Screening and characterization of high performance synthetic-based drilling fluids degrading bacteria

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Abstract. In this paper, eight degrading bacteria using waste high performance synthetic-based fluids (WHPSF) as sole carbon source were isolated. Genetic analysis showed that these strains belonged to 8 species, and distributed in 3 genera. Of them, genus Bacillus was the predomimated one with 6 strains. Based on the COD removal rate, 3 effective strains, i.e. SQ17, SQ8-1 and SQ6-1, were screened; and SQ6-1 had the highest COD degradation rate (40.26%). Growth characteristic analysis indicated that SQ8-1 and SQ6-1 could grow at initial pH value from 4.0 to 10.0, and grow at 10 °C to 40 °C. Moreover, these bacterial strains had strong ability to degrade WHPSF, and both the bacteria amount and COD removal rate increased along with treating time. The results suggested that these bacteria could degrade WHPSF properly, and had good application potential.

1. Introduction
Shale gas is an important fossil energy resource in the world, and the major distribution areas are at Sichuan and Chongqing province in China [1]. Different drilling fluids were needed in the process of gas exploration [2]. Usually, drilling fluids were water-based, oil-based, gas-based and synthetic-based. Recently, a new kind of drilling fluids called high performance synthetic-based fluid (HPSF) was used in the shale gas drilling process. The HPSF consisted of zwitterionic polymer, fatty alcohol derivative and polyacrylamide, etc. [3]. In the process of shale gas drilling, a large amount of waste high performance synthetic-based fluids (WHPSF) with high chemical oxygen demand (COD) was produced [4]. If directly discharged, WHPSF would cause terrible environmental problems [5]; hence, it is very urgent to treat the WHPSF effectively [6].

Treatment of drilling sludge and fluid included physical, chemical and biological methods. Usually, physicochemical techniques had flawed, and perhaps leaded to secondary pollution; and chemical treating techniques were very expensive. While, biological treatment has many advantages, including low cost, no secondary pollution and high efficiency [7-8].

Microorganisms are the most important pollutant decomposers in nature [9]. Several studies showed that many microorganisms could degrade organic pollutants in hydrocarbon, coal tar, oil and other polluted soil or water through artificial enrichment culture. Mohn detected the biodegradation of petroleum hydrocarbons in the arctic tundra soil polluted by diesel fuel [10]. Mohammad screened polycyclic aromatic hydrocarbons tolerant microbial aggregates from 50 fungal and isolated bacterial
strains, and the removal rates reached 41.7%, 31.4% and 36.2%, respectively [11]. There were more than 100 genera and 200 kinds of microorganisms had the ability to degrade petroleum hydrocarbon organic compounds [12]. However, few papers focused on bioremediation of WHPSF [13].

In this paper, WHPSF degrading bacteria were domesticated and isolated, and then identified based on the physiological tests and 16S rRNA gene sequences. The degradation ability was detected so as to screen out the effective degrading strains for treating WHPSF.

2. Materials and methods

2.1. Materials

2.1.1. Sample. High performance synthetic-base drilling fluids and contaminated soil samples were provided by Safety and Environmental Protection Quality Supervision & Testing Research Institute, CNPC Chuanqing Drilling Engineering Co. Ltd.

2.1.2. Medium. Minimal medium (MS): K₂HPO₄ 6 g, (NH₄)₂SO₄ 6 g, NaCl 6g, MgSO₄·7H₂O 3g, CaCl₂·6H₂O 0.08g, water 500 ml, pH 7.4-7.6.
LB medium: Peptone 1.0 g, yeast extracts 0.5 g, NaCl 1.0 g, ddH₂O 1000 ml, pH 7.5

2.2. Research method

2.2.1. Domestication and isolation of bacteria. Five gram of WHPSF contaminated soil were put into 50 ml MS medium with 5% (v/v) WHPSF, and cultured at 30 °C, 160 rpm for 7d. Then 5 ml suspension was transferred to another 50 ml sterile MS medium containing 7.5% (v/v) WHPSM, and cultured as described above. Further, 5 ml culture was taken and inoculated to the 3rd 50 ml MS medium containing 10% (v/v) WHPSM, and incubated for 7 d at the same condition. Finally, 10 ml suspension was used to isolate WHPSF degrading bacteria by dilution plate method. Single colony was picked after incubation for 2 d, and purified by streaking. The pure culture was streaked on LB slant and stored at 4 °C.

2.2.2. Identification of bacteria. Physiological test, including Gram staining, microscopic check, and colony morphology observation was performed. Bacterial DNA was extracted by GUTC method [14], and 16S rRNA gene fragments were amplified by using primers 27f (5'-AGA GTT TGA TCCTGG CTG A-3') and 1492r (5'-GGT TAC CTT GTT ACG ACT T-3') [15]. The polymerase chain reaction (PCR) solution consisted of 2 × PCR TaqMix 15.0 μl, template DNA 3 μl, 16S rRNA primer 0.3 μl, and addition of ddH₂O to 30 μl. The PCR cycling conditions were as follows: 1 cycle of 3 min at 94 °C (pre denaturation); 30 cycles of 1 min at 94 °C (denaturation), 1 min at 52 °C (annealing), and 2 min at 72 °C (extension); and a final extension at 65 °C for 5 min. PCR products were detected in 1% agarose gel stained with EB at 150 V for 20 min. PCR products were sequenced by Beijing Qingke zixi biotechnology co. LTD using Sanger dideoxy sequencing.

