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Full Length Research Paper

Identification and bio-corrosion behavior of *Thermoanaerobacter* CF1, a thiosulfate reducing bacterium isolated from Dagang oil field

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A slightly curved rod strictly anaerobic, thermophilic bacterium was isolated from Dagang oil field, China. Its fundamental physiology and bio-corrosion characteristic were characterized. The growth of strain CF1 occurred at 45 to 78°C (optimum 60°C), pH 5.0 to 9.5 (optimum 7.1). The doubling time under optimal conditions was 45 min. Substrates that could be used included D-cellobiose, fructose, glucose, maltose, mannose, trehalose, melezitose, raffinose, ribose, sucrose, xylose and starch. 16S rRNA gene sequence analysis indicated that the isolate was most closely related to *Thermoanaerobacter uzonensis* (99.2%). The G+C content of the genomic DNA of strain CF1 was 33.5 mol%. The major cellular fatty acid contents were 14 : 0 (2.5%), 15 : 0 (65.8%), 16 : 0 (8.6%), 15-methyl 17:0 (8.1%), 14-methyl 17:0 (7.4%), 18 : 1v9c (1.7%) and 18 : 0 (4.8%). The strain reduced up to 1 M thiosulfate to elemental sulfur without sulfide formation. In the presence of thiosulfate, electrochemical method, weight loss and surface analysis showed the corrosion behavior of the isolate. A slight change of corrosion current was detected through electrochemical method. Elemental sulfur was found on the surface of the carbon steel, with less weight loss in the cultivation system.

Key words: Thiosulfate reduction, element sulfur, bio-corrosion, carbon steel.

INTRODUCTION

Microbial thiosulfate reduction is an important process in the geochemical cycling of sulfur species under anaerobic environment (Ravot et al., 1995). Thiosulfate is a highly significant sulfur intermediate, that can be either reduced to produce inorganic sulfur compounds or disproportionate (Jørgensen, 1990), in the anaerobic conditions. The reduction of thiosulfate increases with stratum depth, anaerobic organisms participate prominently in dissimilatory thiosulfate reduction (Lee et al., 2007), indicating that thermophilic anaerobic microbes may be responsible for the function, in deep stratum of oil fields. Numbers of thermophilic microbes can reduce thiosulfate to either elemental sulfur or hydrogen sulfide, or both. Many of them belong to the genus *Thermoanaerobacter* or *Thermoanaerobacterium*, stratum of oil fields. Numbers of themophilic microbes can reduce thiosulfate to either elemental sulfur or hydrogen sulfide. Most species of the genus *Thermoanaerobcter* reduce thiosulfate to hydrogen sulfide, except 3 species. *Thermoanaerobacter italicus* and *Thermoanaerobacter uzonensis* produce both sulfide and elemental sulfur (Kozianowski et al., 1997; Wagner et al., 2008), and *Thermoanaerobacter sulfurigignens* produces elemental sulfur (Lee et al., 2007). It has been proven that the presence of thiosulfate in oil fields may increase the risk of biocorrosion of oil pipelines (Faudon et al., 1995; Duncan et al., 2009). A "thiosulfate shunt" seems to exist in anoxic environment, and may be very

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important to control the corrsion. So, its further control is therefore a key reaction in oil fields.

In this paper, we report on one *Thermoanaerobacter* isolate from Dagang oil field and which is typically for the genus *Thermoanaerobacter*, which reduces thiosulfate to elemental sulfur only without the expected formation of sulfide. We describe its physiological characteristics and biological corrosion with metabolism of thiosulfate.

MATERIALS AND METHODS

Sample collection and bacterial isolation

Strain CF1 was isolated from a reservoir water sample from an off shore oil-producing well (Dagang oil field) in Tianjin. Production fluids of Dagang oil field used for microbial analysis were collected directly from production wellheads into sterile carboys.

Medium, enrichment and isolation

The modification of Hungate technique (Miller and Wolin, 1974) was used in the overall process. Enrichments were performed at 60°C by directly inoculating a 3 ml sample from the reservoir water into a basal medium containing (per litre of distilled water) 1 g KH_2PO_4, 0.5 g K_2HPO_4, 3 g NaCl, 0.5 g $NaNO_3$, 1 g NH_4Cl, 0.05 g $CaCl_2.2H_2O$, 0.2 g $MgCl_2.6H_2O$, 0.5 g Cysteine-HCl, 2 g yeast extract (Oxoid), 1 g Bio-trypticase (Oxoid), 5 g glucose , 10 ml of the trace element solution (Balch et al., 1979) and 0.1 mg resazurin.

