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Biological souring of crude oil under anaerobic conditions

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ABSTRACT

Seawater injection into oil reservoirs for purposes of secondary oil recovery is frequently accompanied by souring (increased sulfide concentrations). Production of hydrogen sulfide causes various problems, such as microbiologically influenced corrosion (MIC) and deterioration of crude oil. Sulfate reducing bacteria (SRB) are considered to be major players in souring. Volatile fatty acids (VFAs) in oil field water are believed to be produced by microbial degradation of crude oil. The objective of this research was to investigate mechanisms of souring, focusing specifically on VFA production via crude oil biodegradation. To this end, a microbial consortium collected from an oil field water separator was suspended in seawater; crude oil or liquid n-alkane mixture was added to the culture medium as the sole carbon source, and the culture was incubated under anaerobic conditions. Physicochemical analysis showed that preferential toluene degradation and sulfate reduction occurred concomitantly in the culture containing crude oil. Sulfide concentrations were much lower in the alkane supplemented culture than in the crude oil–supplemented culture. These observations suggest that SRB are related to the toluene activation and VFA consumption steps of crude oil degradation. Therefore, the electron donors for SRB are not only VFA, but many components of crude oil, especially toluene. Alkanes were also degraded by microorganisms, but did not contribute to reservoir souring.

Keywords: Souring, sulfate reducing bacteria, microbiologically influenced corrosion

INTRODUCTION

In oil recovery, three classical steps are operated. Primary recovery depends on natural drive. Secondary recovery relies on the injection of water or gas into the reservoir to pressurize the oil well to improve oil production. Tertiary recovery involves different techniques to improve efficiency in the reservoir [1)]. The recovery percentage of the crude oil by geopressure (primary recovery ratio) is estimated around 10 % [2)]. In the secondary oil recovery process, seawater is commonly used as injection water. On average 15 to 60 % of the oil initially in place in the field can be recovered by injection [2)]. Seawater injection is known to cause souring (sulfide production in oil reservoirs) frequently. Souring causes several problems, such as microbiologically influenced corrosion of tubing material, and deterioration of crude oil. Sulfate reducing bacteria (SRB) are recognized as one of the souring inducing microorganisms in oil fields and the most SRB belong to Deltaproteobacteria and Firmicutes [3)]. SRB derive energy for growth by coupling the oxidation of organic electron donors in oil field with the reduction of sulfate to sulfide. Most common study showed electron donors for SRB in oil fields were VFAs [4)]. VFAs in oil field water were considered to be produced by microbial degradation of crude oil components. Crude oil is composed of many components such as alkanes, naphthenes, aromatics, nonhydrocarbon components, and so on. Recently, anaerobic degradation of crude oil components especially alkanes and aromatics were well studied. These compounds are degraded mainly via fumarate addition to form alkylsuccinate or (alkyl) benzylsuucinate, and finally transformed to acetyl-CoA [5, 6, 7)]. Fumarate addition is catalyzed by benzylsuccinate synthase (BSS) or alkylsuccinate synthase (ASS). BSS is encoded by bss [8)]. To identify the component of the crude oil which causes souring, most possible components alkanes (C5~C17), aromatics (benzene, toluene, ethylbenzene, xylene), 2,4-xylenol and naphthenic acids (cyclopentane carboxylic acid and cyclohexane carboxylic acid) were diluted with biologically inert oil 2,2,4,4,6,8,8-heptamethylnonane (HMN) and incubated microbes sampled from the oil well. Crude oil presented in the same well was also investigated. After 91-day incubation, acetate was produced by anaerobic degradation of alkanes and aromatics. Remarkable consumption of toluene and ethylbenzene was observed.

EXPERIMENTAL PROCEDURE

Biological conversion of crude oil under the anaerobic condition

The oil field water sample was collected from oil field platform in Akita prefecture (Japan). The water was taken from the water-oil separator of the plant. The crude oil reservoirs located at depth of 1300-1500 m. The temperature of the field water sample on the ground was around 25°C. Microbes in the oil field water sample were concentrated by centrifugation at 11,000 g for 15 min in a 500 ml centrifuging tube. Then, the pellet was resuspended in 50 ml clean natural seawater collected at Ogasawara (Tokyo, Japan), about 1000 km far from Tokyo. Concentration of microbes in the concentrated sample was 5.04×10^7 cells/ml which measured by direct counting by microscope after staining by DAPI (4' 6-diamidino-2-phenylindle) [10)].

Five mixtures of carbon source examined were;

- AL: alkanes (C5~C17)
- AR: aromatics (benzene, toluene, ethlbenzene, xylen)
- CR: crude oil
- XY: 2,4-xylenol
- NA: naphthenic acids (cyclopentane carboxylic acid, cyclohexane carbox-ylic acid)

AL, AR, and NA contain same volume of each component. Each mixture was diluted 100 times (vol/vol) with HMN [11)]. Five milliliter of diluted mixtures were overlaid on 50ml seawater supplemented microbes collected from oil field water.

