

# A thermophilic nitrate-reducing bacterium isolated from production water of a high temperature oil reservoir and its inhibition on sulfate-reducing bacteria

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**Abstract:** A thermophilic spore-forming facultative anaerobic bacterium, designated as Njiang2, was isolated from the production water of a high temperature oil reservoir (87°C). The physiological, biochemical and 16S rRNA gene based phylogenetic analysis indicated that Njiang2 belonged to the genus *Anoxybacillus*. Njiang2 could significantly inhibit H<sub>2</sub>S production when co-cultured with *Desulfotomaculum* sp under laboratory conditions, which implied its great potential in mitigation of brine souring in the oil reservoir and in control of biocorrosion caused by sulfate-reducing bacteria. As far as we know, this might be the first report of *Anoxybacillus* sp. isolated from high temperature oilfield.

**Keywords:** *Anoxybacillus*, thermophilic, 16S rRNA gene analysis, nitrate-reducing bacterium, souring mitigation, microbial influenced corrosion

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**Received:** February 19, 2016 ; **Accepted:** July 7, 2016 ; **Published Online:** November 17, 2016

**Citation:** Liu J-F, Wu W-L, Yao F, *et al.* 2016, A thermophilic nitrate-reducing bacterium isolated from production water of a high temperature oil reservoir and its inhibition on sulfate-reducing bacteria. *Applied Environmental Biotechnology*, vol.1(2): 35–42. <http://dx.doi.org/10.26789/AEB.2016.02.004>.

## 1. Introduction

Oil reservoir souring and microbial induced/influenced corrosion (MIC), mainly caused by sulfate-reducing bacteria (SRB), are being given more and more attentions by the oil industry, and control on the growth and activity of SRB is of great importance. Studies demonstrate that supplement of nitrate to injection water is an effective me-

thod to inhibit H<sub>2</sub>S production and control the activities of SRB in the oil production system. Nitrate addition can stimulate the nitrate-reducing bacteria (NRB), a competitive group of anaerobic bacteria, which out-compete SRB and hence suppress the activities of SRB and/or remove sulfide<sup>[1-3]</sup>.

The mechanism on the mitigation of souring by injection of nitrate or nitrite has been investigated<sup>[4-10]</sup>. Obviously, NRB plays an important role in mitigation

souring and control of MIC, and more and more attentions have been paid to the investigation and isolation of nitrate-reducing bacteria inhabiting in oil reservoirs recently. Up to now, various microorganisms capable of reducing nitrate have been isolated from oil reservoirs, including *Geobacillus*<sup>[11]</sup>, *Deferribacter* thermophiles<sup>[12]</sup>, *Anaerobaculum thermoterrenum*<sup>[13]</sup>, *Marinobacter aquaeolei*<sup>[14]</sup>, *Denitrovibrio acetiphilus*<sup>[15]</sup>, *Garciella nitratreducens*<sup>[16]</sup>, *Thauera* and *Sulfurospirillum* spp.<sup>[9]</sup>, *Marinobacter*, *Marinobacterium*, and *Halomonas* spp.<sup>[17]</sup>. More recently, the nitrate-reducing community has been investigated based on PCR amplification of *napA* genes and nitrate-reducing bacterial communities have been recognized to be of rich diversity in production water from oil reservoirs<sup>[18]</sup>. However, the current knowledge about physiological properties of these nitrate-reducing bacteria is still limited.

Considering the generally anaerobic and thermophilic property of oil reservoirs underground, thermophilic nitrate-reducing anaerobes or facultative anaerobes have great potential in mitigating oil reservoir souring. This study mainly focuses on the isolation and evaluation of thermophilic nitrate-reducing bacteria from high temperature oil reservoir, and the morphological, physiological and phylogenetic characteristics as well as cellular fatty acids. Inhibition activity to SRB was also tested under laboratory conditions.