Physiological studies

Physiological characteristics of strain CF1 were tested with different carbons, and a range of temperature and pH. The growth was determined by counting and by measuring the increase in optical density at 600 nm. The substrates were tested in the presence or absence of sodium thiosulfate (20 mM). For electron acceptors tests, 20 mM sodium thiosulfate, 20 mM sodium sulfite, 20 mM sodium sulfate and 2% (w/v) elemental sulfur were added to the medium (Ravot et al., 1999). To test the tolerance of thiosulfate, a ranging concentration of sodium thiosulfate was added to the medium. The end products from glucose fermentation were determined by gas chromatography (Chrisotomos et al., 1996).

Phylogenetic analysis

The 16S rRNA gene was selectively amplified from genomic DNA by PCR using 27F and 1492R as the primers. The productions were recovered by a DNA Recovery Kit, and ligated to pMD-19T cloning vector and transformed into *Escherichia coli* JM109. Three positive clones were selected randomly for sequencing analysis. Sequence analysis and phylogenetic tree constructions were done using CLUSTAL_X 1.83 and MEGA 4.1 software, and the GenBank accession number was HM228409.

The DNA G+C content and cellular fatty acid contents

Genomic DNA was extracted using a large-scale phenol/ chloroform-based procedure essentially as described by Wilson (1997). The DNA G+C content was measured by using HPLC (Mesbah et al., 1989). The celluar fatty acids were analyzed by gas chromatography (Sasser, 2001).

Polarization study

Q235 carbon steel (Cangzhou City Rui Pipe Co., Ltd. China) was used as a working electrode in this study, which composed of the following elements with a mass ratio of 0.22% C, 0.05% Si, 0.48% Mn, 0.012% P, 0.022% S. It was plugged in araldite with an exposed area of 1.0 cm^2. The specimens were immersed at 60°C in a sterile medium, as well as the isolation inoculated in basal medium with 250 mM sodium thiosulfate. A platinum plate and a saturated calomel electrode (SCE) were used as a counter electrode and a reference electrode respectively (Kuang et al., 2007). The Tafel polarization curves were obtained according to Anandkumar et al. (2009).

Method for weight loss detection

Size of 1 to 5 cm of Q235 carbon steel coupons were polished, degreased with aceton and air dried. The carbon steels were immersed in 250 ml of isolation medium incubated at 60°C. Duplicate systems were maintained, one inoculated with 5 ml of strain CF1 and the other was blank control. Finally, the carbon steels were removed in pickling solutions, washed with water and dried and the average loss weight of the coupons in each culture and uninoculated control were calculated.

X-ray diffraction (XRD) analysis

The carbon steel specimens were taken out and dried in cool air. The corrosion products were observed by XRD (X' Pert MPD PRO) analysis.

RESULTS

Petroleum reservoir characteristics

The oil field is exploited using water-flooding. The water for injection is separated from the oil produced fluid and recycled. The formations are situated 1965 to 1976 m below the sea floor and have a temperature of 75°C. The formation water of sodium hydrocarbonate type has a low salinity (4780 mg/l) and a pH of 8.3 with low concentration sulfate (8 mg/l).

Physiological studies

A strictly anaerobic, thermophilic bacterium, designated strain CF1, was isolated. The cells were Gram-positive rod-shaped, straight to slightly curved approximately 0.5 µm in width and 2 to 6 µm in length (Figure 1). Occasionally, cells up to 25 µm in length were observed. Shorter or longer cells frequently contained intermittent coccoid cells (Figure 1), spores were oval and located at the terminal of the mother cell which have the typical characteristics of *Thermoanaerobacter* (Wiegel and Ljungdahl, 1981). Physiological analysis showed that yeast extract was necessary for growth on non-

