oxygen was found to be 70.29 ± 0.38; 1.36 ± 0.01; 5.12 ± 0.05; 0.83 ± 0.03 and 17.97 ± 0.06, respectively. From the latter, the coal had 5.09 ± 0.00% of ash, 44.15 ± 0.09% of volatile carbon and 50.76 ± 0.1% of fixed carbon. The heat content was 12,410.65 ± 80.6 BTU/lb [29].

From a CBM well that is in the same seam as where the coal was collected, the formation water samples were gathered from a depth of 3000 ft. At the sampling site, temperature was measured immediately after the formation water came to the surface. Fresh formation water was handled differently depending on their final use. Regarding those for chemical analysis, no chemicals were added. For analysis of total organic carbon (TOC), the formation water was added to glass vials containing HCl. On the way from the San Juan basin to our laboratory in Carbondale, IL, water samples dedicated for chemical composition analysis were kept on ice. In terms of those dedicated for microbial analysis, the formation water was supplemented with sodium sulfide (Na_2S) at 0.25 g/L and resazurin at 1 mg/L to maintain anaerobic conditions. During transportation back to our lab, these water samples were not put on ice for the purpose of keeping the microbes alive.

Once the samples reached our labs in Carbondale, the on-ice samples were transferred to the Carbondale Central Laboratory (CCL, Carbondale, IL, USA) immediately for chemical analysis. Samples for microbial analysis were treated in two ways. First, nine one-liter samples were filtered through 0.2 μm membrane filters (90 mm, Whatman™, Freiburg, Germany). Three resulting membranes were used for DNA extraction using Powerwater DNA extraction kit (Mo Bio, Carlabad, CA, USA) following manufacturer recommended procedures. These DNA samples were stored at −20 °C before use. The remaining six membranes were used to set up microcosms as described in the following. Some water samples were used to make glycerol frozen stocks. Briefly, the formation water was concentrated 80 times through centrifugation at 4 °C. The concentrated samples were then used to make frozen stocks with glycerol at 20%. These stocks were stored at −80 °C before use. The remaining water samples were kept at −20 °C for later use.

Table 1
Chemical composition of the formation water.

Parameter	Unit	Formation water	Filtered formation water
Temperature	(°C)	41–44	NA
pH		8.19	NA
Free ammonia	mg/L	0.23	NA
Total ammonia	mg/L	1.78	NA
Total Nitrogen-N	mg/L	2.4	NA
Chemical oxygen demand (COD)	mg/L	2497	NA
Hydrogen sulfide	mg/L	33	NA
Fluoride	mg/L	4	NA
Nitrite	mg/L	< 0.5	NA
Nitrate	mg/L	0.2	NA
Phosphate	mg/L	< 0.75	NA
Total phosphate-P	mg/L	0.171	NA
Sulfate	mg/L	< 0.75	NA
Chloride	mg/L	161	NA
Iron	mg/L	1.11	NA
Total dissolved organic carbon	mg/L	1.15	NA
Alkalinity as $CaCO_3$	mg/L	1280	NA
Aluminum	μg/L	86.7	25
Boron	μg/L	971	875.1
Cobalt	μg/L	< 1	< 1
Copper	μg/L	4.3	< 1
Manganese	μg/L	22.3	12
Molybdenum	μg/L	6.6	3.2
Nickel	μg/L	4.2	< 1
Selenium	μg/L	< 1	< 1
Tungsten	μg/L	7.2	6.3
Zinc	μg/L	21.1	1.6
Magnesium	mg/L	1.1	< 1
Sodium	mg/L	721	703
Calcium	mg/L	13.6	12.5
Potassium	mg/L	6	5.3

2.2. Chemical analysis

At CCL, concentrations of dissolved metals, such as: Na, K, Ca, Mg, Fe, Al, Co, Mn, Zn, W, Cu, Cu, Ni, Se, B, Mo were analyzed according to EPA method 200.8 through use of Inductively Coupled Plasma – Mass Spectrometry (ICP-MS) (Table 1). Concentrations of anions, such as: Cl^-, SO_4^{2}, PO_4^{3-}, NO_3^- were determined according to EPA method 300.0 through use of Ion Chromatography (IC). HCO_3^- concentration was determined following SM320B. TOC content was measured according to SM5310B. In addition, since nitrogen is especially important for microbial activities, ammonia-nitrogen concentration was determined by using an ion selective ammonia electrode following EPA method 350.3. Total nitrogen concentration was measured by using a Hach Kit TNT827 (Hach, Inc.). Furthermore, since dissolved sulfide above certain concentrations may be toxic to microbes, content of dissolved H_2S was determined according to EPA 376.2.

2.3. DNA sequencing

Following DNA extraction, DNA samples were quantified using a Nanodrop spectrophotometer. Those with excellent quality (A_{260}/A_{280}: 1.8–2.0) and high concentrations (30–50 ng/μl) were sent for sequencing according to procedures reported by our lab [25]. In short, to determine the overall diversity of the microbial population, the 16 S rRNA gene V4 variable region PCR primers F515 (5′-CACGGTCGKCGGCGCCATT-3′) and R806 (5′-GGACTACHVGGGTWTCTAAT-3′) [30,31] 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. Next generation DNA sequencing was conducted at Molecular Research (Shallowater, TX, USA) on an Ion Torrent PGM following the manufacturer's guideline. Sequence data (15–20,000 reads/assay) were processed using an in-house proprietary analysis pipeline. In summary, sequences with ambiguous base calls and with homopolymer runs exceeding 6 bp, barcodes, primers, and sequences < 150 bp were removed. Following the removal, sequences were denoised and chimeras were removed using UCHIME [32] implemented in the open-source software Mothur (v.1.33.3) [33]. Final operational taxonomic units (OTUs) defined by clustering at 3% divergence (97% similarity) [34,35] were taxonomically classified using BLASTn against a database derived from RDPII and NCBI and compiled into each taxonomic level into both "count" and "percentage" files. Count files contain the actual number of sequences while the percentage files consist of the relative (proportion) percentage of sequences within each sample that map to the designated taxonomic classification.

2.4. Developing nutrient solutions for stimulating biogasification

2.4.1. Inoculum development

Each of the six membranes containing collected microorganisms was used to set up one microcosm. Each microcosm comprised 10 g of ground coal (< 40 mesh) in 50 mL filtered formation water samples. Trypticase peptone (2 g/L) and yeast extract (2 g/L) were added to three microcosms for stimulating microbial growth. The other three were used as controls without any peptone and yeast extraction supplementation. All serum bottles (100 mL) were then capped by butyl rubber stoppers and sealed by aluminum crimps. All bottles were 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 maintained in dark at 43 °C. The headspace gas content and volume were monitored periodically as detailed below.