## 2. Materials and Methods

### 2.1 Sample Collection

Oil/water mixture sample from the H88 block (2400 m of depth, 87°C), Jiangsu Oilfield, China, was taken directly from production well-heads into sterile 500 ml serum bottles, and immediately sealed with rubber stoppers before transporting back to our laboratory. The water phase, after separated from oil, was used for bacteria isolation. The high salinity production water was mainly composed of chloride, sulfate, potassium and sodium. The main characteristics of the samples are presented in Table 1.

### 2.2 Enrichment and Isolation

For the enrichment of microorganisms, the basal medium was used ( $\text{g}\cdot\text{L}^{-1}$ ) which contains: sodium acetate 3.0, KCl 0.33,  $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$  0.33,  $\text{NH}_4\text{Cl}$  0.33, NaCl 5.0,  $\text{KH}_2\text{PO}_4$  0.33,  $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$  0.02, yeast extract 0.5,  $\text{NaHCO}_3$  0.3,  $\text{KNO}_3$  2.0,  $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$  0.5, Vitamin solution 3.0 mL, trace element solution 3.0 mL. The vitamin solution contained (mg/L): pyridoxine-HCl, 100; nicotinic acid, 50.0; thioctic acid, 50.0; aminobenzoic

**Table 1.** Characteristics of sample collected from high temperature Jiangsu oil reservoir

Item	Value
Depth (m)	2400
Temp (°C)	87
Water flooding time (years)	3
Mineralization	15165
Water type	$\text{NaHCO}_3$
pH	6.6
$\text{Cl}^-$ ( $\text{mg}\cdot\text{L}^{-1}$ )	4953.1
$\text{SO}_4^{2-}$ ( $\text{mg}\cdot\text{L}^{-1}$ )	2924.6
$\text{PO}_4^{3-}$ ( $\text{mg}\cdot\text{L}^{-1}$ )	4.30
$\text{NO}_3^-$ ( $\text{mg}\cdot\text{L}^{-1}$ )	25.3
$\text{K}^+$ , $\text{Na}^+$ ( $\text{mg}\cdot\text{L}^{-1}$ )	5329.4
$\text{Ca}^{2+}$ , $\text{Mg}^{2+}$ ( $\text{mg}\cdot\text{L}^{-1}$ )	40.84
$\text{S}^{2-}$ ( $\text{mg}\cdot\text{L}^{-1}$ )	57.7

acid, 50.0; thiamine-HCl, 50.0; p-Ca-D(+)-pantothenate, 50.0; riboflavin, 50.0; folic acid, 20.0; D(+)-biotin, 20.0; and vitamin B12, 1.00. Vitamin and trace element stocks solutions were made according to Wang *et al.*<sup>[19]</sup>. The medium was prepared and autoclaved and then put under positive pressure of flow of nitrogen anaerobically and its pH was adjusted to 7.0 with NaOH or HCl before dispensation into 120 mL serum bottles with the headspace (20 mL) purged and filled with pure  $\text{N}_2$ .

The enrichment was conducted by inoculation of the produced water sample (10% v/v) into the basal medium and then incubation at 60°C for 6 days. The fresh culture obtained was inoculated in the same medium and incubated under the same conditions and these procedures repeated for 3 times before it was used for microbial isolation.

The pure isolate was obtained after serial dilution and confirmed by its uniformity both in micromorphological appearance and macromorphological colonies formed anaerobically on solid medium (basal medium supplemented with 1.5% Gellan Gum, Sigma) in Hungate tubes at 60°C.

### 2.3 Morphological and Physiological Characterization

Cellular morphology was determined by light microscope, and colony morphology was determined with colonies formed anaerobically on solid medium at 60°C. The formation of spores was observed with culture after 5 days of incubation. To determine optimal growth temperature, salinity and pH, incubation experiments were performed with basal medium in-

oculated with isolated strain under anaerobic conditions. The temperature range was determined by incubation test at different temperature ranging from 45°C to 70°C with 5°C intervals. In order to determine the optimum NaCl concentration for growth, the concentration of NaCl in the basal medium were set to be 2.0, 3.0, 4.0, 5.0, 8.0, and 10.0% (w/v) while that of the other components of the medium were kept unchanged. The pH range of growth was examined with cultivation test on Nutrient Broth medium with pH from 5.0 to 10.0 at an interval of 1.0 pH unit. These physiological studies were conducted in constant temperature incubators without shaking at 60°C. Growth was determined by measuring the optical density of the culture medium spectrophotometrically at 600 nm.