Chemical analysis

Sulfide concentration was measured by the methylene blue method (NANOCOLOR Standard Experiment Sulphide, Marchereynagel Cop.). Anions were measured by ion chromatography (LC-10AD, Shimadzu Corp.). Organic acids were measured by high performance liquid chromatography (column: SCR102H, detector: CSS 10A, Shimadzu Corp.). Concentration of crude oil components in HMN was measured by gas chromatography (GC 2014, Shimadzu Corp.) with flame ionization detector (325°C) using helium as the carrier gas. Column used in this experiment was HPPONA 50 mm x 0.2 mm (0.5 µm film thickness). The column temperature was initially held at 40°C for 20min, then programmed to 70°C at a rate of 1.5°C min^{-1} ,then to 290°C at 4°C min^{-1}, then to 320°C at 2°C min^{-1} with a final hold time of 30 min. Trans-2-heptene was used as an internal standard substance. Crude oil components dissolved in the HMN were extracted with 2.5 ml hexane for 24h. The organic phase was then dried with anhydrous sodium sulfate and analyzed by gas chroma-tography as described above.

Microbial analyses

PCR amplification of the bssA was performed using primers 1213F: 5'-GACATGACCGAYGCCATYCT-3' 1987R: 5'-TCRTCGTCRTTGCCCCAYTT-3' [9]. PCR condition was as follows: 95°C for 3 min followed by 40 cycles of 95°C for 45 s, 55°C for one min, 72°C for two min, and final extension step at 72°C for 10 min. The amplicon of the bssA was confirmed by agarose gel electrophoresis. The PCR products (ca. 793 bp) were purified with a FastGeneTM Gel/PCR Extraction kit (NIPPON Genetics Co.), and subsequently cloned into pGEM vector using TA cloning kit (Promega) and E. coli XL1 blue was transformed with the plasmid. 20-56 colonies were randomly selected from clone libraries. Inserted DNA fragments were amplified with primers pGEMT seq+ and pGEMT seq [12], and subjected to restriction fragment length polymorphism (RFLP) analysis using HaeIII and MspI. The nucleotide sequences of a part of the bssA were determined by dye terminator cycle sequencing with a BigDye terminator v3.1 cycle sequence kit (Applied Biosystems Corp.) and a capillary sequencer (3730xl DNA Analyzer, Applied Biosistems Corp.). Nucleotide sequences were compared with the Basic Local Alignment Search Tool (BLAST) [13].

RESULTS

VFA production and souring

Mixture of the four crude oil components (alkanes, aromatics, 2,4-xylenol, naphthenic acids) and crude oil 1/100 diluted with biologically inert HMN and overlaid on the seawater supplemented microbes of the oil field water were incubated in the high pressure vessel under 1 MPa at 25 °C for 91 days. No pressure change was observed in five vessels. 1.5 milliliter of the seawater phase was sampled weekly and subjected for chemical analysis. Production of acetate was observed in AL, AR and CR. Concentration of other VFA in all conditions was less than the detection limit of the HPLC. This result confirmed that acetate could be produced from the crude oil components under the absolutely anaerobic condition. On the other hand, no acetate was produced from XY and NA (data not shown). Acetate concentration in AL, AR and CR was fluctuated during the experiment. After 49 days incubation, acetate concentration was under the detection limit in AL and AR. Concentration of acetate in CR was lower than AL and AR during whole experimental period.

Initial concentration of the sulfate originated from the Ogasawara seawater was 27 mM. No decrease of sulfate and increase of sulfide were observed in XY and NA (data not shown). Therefore, we concluded that involvement of XY and NA was negligible for souring. Sulfide concentration reached 2.22 mM at day 63 in AR and 0.02 mM at day 49 in AL. On the other hand, no sulfide production observed in CR. Sulfate concentration was decreased by 6.3 mM in AR and 1 mM in AL. On the other hand, significant sulfate decrease was not observed in CR although crude oil contains

alkanes and aromatic compounds. Sulfur which did not present as sulfide and sulfate might be assimilated by the microbes.