To recover the frozen stocks, three sets of microcosms were established. The first group of two contained 10 g of ground coal and 50 mL filtered formation water. In this study, the formation water was filtered through 0.2 μm before use. This is to eliminate any variation that could be caused by suspended solids in the water samples. The second set of two contained all in the first group, but with yeast extract and trypticase peptone, each at 2 g/L. The third set of two comprised all components in the second group, but with sodium acetate and sodium formate added at 5 g/L for each. All microcosms were kept static at 43 °C in dark. Once the microbial community was ready as indicated by active methane release, these cultures were used for the experiments described in the following.

2.4.2. Two-level factorial design

To identify critical factors that affect methane release from coal, we started with a two-level factorial design through use of Design of Expert (DOE, StatEase, Inc. Minneapolis, MN). A total of nine parameters were selected and tested at two levels with total methane production (ft^3/ton) and methane content (%) defined as responses (Table 2). To minimize the complexity of the experiment design, a Min Run Res IV design- a Min Runs plus 2 model was adopted with a total of 20 runs. The parameters tested were: (1) iron powder (< 10 μm, 0 or 100 mM), (2) Tween 20, 30% or 50% of critical micelle concentration (CMC, 0.06 mM), (3) sodium dodecyl sulfate (SDS), 30% or 50% of its CMC of 7 mM, (4) methanol (0 or 100 mM), (5) ethanol (0 or 100 mM), (6) 2-propanol (0 or 100 mM), (7) sodium formate (0 or 100 mM), (8) sodium acetate (0 or 100 mM), and (9) trace minerals (0 or 0.90 mL). The trace mineral solution at 0.9 mL was added to ensure that the modified formation water contained the same concentrations of minerals or metals as those in the reported MS medium [36].

The 20 microcosms were set up in the same way as detailed above in terms of coal loading and volume of formation water. The microbial community regenerated from the frozen stocks was used as the inoculum at 10% of the final volume. Each microcosm also contained yeast extract and trypticase peptone at 2 g/L. Among the microcosms, the difference was the presence or absence of different additions as shown in Table 1. All microcosms were maintained at 43 °C. Headspace gas was withdrawn at day 10, 15, 20, 25 and 30 for measuring newly produced gas volume and gas content as detailed below. The final cumulative methane yield and methane content on day 30 were presented in Table 1. These data were then subject to statistical analyses through use of the DOE software.

2.4.3. Box-behnken design

After analyzing results generated from the 20 reactors, four out of nine parameters were identified as statistically significant for the two responses. To further determine the optimal value for each parameter, a total of 29 reactors were established based on the Box-behnken design (Table 2). Ethanol and sodium acetate were tested between 10 and 100 mM while methanol and 2-propanol were evaluated at 10 and 50 mM. These reactors were established and monitored in the same way as aforementioned.

2.4.4. Verification of methane production under conditions predicted by the model

Once the optimal value for each parameter and the models were provided by the DOE software, a verification experiment comprising 44 reactors was conducted. These 44 included: (1) four sets of microcosms consisting of filtered formation water + coal + the developed recipe, unfiltered formation water + coal + the developed recipe, filtered formation water + coal without the recipe and unfiltered formation water + coal without the recipe. Each set had five replicates; (2) four sets of microcosms containing filtered formation water + coal and with the addition of either ethanol, methanol, 2-propanol or sodium acetate; and (3) four set of bioreactors containing the filtered formation water without coal, but with either ethanol, methanol, 2-propanol or sodium acetate. For the latter two sets, each had three replicates. Where needed, the four compounds were used at 27 mM for ethanol, 50 mM for methanol, 10 mM for 2-propanol and 100 mM for sodium acetate. Same as the other microcosms, these were maintained at 43 °C in darkness.

2.5. Analysis

Headspace gas analyses were conducted in the same way as reported in our previous work [24]. Briefly, to maintain a one-atm pressure in each reactor and release overpressure caused by microbial activities, a stainless steel needle was inserted to each microcosm headspace at different time points. The needle was connected to a 50 mL gas tight syringe. Gas volume in the syringe was recorded and used for calculation of methane yield. The molar content of methane in the reactor headspace was analyzed though a 17A GC (Shimadzu, Columbia, MS, USA). This GC was equipped with a 60 m × 0.53 mm RT-Msieve 5A porous layer molecular sieve (Restek, Bellefonte, PA, USA) and a flame ionization detector with argon being the carrier gas with a flow rate of 10.1 mL/min. 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. Calibration curves for methane (5–99%) was established using standard gases (Air Liquide, Plumsteadville, PA, USA).

3. Results

3.1. Chemical composition of the formation water

As shown in Table 1, the formation water had a temperature between 41 and 44 °C and pH of 8.19. The low concentrations of nitrate (0.2 mg/L) and sulfate (< 0.75 mg/L) demonstrated that the water was

Table 2
Two level factorial design matrix and the results obtained.

Run	Factor 1	Factor 2	Factor 3	Factor 4	Factor 5	Factor 6	Factor 7	Factor 8	Factor 9	Response	
	Iron	Tween 20	SDS	Methanol	Ethanol	Isopropanol	Sodium Formate	Sodium Acetate	Trace Mineral	Total Production	Methane Content
	mM	%CMC	%CMC	mM	mM	mM	mM	mM	ml	ft^3/ton	%
1	100	30	30	100	0	100	100	0	0	225.9	49.5
2	100	50	50	100	100	100	0	0	0	335.5	69.0
3	0	30	50	100	0	0	0	0	0	1.8	0.5
4	0	30	50	0	100	100	100	0	0	236.0	52.4
5	0	50	30	100	100	0	100	100	0	421.5	66.7
6	100	30	50	0	0	100	0	0	0.9	68.3	24.3
7	0	50	50	0	100	0	0	100	0.9	121.8	34.1
8	100	50	30	100	0	0	0	100	0.9	389.8	74.9
9	0	30	30	0	0	0	100	100	0.9	318.7	75.6
10	0	50	50	0	0	100	100	100	0	73.1	21.2
11	0	30	30	0	100	100	0	100	0	716.9	73.2
12	0	50	30	0	100	0	100	0	0.9	196.5	39.9
13	0	50	30	100	0	100	0	0	0.9	342.4	63.2
14	100	50	50	100	0	0	100	0	0.9	56.5	18.1
15	0	30	50	100	100	100	100	100	0.9	162.7	29
16	100	50	30	0	100	100	100	100	0.9	249.3	51.5
17	100	30	50	0	100	0	100	100	0	107.7	35.1
18	100	30	30	100	100	0	0	0	0.9	554.4	79.4
19	100	50	30	0	0	0	0	0	0	3.2	1.2
20	100	30	50	100	0	100	0	100	0	84.8	20.7