The ability of Njiang2 in utilizing different carbohydrates was examined by incubation in the Peptone Water Medium with carbohydrate at a final concentration of 0.75%–1% (w/v) under aerobic conditions. The carbon source, D-cellobiose, D-fructose, L-rhamnose, L-arabinose, L-D-xylose, glucose, sucrose,  $\alpha$ -lactose, mannose, L-sorbose, D-ribose, D-galactose, D-mannitol, sorbitol, and starch were separately sterilized as stock solutions. Gram staining, hydrolysis of starch, gelatin and casein, citrate and urea utilization, reduction of nitrate to nitrite, Voges-Proskauer test, Methyl red test, indole and hydrogen sulfide production tests were carried out by the methods according to Microbiology Experiment<sup>[20]</sup>. Air bubble production using 3% (v/v) H<sub>2</sub>O<sub>2</sub> solution was used for evaluating the activity of catalase. The oxidase reaction was examined with agar plate containing 1% (w/v) N, N, N', N'- tetramethyl-*p*-phenylenediamine<sup>[21]</sup>.

#### 2.4 16S rRNA Gene Sequencing and Phylogenetic Analysis

The extraction and purification of DNA was performed using AxyPrep<sup>TM</sup> Bacterial Genomic DNA Mini-prep Kit (Axygen Bioscience, Inc., CA, USA) according to the manufacturer's protocol. Resulting genomic DNA was immediately preserved at -20°C before usage. The 16S rRNA gene was amplified by PCR using 27F (5'-AGAGTTTGATCCTGGCTCA-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') primers with the program as follows: initial denaturation at 95°C for 180 sec, 30 cycles of 94°C for 40 sec, annealing at 52°C for 45 sec, and 72°C for 90 sec, and a final elongation step of 72°C for 10 min. Two replicates of 25  $\mu$ L PCR reaction mixture were made, each of which contains 2 $\times$ PCR MasterMix 2  $\mu$ L, ddH<sub>2</sub>O 11

$\mu$ L, two primers 1  $\mu$ L, respectively and template DNA 2  $\mu$ L. The sequence of the PCR products of 16S rRNA gene was determined by BGI sequencing company after purification. The results are presented as a phylogenetic tree which was made with MEGA version 5.0. The tree was rooted with 16S rRNA gene sequence from *Geobacillus thermodenitrificans*. 16S rRNA gene from Njiang2 is available in NCBI under the accession number KF421130.

#### 2.5 G+C Content Determination and Cellular Fatty Acid Analysis

The genomic DNA of Njiang2 was extracted from overnight culture with basal medium mentioned above and purified before the determination of G+C content by thermal denaturation temperature method<sup>[22]</sup> using *Escherichia coli* DNA as standards. Denaturation profiles were determined at 260 nm using a thermo-programmable Lambda35 UV/VIS spectrophotometer equipped with a temperature controller.

We used the Sherlock Microbial Identification System (MIDI) to determine the cellular phospholipid composition of Njiang2. Briefly, fresh cells in the mid-exponential phase were collected and put into a clean glass tube. The glass tube containing cells was then added with 1.0 mL saponification solution (45 g NaOH, 150 mL CH<sub>3</sub>OH, and 150 mL dH<sub>2</sub>O) before it was vortexed for 5–10 s and afterwards heated in a boiling water bath at 100 °C for 30 min. After saponification, the tube was added with 2.0 mL methylating reagent (a mixture of 275 mL methyl alcohol and 325 mL 6.0 N hydrochloric acid), and placed in 80 °C water bath for 10 min for methyl esterification. The esterified mixture, after cooled to room temperature, was extracted with 1.25 mL extraction reagent (200 mL methyl *tert*-butyl ether and 200 mL hexane) and the extracts were collected and washed with 3.0 mL base solution (10.8 g NaOH dissolved in 900 mL dH<sub>2</sub>O) before GC analysis. Cellular Fatty Acid Methyl Esters (CFAMEs) were identified and quantified with Sherlock Microbial Identification System Software (V6.0).