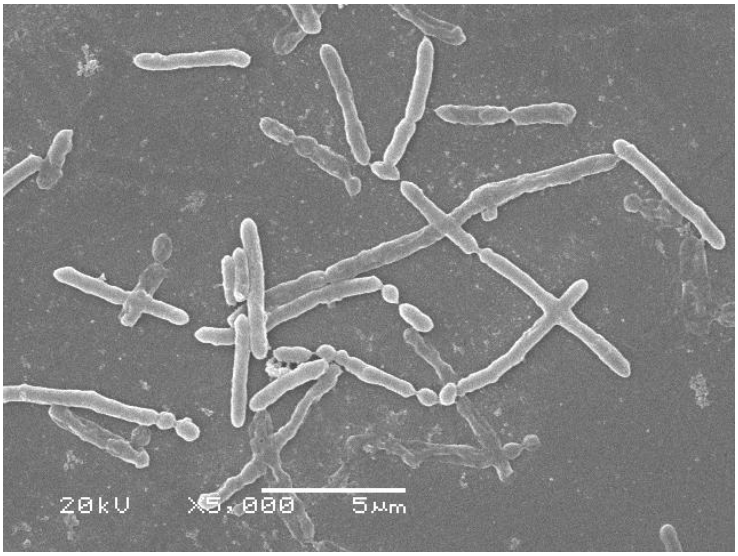

Figure 1. Electron micrograph of BF1.

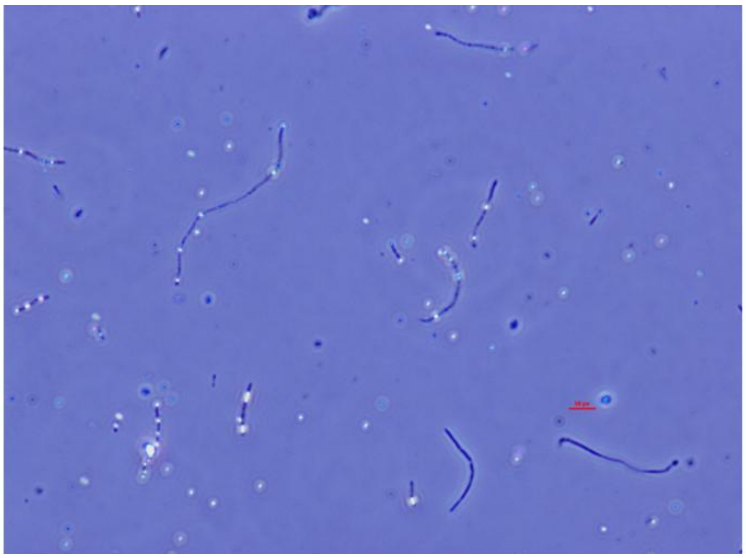

Figure 2. Morphology of cells and intracellular sulfur deposits were observed in strain CF1 (The lights pots in cell are sulfur deposits).

proteinaceous substrates. Using glucose as carbon sources, growth occurred at 45 to 78°C (optimum 60°C), at pH 5.0 to 8.5 (optimum 7.1), at NaCl 0 to 4.5% (optimum 0.5%).

At the optimum, the shortest observed doubling-time was 45 min. Some sugars were used as energy sources, the substrates were the following: D-cellobiose, fructose, glucose, maltose, mannose, trehalose, melezitose, raffinose, ribose, sucrose, xylose and starch. Strain CF1 could not utilize fumarate, lactose, D-galactose, inulin, melibiose, L-sorbose, glycerol, rhamnose, sorbitol, arabinose, sodium carboxymethylcellulose, xylan. When grown with 2 g glucose l^{-1} and 1 g yeast extract l^{-1}, the fermentation end products were detected with 0.29 g ethanol l^{-1}, 0.07 g acetic acid l^{-1}, some CO_2 and H_2, after cultivation for 3 days. The presence of thiosulfate did not affect the substrate utilization pattern. The effect of electron acceptors analysis showed that the strain CF1 used thiosulfate but not sulfur or sulfate. It could be resistant to 1.2 M thiosulfate and 75 mM sulphite. Cultivated with thiosulfate, intracellular sulphur deposits were observed in strain CF1 (Figure 2), and no H_2S was