Substrate consumption

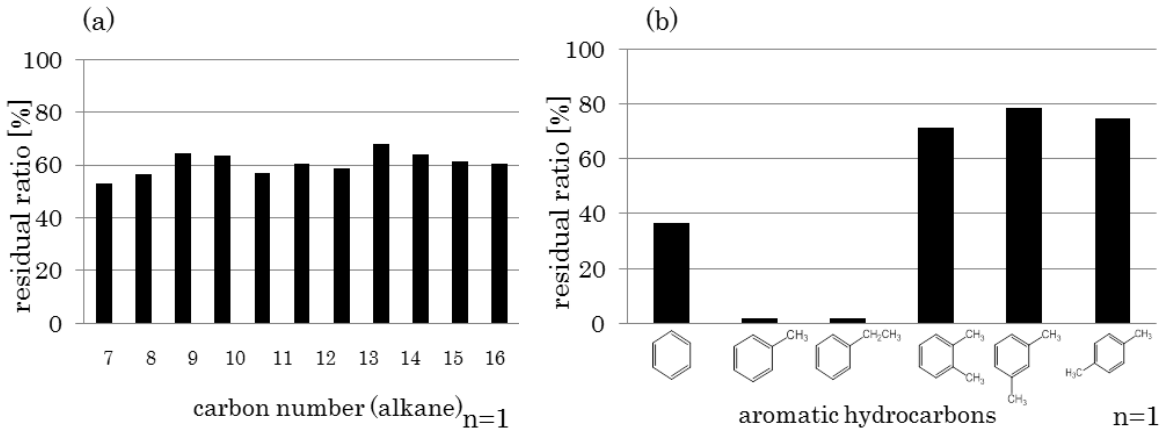

Fig. 1 (a): Remaining alkanes after incubation in AL (b): Remaining romatic hydrocarbons after incubation in AR. n=1

The concentration of carbon sources was measured before and after 91 days incubation. Concentration was determined by internal reference method and residual ratio was calculated (Fig.1 (a), (b)). No change of substrates concentration in XY and NA was observed (data not shown). In AL, concentration of pentane (C5) and hexane (C6) were unable to be detected. Pentene and hexane might be completely consumed or evaporated during the experiment. No preference on utilization of carbon number was observed from heptane (C7) to heptadecane (C17) in AL. Residual ratio for alkanes (C7-C17) was 40-70% after 91 days incubation. On the other hand, almost complete consumption was observed for toluene and ethylbenzene in the aromatics mixture. Production of acetate and sulfide, and change of sulfate was not observed in the CR. However, Drastic decrease of alkanes (C>18) and aromatic compounds except benzene were observed in CR. Residual ratio for alkanes (C7~C17) was 60-88%.

Detection of bssA

To investigate the biological pathway of fatty acid production under absolute anaerobic condition, detection of bssA gene was conducted. The bssA fragment was amplified as a expected size in AL, AR and CR by PCR. PCR products were analyzed by clone library method. 32 colonies in AL, 56 colonies in AR and 21 colonies in CR were picked up and classified 8 groups in AR, 3 groups in AL and CR by RFLP. As a result of sequencing, all clones were affiliated with bssA belongs to *Desulfobacula toluolica*. *Deslfobacula toluolica* is known as toluene degrading SRB [1)]11]. However, similarities were low (85%). To determine whether the microbes which encode bssA belongs to SRB or not, isolation and characterization of those microbes is necessary. This bssA was detected not only in AR and CR but also in AL although toluene was not added as carbon source in AL. BssA was not observed in XY and NA.

Analysis of microbial consortia

Microbial communities were compared between day 0 (OFW) and 49 (AL, AR and CR). After incubation, the portion of *Deltaproteobacteria* increased and large portion of *Deltaproteobacteria* was

classified Desulfotignum sp. Three OTUs (operational taxonomic units) affiliated with Desulfotignum were detected in AL, AR and CR. The closest species of these OTUs were *Desulfotignum toluenicum* (toluene degrading SRB) [14]. The portion of Clostridia also increased in AL, AR and CR. Dominant species of Clostridia detected in this study was *Fusibactetr* sp. *Fusibacter* sp. was known as fermenting bacteria. The portion of Bacteroidia also increased in AL, AR and CR. Increase of *Acinetobacter* sp. belongs to *Gammaproteobacteria* was also observed in AL, AR and CR. Amplification of 16SrRNA gene was not observed in XY and NA.

CONCLUSIONS

Crude oil and alkane mixture (C6–C17) were used to demonstrate souring in the lab under anaerobic and microaerobic conditions. The findings can be summarized as follows;

1. Microaerobic conditions did not promote souring.
2. Sulfide production (souring) was observed when the crude oil was incubated with seawater supplemented with microbes separated from oil field water.
3. Temporal production of sulfide was observed when the alkane mixture was used.
4. Toluene in crude oil was consumed selectively.
5. A drastic change in the microbial consortia was observed when crude oil or alkane mixture was incubated with the seawater; however, the difference in consortia between the two substrates was small.
6. Several kinds of SRB and fermentative bacteria were identified by PCR-DGGE sequencing analysis.
7. The relative abundance of SRB increased along with souring.

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