#### 2.6 Inhibition of Sulfide Production by SRB

To evaluate the ability of Njiang2 in inhibition of SRB for its sulfide production, incubation experiment with 3 treatments were conducted: (1) SRB only, (2) SRB+ nitrate, and (3) SRB +nitrate+Njiang2. The SRB used here was *Desulfotomaculum* sp, a typical SRB isolated from Jiangsu Oilfield, and the incubation medium was

composed of (g·L<sup>-1</sup>): Na<sub>2</sub>SO<sub>4</sub> 1.25, MgCl<sub>2</sub>·6H<sub>2</sub>O 0.33, KH<sub>2</sub>PO<sub>4</sub> 0.33, CaCl<sub>2</sub> 0.02, NH<sub>4</sub>Cl 0.50, KCl 0.33, yeast extract 0.10, C<sub>3</sub>H<sub>5</sub>O<sub>3</sub>Na 3.50, NaCl 5.0, L-Cysteine hydrochloride anhydrous 0.20, Na<sub>2</sub>S·9H<sub>2</sub>O 0.5, Resazurin 0.002. All these experiments were conducted at 65°C and the H<sub>2</sub>S produced was determined by colorimetric method<sup>[23]</sup> briefly as follows: 0.5 mL sample solution taken was transferred into a tube, followed by 5 mL of zinc acetate solution (20 g·L<sup>-1</sup>). The obtained mixture was placed in a water bath maintained at 20°C for 10 min, followed by addition of 1.0 mL of *N, N*-dimethyl *para*-phenylenediamine hydrochloric acid solution (1.0 g·L<sup>-1</sup>, 40% hydrochloric acid solution as solvent), after which 2.0 mL of ferric chloride solution (27 g·L<sup>-1</sup>, 40% hydrochloric acid solution as solvent) were added and blended. The mixture was placed into the water bath at 20°C for 10 min. Then, zinc acetate solution was added to the mixture to make the final volume to 10.0 mL before it was analyzed spectrophotometrically at 670 nm. Every treatment was conducted with three replicates and the mean value was presented.

### 2.7 Resistance to Antibiotics

The ability of Njiang2 to resist antibiotics was evaluated by Sensi-discs method<sup>[24]</sup> on Nutrient agar plates containing 1.8% agar (w/v). The filter-sterilized antibiotics (all at 10 µg·L<sup>-1</sup>) of Penicillin, Chloramphenicol, Streptomycin, Vancomycin, Gentamicin, Erythromycin and Kanamycin were used. After these plates were incubated at 60°C for 24 h – 48 h, zones of inhibition were measured.

## 3. Results

### 3.1 Phenotypic Characteristics

All the phenotypic characteristics of strain Njiang2 are listed in Table 2. The Njiang2 colonies formed after cultivation on Nutrient Agar plates for 16 h with appearance of cream, smooth and circular with a diameter of 2–3 mm. Cells of Njiang2 in liquid culture were stained Gram-positive, appeared as single, rods (0.3 – 0.5 µm in diameter and 2.0 – 4.0 µm in length). Growth of Njiang2 was observed at 45°C – 70°C with an optimum at 65°C, pH 6.0 – 9.0 with an optimum at pH 7.0, NaCl concentrations of 0% – 2.5% (w/v), with an optimum rang of 0.5% – 1%.