from a highly reducing environment. The total nitrogen (N) and total phosphorous (P) content being only 2.4 and 0.17 mg/L, respectively, revealed the lack of N and P in the formation water. The formation water, however, contained a high concentration of organic compounds as reflected from the chemical oxygen demand (COD) of 2497 mg/L. The dissolved total organic carbon (TOC) content was 1.15 mg/L. Thus, it is possible that a majority of organic matter was not water soluble. To detect metals, the formation water was filtered before analysis. Large differences were observed for some minerals, such as Zinc, 21.1 μg/L in unfiltered versus 1.6 μg/L in the filtered samples. The same was true for manganese, nickel, copper and aluminum. Thus, these metals existed in both water insoluble and soluble phases.

3.2. Microbial composition

According to DNA sequencing results, the microbes in the formation water were composed of 68% of bacteria and 32% of archaea. Among bacteria, a possible total of 294 species were distributed within 61 orders (Fig. 1a). The three dominant orders were *Thermoanaerobacterales* (16.1%), *Synergistales* (13.9%) and *Bacillales* (13.8%). The Archaea kingdom comprised five orders (Fig. 1b). The order of *Methanobacteriales*, *Methanosarcinales*, *Methanomicrobiales*, *Methanocellales*, and *Thermoproteales* was 96.1%. 2.5%, 1.0%, 0.2% and 0.2%, respectively.

3.3. Inoculum development

As shown in Fig. 2a, the freshly collected cells were able to produce methane from coal. However, this was only possible when yeast extract (YE) and trypticase peptone (TP) were supplemented. Without these two ingredients, marginal amount of methane was released. The same observation was obtained for the cultures derived from the frozen stocks (Fig. 2b). Compared with the fresh cells, it took approximately 20 more days for the frozen stocks to be active. Again, YE and TP were needed to regenerate the microbes. Besides these two nutrients, addition of sodium acetate and sodium formate seemed unnecessary.

3.4. Nutrient recipe development

To determine the optimal nutrient recipes for stimulating the microbial activities, we took a three-stage process. For the first stage, we screened nine parameters to identify the most significant ones (Table 2). According to the half-normal probability plot (Fig. 3), the two surfactants, Tween-20 and SDS were shown to exert negative effects. Among those that had positive effects on methane production from coal, all were statistically significant ($p < 0.05$) except iron (Table 4). Among those that were critically important for methane release, the relative contribution was: ethanol > 2-propanol > trace minerals > sodium acetate > methanol > sodium formate. Regarding the second response, methane content, similar results were attained. Thus, for the second stage of parameter optimization, we only focused on four parameters as shown in Table 3. Since trace minerals were important, they were added to all microcosms to ensure a concentration the same as those in the MS medium. Sodium formate was eliminated from further studies since its positive effect was relative small.

For parameter optimization, we used a Box-behnken design (Table 3) to statistically analyze and determine the optimum value for the four critical parameters: ethanol, sodium acetate, methanol, and 2-propanol. For the first two compounds, the range tested was 30–100 mM. For the second two, the highest concentration was 50 mM for each. This is to consider that high concentration of alcohol may be toxic to the cells. Ethanol was tested at the highest concentration since it was found to have the most significant effect on methane release from Illinois bituminous coal [37].

As shown in Table 5, results of methane production in ft^3/ton were fitted perfectly with a reduced quadratic model with $R^2 = 0.91$. The p value of the model was less than 0.0001, which portrayed its significance. Among the four parameters, except 2-propanol, the other three were all statistically significant. When the concentrations of methanol and 2-propanol were fixed at 50 and 10 mM, respectively, increase of sodium acetate concentration led to increased methane production. Effect of ethanol, however, had a different trend. Maximum methane release was observed when content of ethanol was between 19 and 37 mM (Fig. 4A). In addition, the interaction between methanol and 2-propanol was interesting. When methanol content was fixed at 10 mM, increased content of 2-propanol resulted in increased methane production. The effect of sodium acetate remained the same, but the content of ethanol needed to be between 37 and 46 mM in order to maximize methane generation. Thus, alcohols had important roles in converting coal to methane. Similar phenomena were observed for