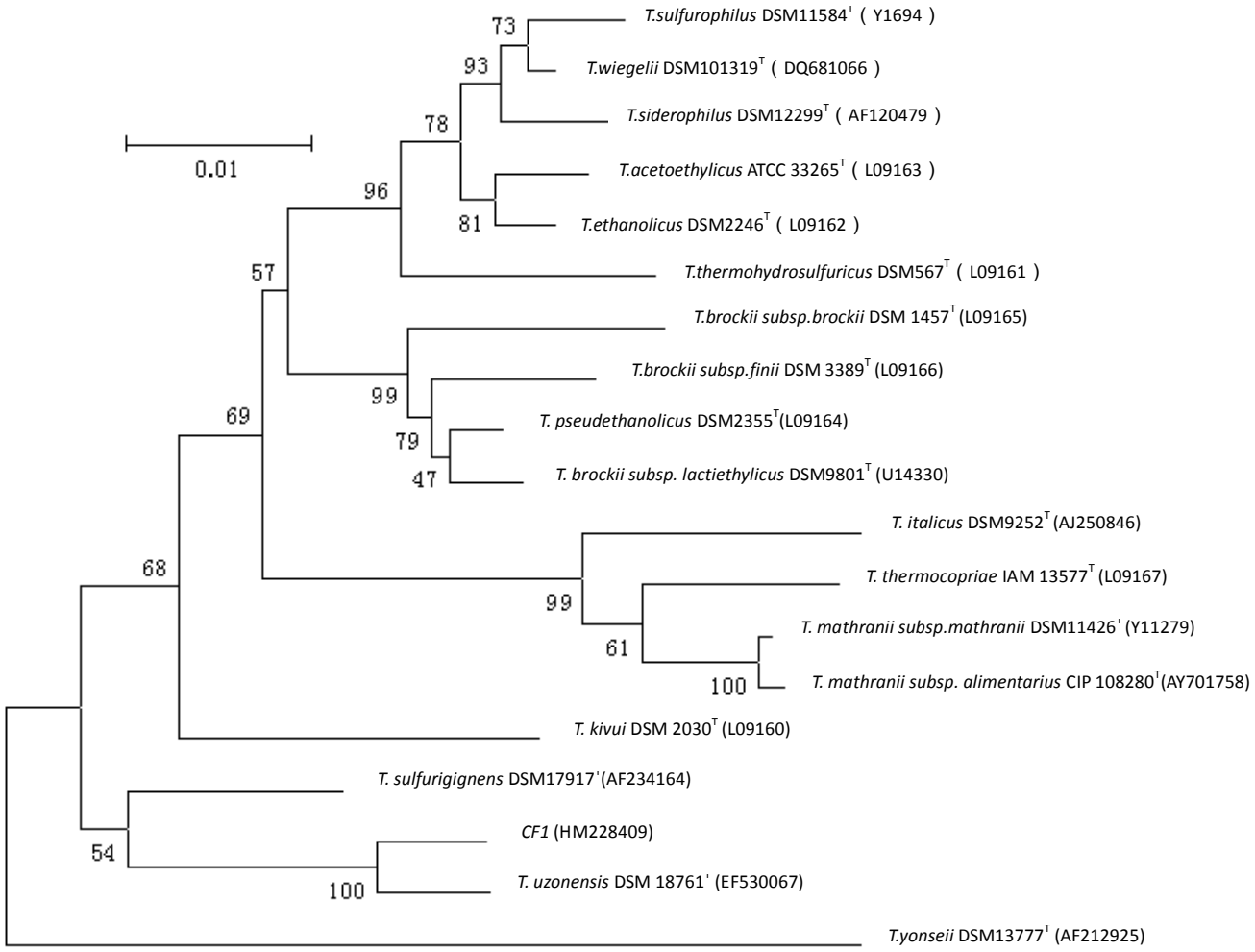

Figure 3. Phylogenetic tree of CF1 and its related bacteria constructed using 16S rRNA sequences.

detected.

Molecular identification

The 16S rRNA sequence analysis indicated that strain CF1 was related to members of the genus *Thermoanaerobacter*, the closest phylogenetic relatives with validly published names were *T. uzonensis* DSM 18761^T (99.2% similarity) and *T. sulfurigignens* DSM 17917^T (97.4% similarity) (Figure 3).

The DNA G+C content and cellular fatty acid contents

The G+C content of DNA of isolate CF1 was 33.5 mol% (as determined by HPLC). The major cellular fatty acid contents for strain CF1 were very different with *T. uzonensis* DSM 18761^T. They were 14: 0 (2.5%), 4, 8, 12-trimethyl 16:0 (0.4%), 12-methyl 14: 0 (0.3%), 15: 0 (65.8%), 16: 0 (8.6%), 16: 1 v 9c (0.4%), 15-methyl 17: 0 (8.1%), 14-methyl 17: 0 (7.4%), 18 : 1 v 9c (1.7%) and 18 : 0 (4.8%) (Table 1).

Polarization study

In the polarization study (Figure 4), E_{corr} of the control system was -0.737 V vs SCE, and in the presence of inoculating bacterial isolate strain CF1, E_{corr} was -0.674V vs SCE. The corrosion current was slightly higher in the presence of bacteria, but not obviously. It was 3.528e-005 A/cm^2 in the control and 3.5746e-005 A/cm^2 in the presence of strain CF1.