This strain can use a wide variety of carbon sources including citrate, maltose, mannose, glucose, D-fru-

ctose, sucrose, D-cellose, D-ribose, D-mannitol, D-xylose and starch for growth. However, no growth was observed with L-sorbinose, L-rhamnose, L-arabinose, α-lactose, galactose and sorbitol as substrates. It is tested positive for oxidase, urease and catalase. Gelatin and starch could be hydrolyzed and nitrate be reduced to nitrite by the isolated strain. Njiang2 fails to hydrolyze lipid and casein and shows negative test in indole production, Methyl red and hydrogen sulfide production. The Voges-Proskauer test was positive. As showed in Table 2, this strain was distinguished from other closest neighbor *Anoxybacillus* species by some characteristics.

**Table 2.** Comparison of the phenotypic characteristics of strain Njiang2 and related species

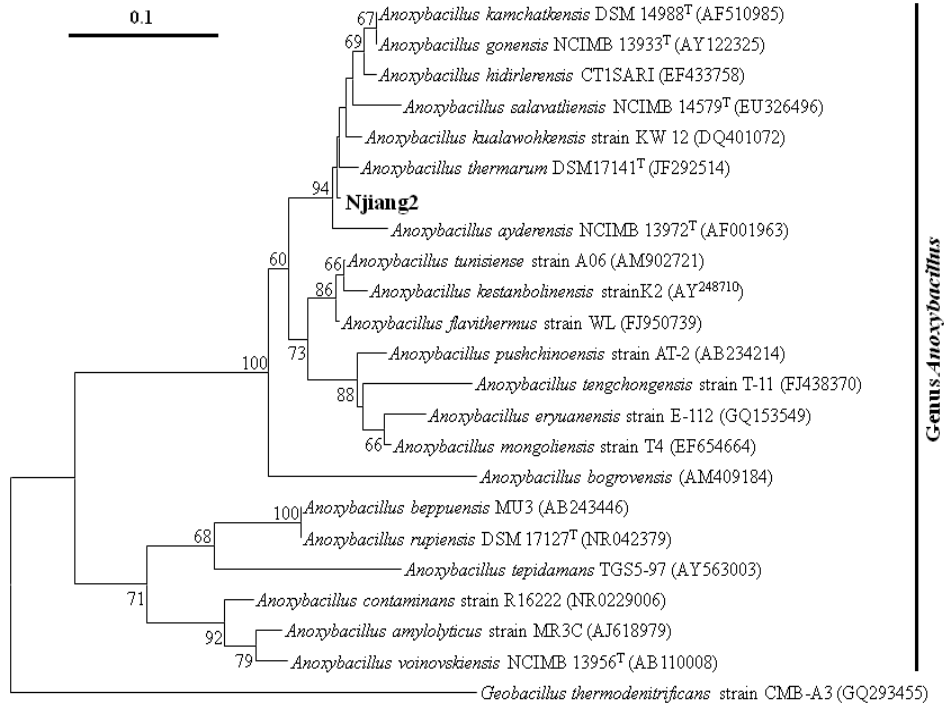
Characteristic	1	2	3	4	5
Relation to O <sub>2</sub>	Facultative anaerobe	Aerobe	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe
Temperature (°C)	45–70	55–67	37–69	37–70	37–66
Optimum temperature(°C)	60–65	65	60	55–60	57–62
pH	6.0–9.0	6.0–7.5	5.5–9.5	5.5–9.5	5.7–9.9
Optimum pH	7.0	7.2	8.0–9.0	7.0	6.8–8.5
NaCl(3%, w/v)	–	–	+	–	ND
Motility	–	+	+	+	+
Hydrolysis					
Gelatin	+	–	+	+	–
Starch	+	–	+	–	–
Casein	–	–	ND	ND	–
Oxidase	+	–	+	+	–
Catalase	+	+	+	+	–
Nitrate reduction	+	–	+	+	ND
Substrates utilized					
L-Arabinose	–	ND	–	–	–
D-xylose	+	–	–	+	–
Glucose	+	–	+	+	+
Sucrose	+	–	ND	–	+
α-lactose	–	–	–	–	–
Mannose	+	–	+	–	–
D-ribose	+	–	ND	ND	+
D-galactose	+	–	+	ND	ND
D-mannitol	+	ND	+	–	+