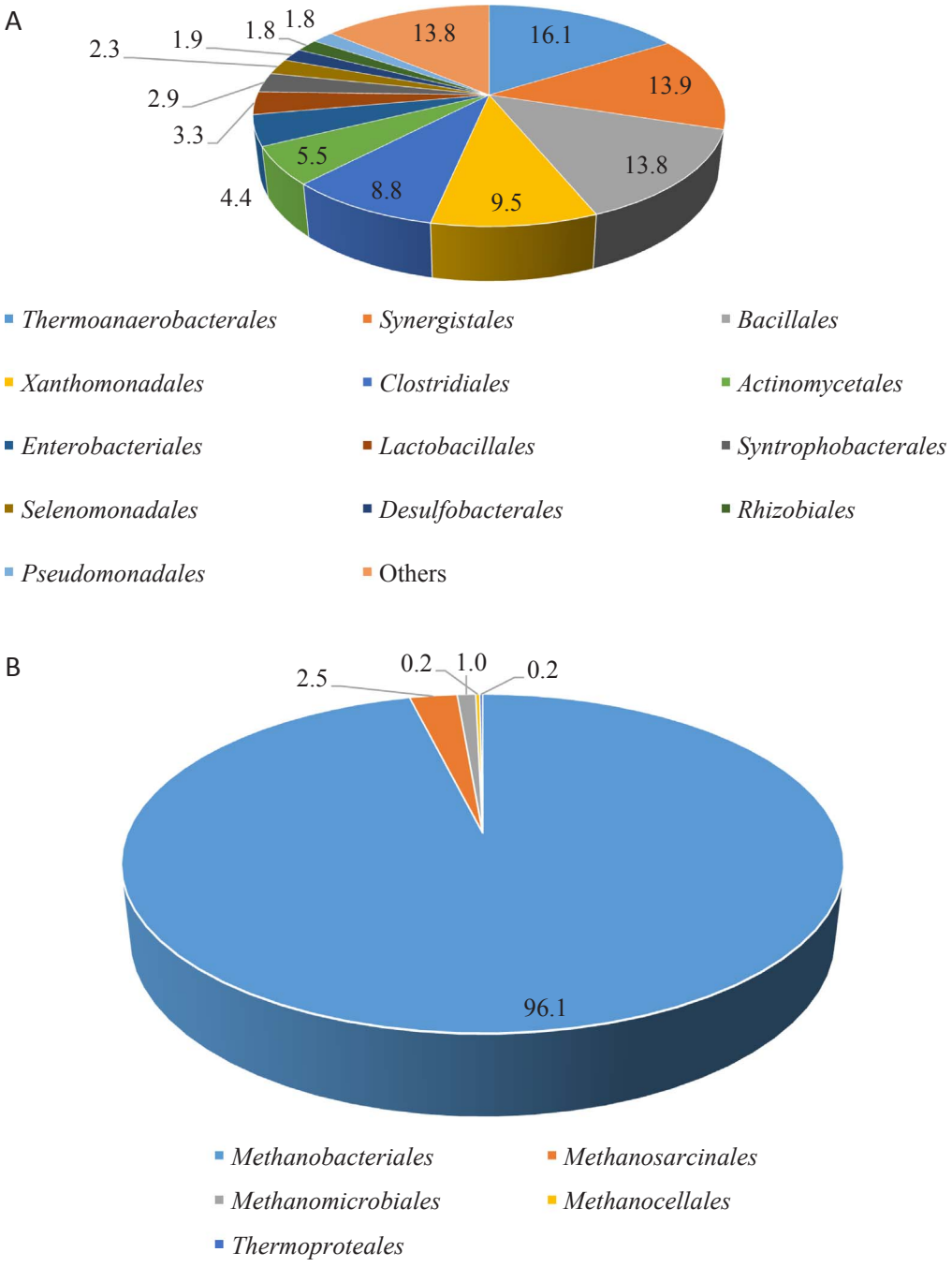

Fig. 1. Microbial composition of the formation water based on next generation DNA sequencing results. A: Bacterial kingdom at the Order level; B: Archaeal kingdom at the Order level.

methane content (Fig. 4b). The only difference was that maximum methane content was achieved when ethanol concentration was at the low end. Based on these analyses, the optimal value for ethanol, methanol, 2-propanol and sodium acetate was 27, 50, 10 and 100 mM, respectively and the predicted methane yield was 770.36 ft^3/ton with a methane content of 80.28%. The models for methane yield and content were represented by Eqs. (1) and (2).

$$\begin{aligned} Total\ production\,(ft^3/ton) = {} & -110.62 + 16.13 \times Ethanol \\ & + 6.66 \times Methanol + 3.44 \times Isopropanol \\ & + 5.02 \times Sodium\ acetate \\ & -0.07 \times Ethanol \times Methanol \\ & -0.06 \times Ethanol \times Sodium\ acetate \\ & -0.16 \times Methanol \times Isopropanol \\ & -0.12 \times Ethanol^2 \end{aligned} \quad (1)$$

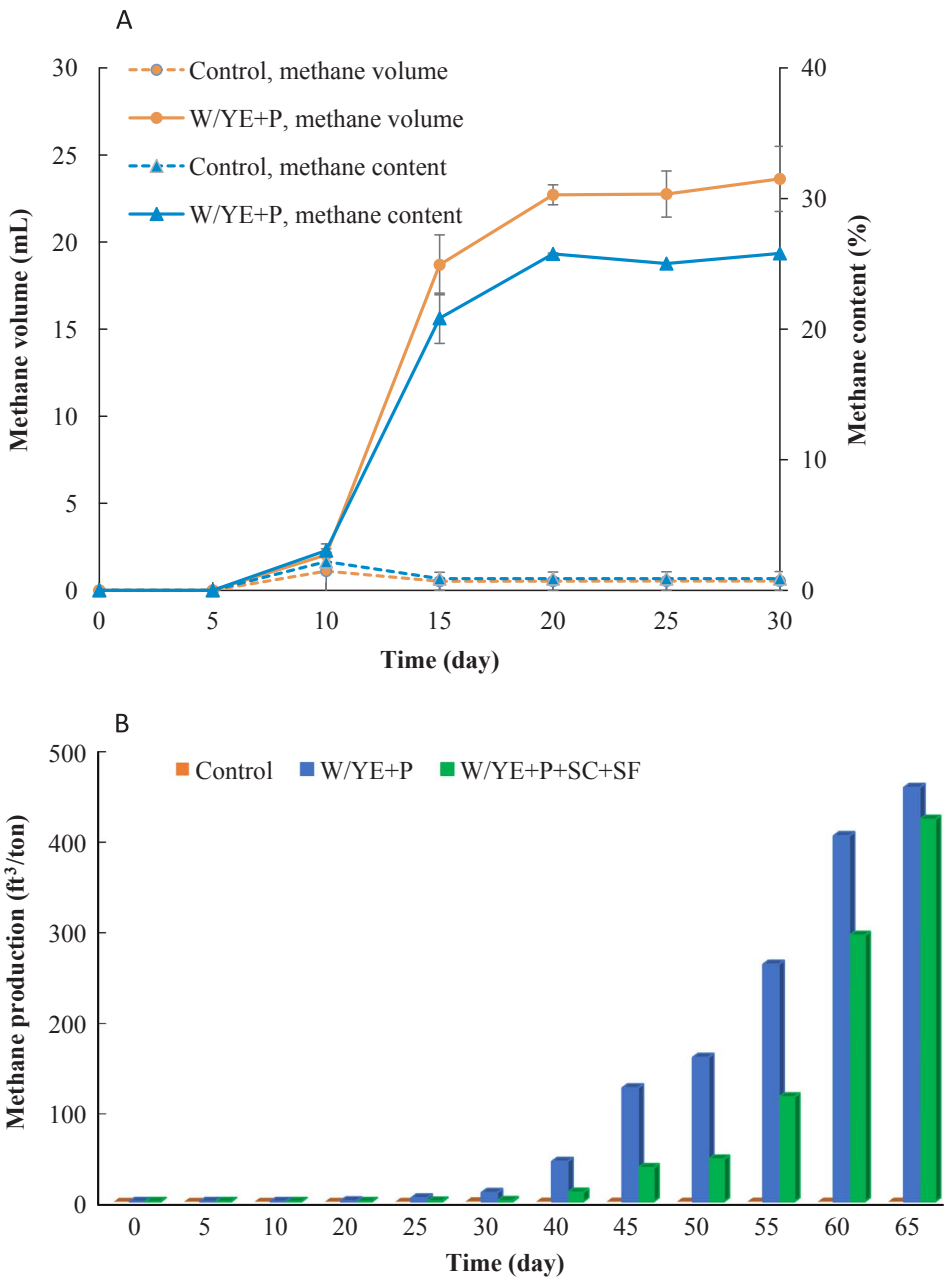

Fig. 2. Methane release from coal. A: freshly collected cells from the formation water; B: cells derived from the frozen stocks prepared from microorganisms initially in the formation water.