Weight loss analysis

The corrosion rate was investigated by weight loss method. After being cultivated, a thin layer of hard and black film was found on the outer part of the carbon steel in the inoculated system. In the control system, the

Table 1. Cellular fatty acid contents (%) of strain CF1 and *Thermoanaerobacter uzonensis* (ND: not found).

Fatty acid composition	Strain CF1	*T. uzonensis*
iso-13 : 0	ND	1.4
14 : 0	2.5	1.7
4,8,12-trimethyl 16:0	0.4	ND
iso-15 : 0	ND	53.5
12-methyl 14:0	0.3	ND
anteiso-15 : 0	ND	5.3
15 : 0	65.8	11.8
16 : 0	8.6	7.3
10-methyl 16 : 0	ND	7.3
16:1v9c	0.4	ND
iso-17 : 0	ND	2.8
15-methyl 17:0	8.1	ND
14-methyl 17:0	7.4	ND
18 : 1v9c	1.7	3.9
18 : 1v7t	ND	ND
18 : 0	4.8	5.0
Total	100	100

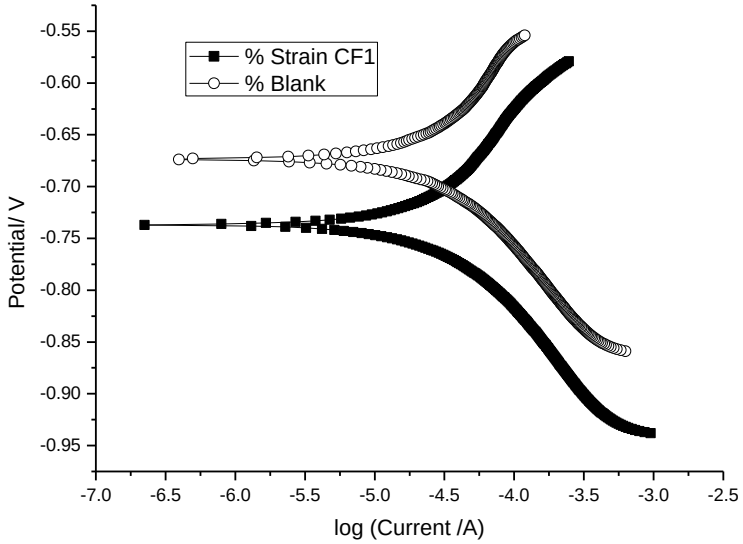

Figure 4. Potentiodynamic polarization curves of Q235 carbon steel in cultivated and control system after 7 days.

weight loss was 65 mg, whereas in the presence of strain CF1, the weight loss was 50 mg (Table 2). It indicates that the corrosion rate was less (0.0033 mm/year) in the presence of strain CF1 than the control system.

XRD analysis

The corrosion products (crystalline or amorphous), thought to be related to microbial activites of strain CF1, on the carbon steel surface were revealed by X-ray diffraction (X' Pert MPD PRO). Peaks of higher intensity of Fe_2O_3, element sulfur, $Fe_3(PO_4)_2$ and $Fe(OH)_3$ in the experimental system were noticed (Figure 5). Comparing with the inoculated system, similar results were found in the control system, except for elemental sulfur.

DISCUSSION

One thermophilic bacterium (CF1) was isolated and studied in this paper. Its 16S rRNA gene sequence

Table 2. Corrosion rate of carbon steel.

S/N	System	Weight loss (mg)	Corrosion rate (mm/year)
1	Control uninoculated medium	65±2	0.0042
2	Experimental system CF1	50±1	0.0033