1, strain Njiang2; 2, *A. thermarum* DSM 17141<sup>T[25]</sup>; 3, *A. Salavatiensis* DSM 22626<sup>T[26]</sup>; 4, *A. gonensis* NCIMB 13933<sup>T[27]</sup>; 5, *A. Kamchatkensis* DSM 14988<sup>T[28]</sup>; + Positive, – Negative, ND not determined

### 3.2 Phylogenetic Analysis

The 16S rRNA gene sequence of Njiang2 was compared with its closely related bacteria and the results are presented in phylogenetic tree (Figure 1). The new isolate had a sequence similarity ranging from 94% to 99% with strains of *Anoxybacillus* species. Its closest

relatives are *A. thermarum* DSM17141<sup>T</sup>, *A. salavatiensis* NCIMB 14579<sup>T</sup>, *A. gonensis* NCIMB 13933<sup>T</sup>, *A. kamchatkensis* DSM 14988<sup>T</sup> and the more distant one was *A. voinovskiensis* NCIMB 13956<sup>T</sup>. According to 16S rRNA gene sequence analysis, the new isolate was considered to be members of the genus *Anoxybacillus*.



**Figure 1.** 16S rRNA gene based neighbor-joining phylo tree of strain Njiang2 and relatives from genus *Anoxybacillus*. The tree was rooted with *Geobacillus thermodenitrificans*. 10% substitutions per nucleotide position are given on the scale bar.

### 3.3 G+C Content and Fatty Acid Composition

The G+C content was determined by thermal denaturation temperature method and the result showed that the DNA G+C content of Njiang2 was 48.8%, which was different from its closest relatives *A. gonensis* (57%), *A. salavatiensis* (45.1%), *A. kamchatkensis* (42.3%) and *A. thermarum* (53.5%).

The results of phospholipid analysis are listed in Table 3. Njiang2 is composed mostly of branched saturated fatty acids and little amounts of *anteiso*-fatty acids. The main components of fatty acids are *iso*-C<sub>15:0</sub> (33.4%) and *iso*-C<sub>17:0</sub> (25.7%), which agree well with fatty acid profiles of previously identified and published *Anoxybacillus* species. *Iso*-C<sub>15:0</sub>, followed by *iso*-C<sub>17:0</sub> and *anteiso*-C<sub>17:0</sub> are the major fatty acids of Njiang2 and its closest relatives *A. gonensis*, *A. salavatiensis* and *A. kamchatkensis*. However, Njiang2 has high amounts of *iso*-C<sub>16:0</sub> and C<sub>16:0</sub>, similar to

**Table 3.** Cellular fatty acid compositions of Njiang2 and related *Anoxybacillus* type strains

Fatty acids	1	2 <sup>a</sup>	3 <sup>a</sup>	4 <sup>a</sup>	5 <sup>a</sup>
C <sub>14:0</sub>	–	1.25	–	–	0.9
<i>iso</i> -C <sub>14:0</sub>	1.48	1.18	5.19	–	1.6
<i>iso</i> -C <sub>15:0</sub>	33.39	65.19	46.91	46.6	9.1
<i>anteiso</i> -C <sub>15:0</sub>	2.04	2.64	2.81	2.9	7.7
C <sub>15:0</sub>	–	1.12	0.42	–	2.6
<i>iso</i> -C <sub>16:0</sub>	12.36	5.99	1.23	8.7	20.7
C <sub>16:0</sub>	9.96	2.38	19.41	–	21.0
<i>iso</i> -C <sub>17:0</sub>	25.74	11.96	15.80	6.6	9.3
<i>anteiso</i> -C <sub>17:0</sub>	5.87	3.29	5.33	25.6	18.9
C <sub>18:0</sub>	2.11	–	0.31	–	3.0
C <sub>18:1<math>\omega</math>9</sub>	3.67	0.23	–	–	–