$$\begin{aligned} \textit{Methan econtent}\,(v\%) = {} & 20.78 + 0.60 \times \textit{Methanol} + 0.89 \times \textit{Ethanol} \\ & + 0.35 \times \textit{Isopropanol} + 0.33 \times \textit{Sodium acetate} \\ & -0.007 \times \textit{Methanol} \times \textit{Ethanol} \\ & -0.004 \times \textit{Ethanol} \times \textit{Sodium acetate} \\ & -0.01 \times \textit{Methanol} \times \textit{Isopropanol} \\ & -0.005 \times \textit{Ethanol}^2 \end{aligned} \quad (2)$$

To confirm these predicted results, we conducted a comprehensive verification experiment using filtered formation water. In addition, to test whether the developed nutrient recipe was also applicable to the formation water in situ, unfiltered formation water was evaluated, too under the same conditions. As shown in Fig. 5, the methane yield from microcosms containing coal, filtered formation water and the recipe was 870.8 ± 58.1 ft^3/ton on day 30. For the unfiltered formation water, the value was 1041.9 ± 38.7 ft^3/ton during the same period. Thus, probably due to the degradable nature of the suspended solids in the formation water, higher methane yield was detected from those unfiltered samples.

Considering the possibility that the added compounds might contribute to methane release, a total of 10 sets of controls were also established (Fig. 5). Without the supplementation of the developed recipe, methane release of 99.8 and 42.8 ft^3/ton was observed from the filtered and unfiltered formation water with coal, respectively. With the addition of ethanol only to the filtered formation water and coal, the day 30 methane yield was 208.3 ft^3/ton. When ethanol was replaced by methanol, 2-propanol or sodium acetate, the methane yield was 211.6, 359.5, 499.8 ft^3/ton, respectively. To understand whether the microbial community converted the added chemicals to methane, these four compounds were added individually to filtered formation water only without coal. Methane yield of 45.5 ft^3/ton was observed from ethanol only, 9.3 ft^3/ton was detected from methanol only, 10.7 ft^3/ton was from 2-propanol only and 2.9 ft^3/ton was from sodium acetate only. It needs to be noted that for all microcosms set up for the verification experiment, the same inoculum was used. And concentrations of the four compounds were tested at the optimal values identified above.

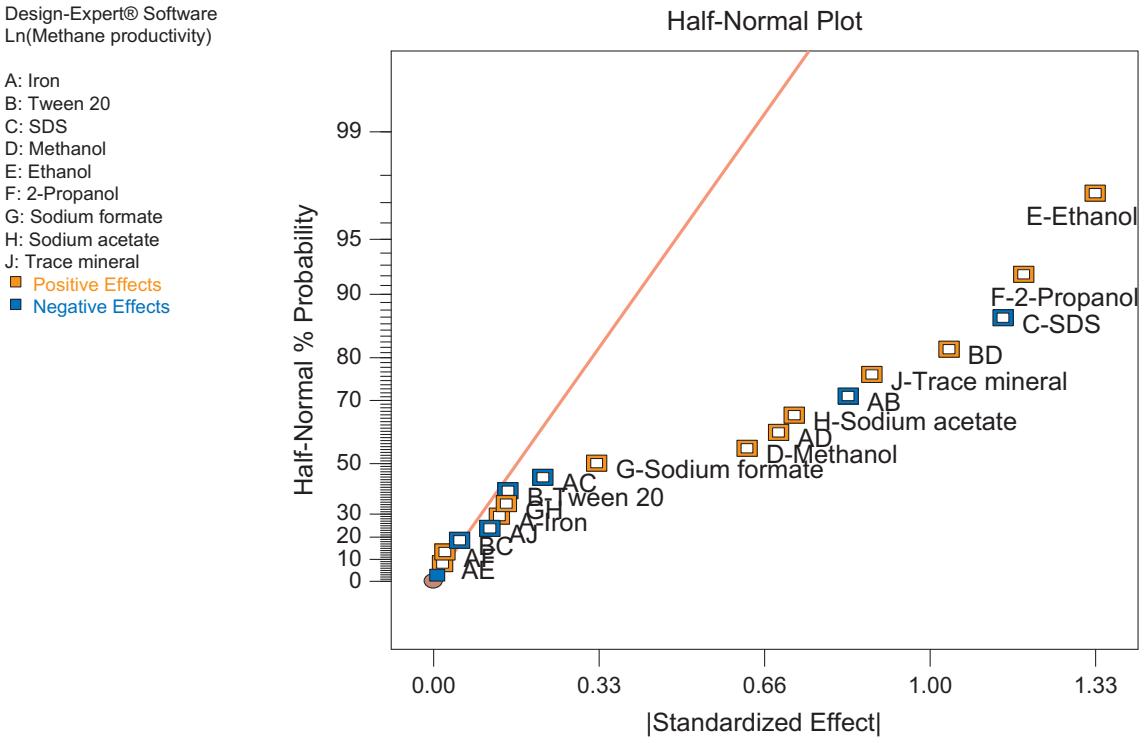

Fig. 3. Half-normal probability plot for cumulative methane production in ft^3/ton.

Table 3
Matrix for the Box-behnken design and the results obtained.