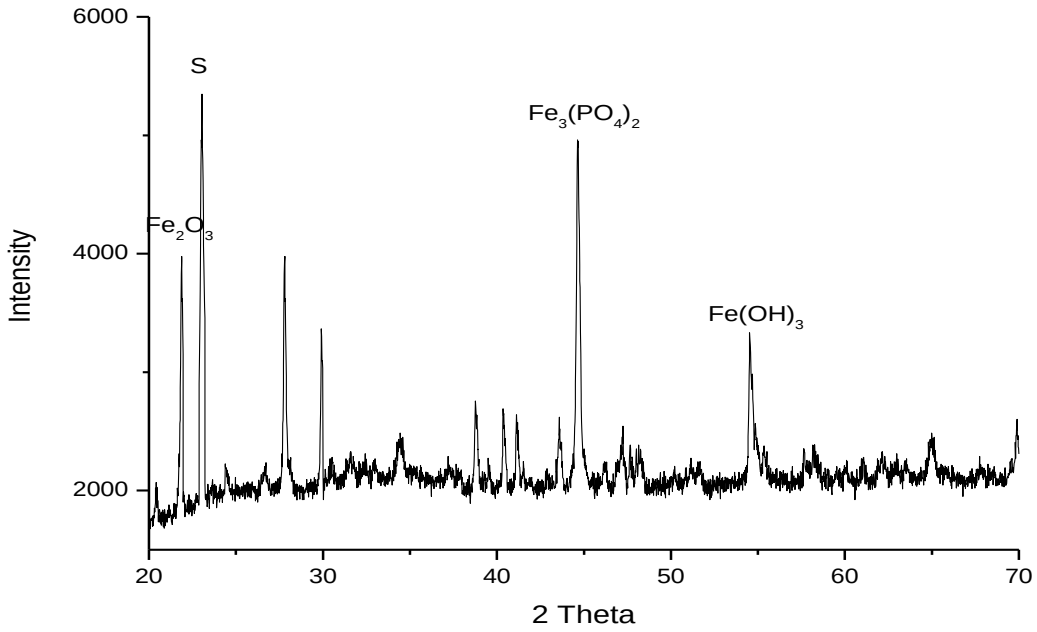

Figure 5. XRD pattern of the corrosion product.

Table 3. Characteristics that differentiate strain CF1 and related species of *Thermoanaerobacter sulfurigignens* and *Thermoanaerobacter uzonensi* (+: yes , -: no).

Characteristic	CF1	*T. sulfurigignens*	*T. uzonensi*
Source	Oil field	Hot spring	Hot spring
Cell size (µm)	0.5×2-6	0.3–0.8×1.2–4.0	0.5×2–5
Temperature rang (optimum °C)	45-78(60°C)	34–72 (63–67)	32.5–69 (61)
pH range (optimum)	5.0-8.5(7.1)	4.0–8.0 (5.0–6.5)	4.2–8.9(7.1)
DNA G+C content (mol %)	33.5	34.5	33.6
Doubling time under optimal conditions (h)	0.75	2.4	0.5
Product from $S_2O_3^{2-}$ reduction	S^0	S^0	S^{2-}, S^0
Toleration of 1 M thiosulfate	+	+	-

analysis indicated that the closest phylogenetic relatives were *T. uzonensis* DSM 18761^T (99.2%) and *T. sulfurigignens* DSM 17917^T (97.4%), respectively. However, the strain CF1 was different with *T. uzonensis* DSM 18761^T and *T. sulfurigignens* DSM 17917^T in many physiological characteristic (Table 3), and fatty acid profile (Table 1). The results suggested that the strain CF1 may be a new species of *Thermoanaerobacter* genus, the exact taxonomic status of it requires DNA hybridization further.

One activity of the strain tolerated high concentration of thiosulphate. It can be resistant to 1.2 M thiosulphate similar with *T. sulfurigignens* DSM 17917^T (Lee et al., 2007), but *T. uzonensi* DSM 18761^T tolerated only up to 200 mM thiosulfate (Wagner et al., 2008). The production was sulfur globules instead of hydrogen sulfide, when it was grown in a medium containing thiosulfate. Thiosulfate is produced from chemical oxidation of sulfide, and is common in oil fields (Jørgensen, 1990; Cline and Richards, 1969). Some reports revealed that

the presence of thiosulfate may increase the risk of biocorrosion of oil pipelines (Faudon et al., 1995; Duncan et al., 2009). We have therefore initiated a study of the bio-corrosion behavior of the strain CF1 undergoing thiosulfate reduction. After being tested, a less corrosive was found. With results presented in this paper, the process of microbial thiosulfate reduction could be less biocorrosive in oil fields, as the “thiosulfate shunt” was controlled and less H_2S was produced.

Conclusion

The main objective of this study is to isolate new thiosulfate reducing and elemental sulfur producing bacteria from oil reservoir and reveal their bio-corrosion process. The results suggested that there might be members of microbes in oil fields that reduce thiosulfate exclusively to elemental sulfur, and do not form sulfide. Further research is necessary to understand the physiological characteristics and the microbial corrosion mechanism of them, and it may be very important to reduce the damage caused by H_2S in oil fields.

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