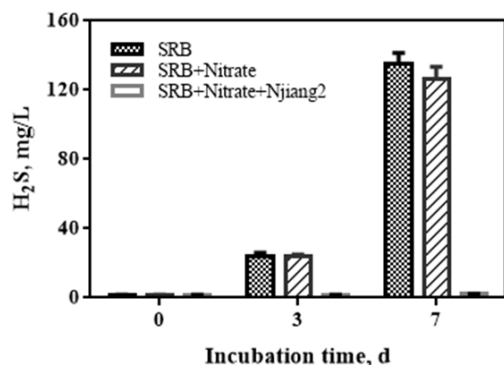
Strains: 1, Njiang2; 2, *A. gonensis* NCIMB 13933<sup>T</sup>; 3, *A. salavatiensis* DSM 22626<sup>T</sup>; 4, *A. thermarum* DSM 17141<sup>T</sup>; 5, *A. kamchatkensis* DSM 14988<sup>T</sup>. a, Data from the corresponding reference of the concerning strain, as, *A. gonensis* NCIMB 13933<sup>T</sup>[27]; *A. salavatiensis* DSM 22626<sup>T</sup>[26]; *A. thermarum* DSM 17141<sup>T</sup>[25]; *A. kamchatkensis* DSM 14988<sup>T</sup>[28].



its closest relative *A. thermarum*. The contents of other fatty acids, such as *iso*-C<sub>14:0</sub> (1.48%), *anteiso*-C<sub>15:0</sub> (2.04%) and C<sub>18:0</sub> (2.11%), are relatively low. In contrast, the content of C<sub>18:1 $\beta$</sub>  is higher in Njiang2.

### 3.4 Inhibition on Sulfide Production

H<sub>2</sub>S produced in 3 different incubation treatments at 65°C were determined and shown in Figure 2. It showed that the H<sub>2</sub>S production in co-culture treatment of SRB and Njiang2 was negligible when compared to the SRB only and SRB with nitrate incubation treatments. The results of DGGE analysis of the microbial community in these 3 treatments also showed that the DNA bands corresponding to SRB appeared undetectable and those corresponding to Njiang2 were very strong, implying the fact that SRB was near completely inhibited by Njiang2.



**Figure 2.** Production of hydrogen sulfide over incubation time under different treatments (Each data point is the mean of three replicates, mean values  $\pm$  SD,  $n = 3$ ).

### 3.5 Antibiotic Sensitivity

Growth of Njiang2 was inhibited by Tetracycline, Streptomycin, Erythromycin, Gentamycin and Penicillin (each at 10  $\mu\text{g}\cdot\text{L}^{-1}$ ) but not by chloramphenicol and Kanamycin (each at 10  $\mu\text{g}\cdot\text{L}^{-1}$ ).

## 4. Discussion

Pikuta *et al.* first characterized the genus *Anoxybacillus* in 2000 and then the type strain of *A. pushchinoensis* was established. This strain was proposed as anaerobic bacterium and a further investigation showed that it was an aerotolerant anaerobe. At present, twenty species in this genus have been isolated and described, including *A. gonensis*<sup>[27]</sup>, *A. Kamchatkensis*<sup>[28]</sup>, *A. thermarum*<sup>[25]</sup>, *A. Salavatliensis*<sup>[26]</sup>. *Anoxybacillus* species were usually isolated from geothermal environments. Here, we report a new isolate

from a high temperature oil reservoir. The 16S rDNA sequence analysis showed the phylogenetic nature of genus *Anoxybacillus* of this isolate. To the best of our knowledge, this report is the first one on the isolation of *Anoxybacillus* from production water of oil reservoir.