Run	Factor 1	Factor 2	Factor 3	Factor 4	Response	
	Ethanol	Methanol	Isopropanol	Sodium Acetate	Total Production	Methane Content
	mM	mM	mM	mM	ft^3/ton	v%
1	10	10	30	55	440.7	61.4
2	10	50	30	55	470.0	63.5
3	55	50	30	10	536.5	60.6
4	55	30	10	100	727.6	72.8
5	100	30	10	55	139.9	45.1
6	55	50	10	55	536.5	63.4
7	10	30	30	100	586.2	67.4
8	55	30	30	55	605.6	66.0
9	55	10	10	55	571.2	62.8
10	55	10	30	100	669.0	72.2
11	10	30	10	55	442.4	62.7
12	100	50	30	55	92.1	31.6
13	55	30	30	55	533.1	64.3
14	55	30	30	55	545.9	65.2
15	100	10	30	55	324.2	55.6
16	55	10	50	55	570.1	68.1
17	55	30	30	55	535.2	64.1
18	55	50	50	55	282.7	48.6
19	55	30	30	55	547.5	63.7
20	55	10	30	10	456.4	60.0
21	55	50	30	100	617.7	71.0
22	55	30	10	10	484.6	58.4
23	10	30	30	10	230.8	46.4
24	100	30	30	10	280.1	54.5
25	55	30	50	10	496.2	61.2
26	100	30	50	55	203.4	50.9
27	55	30	50	100	680.5	70.7
28	10	30	50	55	358.5	59.0
29	100	30	30	100	156.4	42.3

Table 4
Analysis of variance (ANOVA) for the screening experiment.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	45.14	16	2.82	84.79	0.0018	significant
A-Iron	0.08	1	0.08	2.39	0.2198	
B-Tween 20	0.1	1	0.1	3.05	0.1793	
C-SDS	5.88	1	5.88	176.76	0.0009	
D-Methanol	1.78	1	1.78	53.63	0.0053	
E-Ethanol	7.94	1	7.94	238.55	0.0006	
F-2-Propanol	6.32	1	6.32	189.83	0.0008	
G-Sodium formate	0.48	1	0.48	14.55	0.0317	
H-Sodium acetate	2.36	1	2.36	70.95	0.0035	
J-Trace mineral	3.49	1	3.49	104.78	0.002	
AB	3.12	1	3.12	93.75	0.0023	
AC	0.23	1	0.23	6.94	0.078	
AD	3.49	1	3.49	104.99	0.002	
AE	0.14	1	0.14	4.06	0.1372	
AJ	0.058	1	0.058	1.75	0.2773	
BC	8.21E-06	1	8.21E-06	2.47E-04	0.9885	
BD	5.42	1	5.42	162.91	0.001	
Residual	0.1	3	0.033			
Cor Total	45.24	19				

4. Discussion

The process of coal biogasification necessitates the synergistic actions of microorganisms spanning across three major metabolic groups: (1) hydrolytic and fermentative bacteria; (2) acetogenic bacteria, and (3) methanogenic archaea [6]. As revealed above, the microbial community in the San Juan basin formation water did contain bacterial and archaeal species. The top order of Thermoanaerobacterales (phylum: Firmicutes) indicated the presence of anaerobic and thermophilic bacteria that are under the class of Clostridia. *Clostridium* sp. are known fermentative bacteria that degrade cellulose, xylan and polysaccharides [38]. The second order of Synergistales (phylum, Synergistetes) has been identified in the formation water samples collected from the South Sumatra basin (SSB) CBM wells in Indonesia [39] and crude oil reservoirs at north central Louisiana, USA

Table 5
ANOVA analysis for the optimization experiment.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	7.56E + 05	8	94467.69	25.39	< 0.0001	Significant
A-Ethanol	1.48E + 05	1	1.48E + 05	39.77	< 0.0001	
B-Methanol	20518.7	1	20518.7	5.52	0.0292	
C-2-Propanol	8052.91	1	8052.91	2.16	0.1568	
D-Sodium acetate	75694.95	1	75694.95	20.35	0.0002	
AB	17084.32	1	17084.32	4.59	0.0446	
AD	57363.32	1	57363.32	15.42	0.0008	
BC	15967.79	1	15967.79	4.29	0.0514	
A^2	4.13E + 05	1	4.13E + 05	111.04	< 0.0001	
Residual	74406.25	20	3720.31			
Lack of Fit	70844.25	16	4427.77	4.97	0.0659	Not significant
Pure Error	3562	4	890.5			
Cor Total	8.30E + 05	28				

$R^2 = 0.91$; Adj $R^2 = 0.87$.

[40]. The third dominant order of Bacillales (phylum: Firmicutes) was found in the anoxic zone of the paddy soil in Italy [41]. Some members of the Bacillales order are reported with the ability to degrade crude oil, diesel and oxygenated hydrocarbons such as phenol, benzoate and m-hydroxybenzoate as sole sources of carbon [42,43]. The majority of these bacteria have been identified in environment where methanogens are also present. At the phylum level, the first and second predominant phylum: Firmicutes, 44.4% and Proteobacteria, 27.9% have been identified in many other CBM sites [38,44–46]. Thus, this may indicate fermentative and acetogenic bacteria exist ubiquitously in coal basins. Within the archaeal kingdom, the dominant Archaea belong to the order of Methanobacteriales. Members of this order are generally hydrogenotrophic. They can use hydrogen to reduce CO_2 to methane. In addition, some members can use formate, CO or secondary alcohols as electron donors for CO_2 reduction [36].

To report methane yield, different studies have chosen different units, such as μmol [47,27], μmol, mmol or mol per g or kg [9,48–51], cc/100 g [52], or ft^3/ton [20,23–25,37,53–55]. Even though the latter has been used the most, we do need to realize the uncertainty associated with extrapolating laboratory results. One way to minimize the uncertainty is to compare total surface area of coal particles studied in the lab with that of coal in the field. This method was demonstrated by Papendick et al. [26]. But, for this study, since the total surface area of coal in the San Juan basin is unknown, we could not adopt this delicate approach. Using this ft^3/ton unit, however, allows us to compare methane yields under different conditions investigated in this study and to compare results obtained here with those reported in the literature already.

The potential capability in converting coal to methane by the microbial community in the San Juan basin was confirmed by the microcosm study. Both the freshly collected cells and those derived from the frozen stocks released methane from coal although the time needed for the lag phase was different. Without the presence of YE and TP, little amount of methane was observed. The need for these two nutrient sources hinted the lack of nitrogen in the formation water, which was proven by the chemical analysis of these water samples.

YE and TP have been used generally as major sources of nitrogen in numerous recipes for cultivating various microbes. They have been proven to be indispensable in a nutrient solution designed for stimulating methane release from Illinois bituminous coal [23,24,37]. Besides nitrogen, these two ingredients do comprise organic carbons. However, when the microbial community initially developed from the formation water in an Illinois CBM well was incubated with YE and TP only without coal, no methane was detected although CO_2 was observed [55]. Thus, methane released from the microcosms should be from coal itself. Similarly, in this study, negligible amount of methane was observed for control microcosms where YE and TP were included besides ethanol, methanol, isopropanol or sodium acetate (Fig. 5). This again demonstrated that these two ingredients were not converted to methane by the studied microbial community during the experimental period. Compared to results obtained from microcosms where coal was present (Fig. 5), one may argue that a lack of solid material where microbes can form biofilm could be the major explanation for little methane release from cells having no place to attach to. This could be true. But it needs to be noted that all inoculum used in this study was developed from cells initially in suspension. Thus, the role of coal in biogasification certainly needs to be further studied.

In addition to YE and TP, nine parameters were selected for screening. The selection of these nine was mainly considering: (1) the in situ application of the developed recipe; and (2) results reported by other researchers and our own experience in enhanced coal bed methane. For in situ application, temperature and pH of the coal bed cannot be easily modified. Thus, these two factors were not included in the experimental design. What we can do to enhance methane release from coal is to stimulate microbial activities by adding what they may need. For this purpose, we chose these nine parameters which fall into five categories: possible electron donors, such as iron; surfactant, Tween 20 and SDS; organic solvent, ethanol, methanol and isopropanol; carbon source, sodium formate and sodium acetate; and trace minerals to make up what is lacking in the formation water compared to a standard medium recipe for cultivating methanogens [36]. Certainly, other chemicals that belong to these groups can be tested, too. But the chosen ones are featured by their low costs and ease of use. And they serve as the starting point for developing nutrient recipes to stimulate in situ coal bioconversion to methane.

According to the screening results, three alcohols and sodium acetate were demonstrated to be critical for maximum methane production from coal. In our previous studies, we have proven that ethanol had statistically significant effect on increasing methane yield. And that effect is concentration dependent. When used at 100 mM, ethanol increased methane yields at least 24-fold. At 300 mM, however, no positive effect was noted [24]. This could be explained by ethanol toxicity at a higher concentration. Similar observation was reported by another study where ethanol added in the amount of 5 or 10 mg to 10 g coal from Power River Basin increased methane release [27]. The mechanisms of this alcohol on enhancing methane yield, however, are unclear. Ethanol in the microcosms, could be a potential organic solvent and/or carbon source. But the fact that we have detected marginal amount of methane from the same microbial community with the same MS medium and ethanol at 100 mM (Fig. 5), discredits its potential role as a methane contributor. In another study, through ^{13}C tracer tests, ethanol was found to account for 6, 14, and 2.5% of the total carbon flux to methane in anoxic environments of Lake Mendota, Knaack Lake and sewage digester sludge, respectively [56]. Thus, it is highly possible

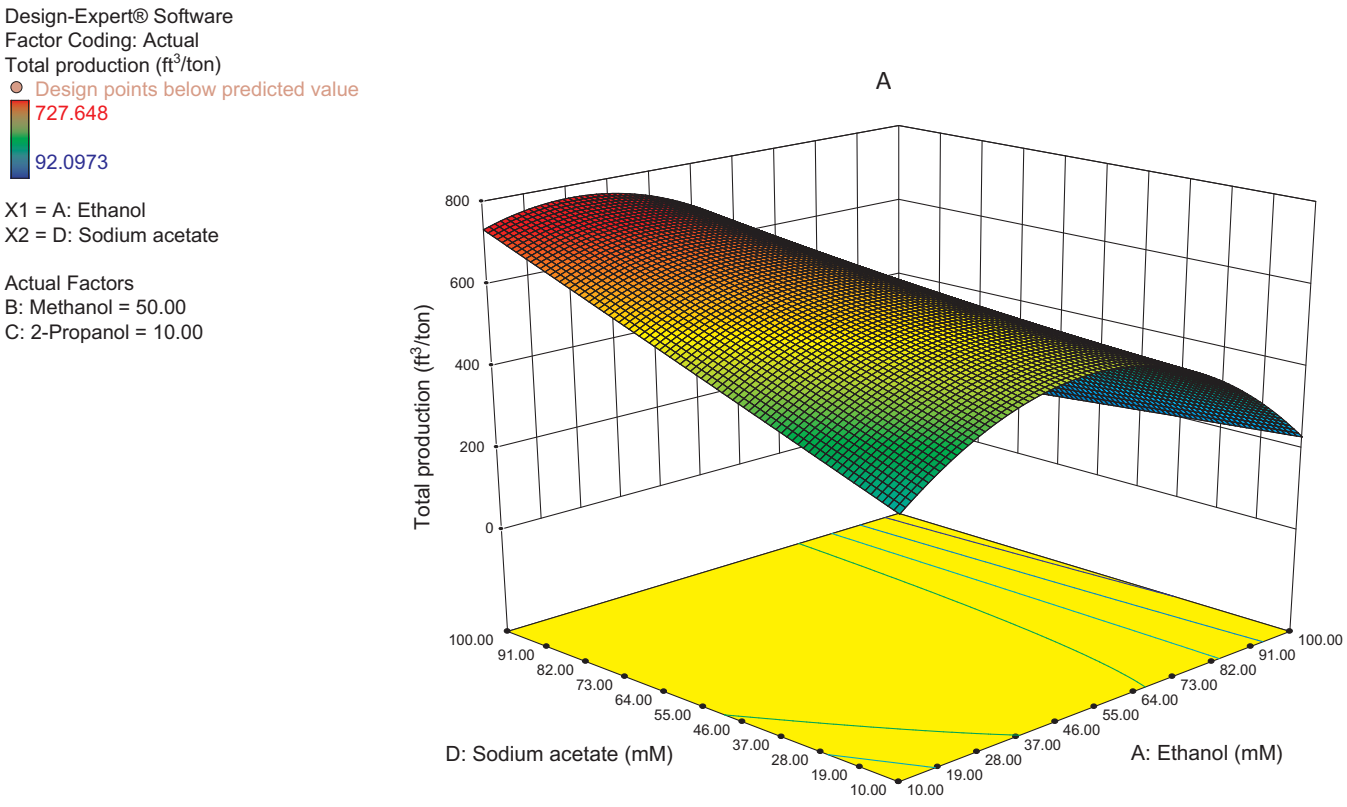

Fig. 4. 3-D response surface. A: methane yield in ft^3/ton; B: methane content in%.

that the positive effect of ethanol is based on its solvation function that might have increased bioavailability of some compounds initially associated with coal.