There are only a few works reported on thermophilic nitrate-reducing bacteria isolated from oil reservoirs<sup>[11,12]</sup>. The typical NRBs are *Geobacillus* and *Deferribacter*. The genus *Geobacillus* can reduce nitrate anaerobically<sup>[11]</sup>. Genus *Deferribacter* is thermophilic and can anaerobically respire with multiple electron acceptors. *D. thermophilus* could reduce manganese and iron by oxidation of organic acids, hydrogen, and complex substrates<sup>[12]</sup>. Njiang2 grows between 37°C and 70°C with an optimal growth at 65°C, pH 6.0 – 9.0 with an optimum at pH 7.0, with NaCl concentration of 0% – 2.5% (w/v) (optimal 0.5%) which shows good agreement with the *in situ* conditions of the original oil reservoir characteristics with temperature of 86°C – 90°C, mineralization of 15000 mg/L and pH of 6.5. Generally, the temperature of oil strata varies from injection well to production well and becomes lower after water injection for years. Thus, it is reasonable to consider Njiang2 as the likely indigenous microorganism to the oil reservoir where it was isolated.

Nitrate-reducing bacteria (NRB) are environmentally significant bacteria which can reduce nitrate in waters containing rich organic matter and nitrate and are frequently detected in oil reservoirs including heterotrophic NRB and sulfide-oxidizing, chemolithotrophic NRB<sup>[6]</sup>. One of the important properties of NRB is its inhibition activity towards SRB in oil reservoir which are believed to be chief culprits of Microbial Influenced Corrosion (MIC). The activities of NRB in oil reservoir can be stimulated by injection of nitrate and sulfide production by SRB consequently inhibited<sup>[29]</sup>. Different NRBs play different roles, the heterotrophic NRB suppresses sulfide production by outcompeting heterotrophic SRB for substrates, whereas, the chemolithotrophic NRB, such as the NR-SOB (nitrate-reducing, sulfide-oxidizing bacteria), not only remove sulfide, but also suppress SRB for their sulfide production<sup>[30]</sup>. Taking into consideration of the facultative and thermophilic growth characteristics and the ability to inhibit sulfide production by SRB even under anaerobic conditions, Njiang2 shows a great potential in mitigation of oilfield souring and on control of biocorrosion caused by SRB.

The result of 16S rRNA gene sequence analysis

shows that Njiang2 is among the species of genera *Anoxybacillus* and has a similarity of 99% with sequences of *A. thermarum* DSM17141<sup>T</sup>. The difference in the G+C content of Njiang2 (48.7%) and *A. thermarum* DSM17141<sup>T</sup> (53.5%) is 4.8%. Notably different phenotypic characteristics are found such as hydrolysis of starch and gelatin, oxidase activity, nitrate reduction reaction, as well as the utilization of sucrose, mannose, and D-mannitol (Table 2). Members of the genus *Anoxybacillus* are characterized by their fatty acid profile with the major component of *iso*-C15:0 and *iso*-C17:0 and Njiang2 is in agreement with this for *iso*-C15:0 (33.39%) and *iso*-C17:0 (25.74%). The composition of fatty acid is quite different between Njiang2 and *A. thermarum* DSM17141<sup>T</sup> as shown in Table 3, especially in content of *anteiso*-C17:0 (5.87% for Njiang2 and 25.6% for *A. thermarum* DSM17141<sup>T</sup>). Based on the phenotypic and biochemical differences mentioned above, Njiang2 is more likely a new species or subspecies of the genus *Anoxybacillus*.

## 5. Conclusion

Njiang2 is a thermophilic spore-forming facultative anaerobic bacterium isolated from high temperature oil reservoir. It is identified by its physiological, biochemical and phylogenetic characteristics to be a member of genus *Anoxybacillus*. Njiang2 can significantly inhibit H<sub>2</sub>S production under laboratory conditions by *Desulfotomaculum* sp, a typical SRB isolated from Oilfield, which implied its great potential in mitigation of brine souring in the oil reservoir and on control of biocorrosion caused by SRB.

## Conflict of Interest and Funding

No conflict of interest was reported by the authors. This work was financially supported by the National Natural Science Foundation of China (Grant No. 41273084, 41530318), the NSFC/RGC Joint Research Fund (No. 41161160560), the Research Foundation of Shanghai (No. 15JC1401400), and the China Petroleum and Chemical Corporation, Sinopec (P14071).

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