Similar to ethanol, methanol could act as a solvent and/or carbon source to microorganisms. In one study, where methanol was tested together with two other solvents: pyridine, and N,N-dimethylformamide (DMF), only DMF at 0.25 vol% produced 346% more methane than the no-solvent control cultures [20]. Specific to the microbial

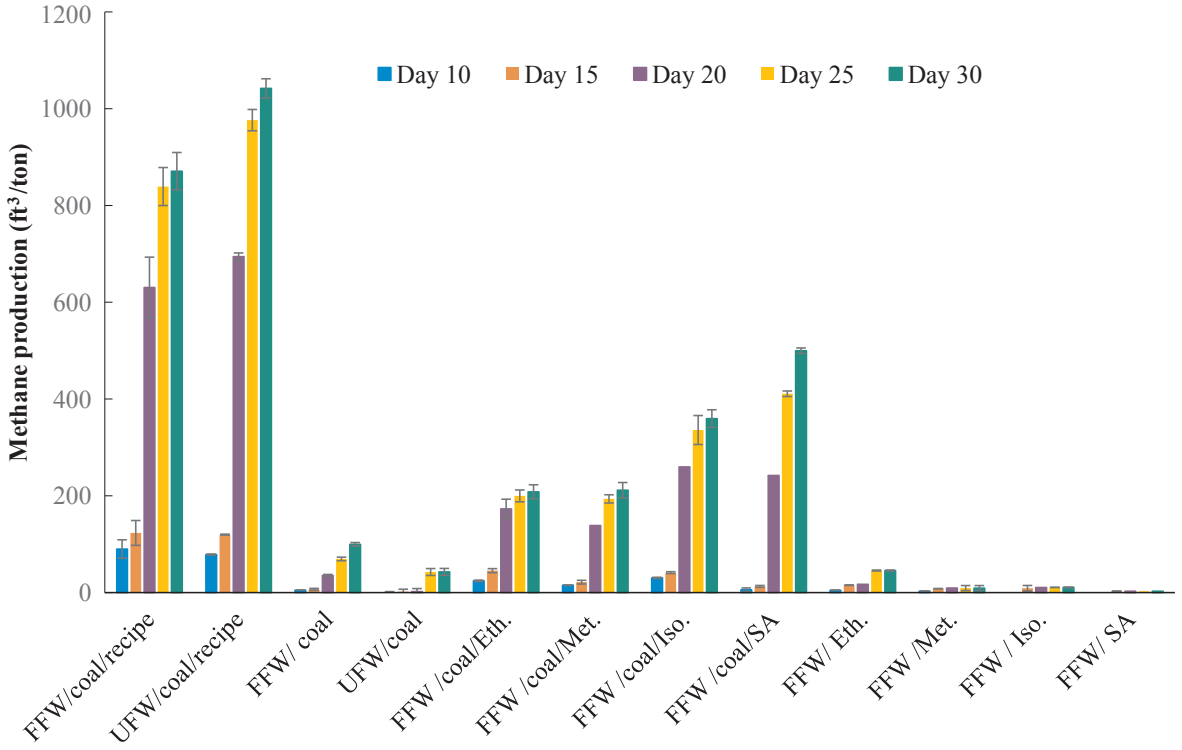

Fig. 5. Results of the verification experiment. FFW: filtered formation water; UFW: unfiltered formation water. Eth: ethanol; Met: methanol; iso: isopropanol; SA: sodium acetate.

community studied here, methanol, 2-propanol, formate or acetate could be an electron donor for CO_2 reduction for the dominant order of Methanobacteriales [36] in the archaea population. This was evidenced in the results from the verification experiment. Compared with those with formation water and coal only, the addition of an individual alcohol or acetate did improve methane yield from coal. These compounds, when used without the presence of coal, however, produced methane only at barely detectable levels. This was especially true for methanol, 2-propanol and sodium acetate. Same as ethanol, the exact role of these three compounds in the coal biogasification process deserves further investigations.

For filtered formation water, the nutrient recipe led to 8.7-fold methane yield increase (Fig. 5). For the majority tests reported here, filtered formation water was used to eliminate variance that could be introduced by the heterogeneous nature of the original formation water. But surprisingly, the developed nutrient recipe resulted in 24.3-fold increase of methane release compared to those without. Thus, this recipe can certainly be used directly in situ.

The need for YE, TP, alcohols and acetate for increased methane release certainly will add up the cost of the nutrient solution. But comparing with recipes disclosed in the literature by different companies, the nutrient solution we developed for the San Juan basin is not too complicated or expensive. For example, amendment constituents reported by Luca Technologies, Inc. included four major categories: vitamins and minerals; multi-nutrients; cell vitality enhancers and tracers. For multi-nutrients, casein hydrolysates, yeast extract, brewer's yeast, soy protein and trypticase peptones were possible choices. In terms of cell vitality enhancers, glycerol, weak organic acids and others were used [28]. However, owing to the low price of natural gas in the current market, comprehensive techno-economic and life cycle analyses need to be conducted before this recipe can be used at a CBM site. It is also noteworthy that the results reported in this study cannot be directly used for estimating methane yield in situ. In this work, even though the formation water and site temperature were adopted, the in situ pressure and stress as well as the hydrological condition could not be replicated due to the need to set up a great number of microcosms. To truly understand the effect of the developed recipe, the nutrient solution needs to be evaluated either strictly in situ or in laboratory setups that exactly simulate the on-site conditions.

5. Conclusion

A formation water-based nutrient recipe was successfully developed for the San Juan basin. This recipe was designed according to the microbial and chemical composition of the formation water. Based on next-generation sequencing, the microbial community in the water samples did have bacterial and archaeal strains that can degrade coal and form methane from compounds derived from coal. Considering the lack of nitrogen in the formation water, yeast extract and trypticase peptone were needed to satisfy the needs of microbial metabolisms. Through a software-aided experimental design including screening and optimization, the optimal concentrations for statistically significant parameters: ethanol, methanol, 2-propanol and sodium acetate were identified and models predicting methane yield and content became available. Adopting these optimal conditions, a methane yield of 870.77 ± 58.10 ft^3/ton was observed when filtered formation water was used. For unfiltered formation water, the yield was 1,041.88 ± 38.70 ft^3/ton. These yields represented 8.7- and 24.3-fold increase for the filtered and unfiltered formation water, respectively when compared to those without the addition of the nutrient recipe.

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