2.2.3. Screening of effective degrading bacteria. Effective degrading bacteria were screened based on the COD removal rate. Each degrading bacteria was prepared as a bacterial suspension in LB medium to obtain 0.6 unit of optical density measured with a visible spectrophotometer at a wavelength of 600nm (OD600). Then 5% (v/v) of Effective degrading bacteria was inoculated into MS medium containing 10% WHPSM , and cultured at 30 °C, 160 rpm for 7 days. The COD removal rates of supernatant were measured, and then the effective degrading bacteria were screened to do further study.

2.2.4. Growth characteristics. Each effective degrading bacteria was prepared as described above (OD600 of 0.6). When doing growth temperature range test, 5 μl bacterial suspension was dotted
inoculated to LB medium plate, and incubated at 10, 20, 25, 30 and 40 °C, respectively. While testing initial growth pH, the pH value of LB medium was adjusted to 4, 6, 7, 9 and 10, respectively, and then incubated at 30 °C for 48 h. The growth status was observed.

2.2.5. Degradation characteristics. The degradation characteristics were evaluated by the dynamic of bacterial quantity and COD removal rate. The tested bacterial suspension was prepared as described above, and the same amount of single strain suspension was taken for bacterial mixture. Then, 5% (v/v) inoculum of single strain and mixed bacteria suspension was inoculated into MS medium containing 10% WHPSM, respectively. The same amount sterilized water was used as control, and culture at 30 °C, 160 rpm. The COD removal rate was determined at 0 d, 1 d, 3 d and 5 d. Meanwhile, 1 ml bacterial suspension was diluted to 10^-6, 10^-7 and 10^-8, and the bacterial quantity was obtained by dilution plate method.

2.2.6. Statistical analysis. The data were analyzed by Microsoft Excel 2010, and the significant difference of bacteria number was analyzed by one-way ANOVA and Duncan tests. The sequences were blast against NCBI database and highly related sequences of type strains were selected manually. Phylogenetic tree were constructed by MEGA7.0 using neighbor-joining method.

3. Results

3.1. Isolation and identification of tested bacteria
Eight strains were isolated in this study, and most bacteria were rod shape, spore-forming, and G^+ (Table 1).

| Strain  | colony morphology       | Gram staining | Cell shape | Spore form |
|---------|-------------------------|---------------|------------|------------|
| SQ17    | circular, light yellow color | +             | brevis     | -          |
| SQ6-1   | circular, orange color  | -             | long rod   | +          |
| SQ8-1   | circular, white color   | +             | long rod   | +          |
| SQ2-6   | circular, milky white color | +           | brevis     | +          |
| SQ7-2   | circular, light yellow color | +           | long rod   | +          |
| SQ8-2   | circular, orange color  | +             | long rod   | +          |
| SQ5-3   | circular, orange color  | +             | long rod   | +          |
| SQ5-4   | circular, orange color  | +             | long rod   | -          |

16S rRNA gene fragments were sequenced and submitted to NCBI, the accession numbers were MN235846 - MN235847 and MN235849 - MN235854. The phylogenetic tree of isolates was constructed (Figure 1). The results showed that 8 bacterial strains were distributed in 3 phylogenetic branches. Strains SQ6-1, SQ8-1, SQ2-6, SQ7-2, SQ8-2 and SQ5-3 belonged to genus Bacillus. Strain SQ5-4 was classified to the genera Fictibacillus, and closely related to Fictibacillus barbaricus V2-BIII-A2^T (99.8%). Strain SQ17 was identified as Arthrobacter subterraneus CH7^T with 99.5% similarity. Strain SQ8-1 was closed to Bacillus oceaniseminis H2^T with 99.2% similarity, and SQ8-2 was closed to Bacillus firmus NBRC 10306^T. Apparently, these WHPSM degrading bacteria were diverse.

3.2. Screening of effective degrading bacteria
The COD degradation ability of the tested strains was measured (Figure 2). Compared with control treatment (CK), The COD of the medium inoculated with bacteria was significantly decreased after incubation at 30 °C for 7 days, and each strain had different COD degradation levelability. Among them, SQ6-1 had the highest degradation ability. The COD of liquid culture decreased from 476.1 mg/L initially to 283.50 mg/L at final, and COD degradation rate was 40.26%. The COD degradation rate of SQ17 and SQ8-1 was 36.62% and 34.86%, respectively; and The COD degradation rate of the other
bacteria was less than 20%. However, the COD of CK treatment remained unchanged, indicating that WHPSM was stable and hardly decomposed in natural condition [16]. Based on the COD removal rate, SQ6-1, SQ17 and SQ8-1 were selected as effective degrading bacteria to do the further studies.

Figure 1. Phylogenetic tree of tested strains and related species constructed on the basis of 16S rRNA gene sequences using the neighbor-joining method. The superscript character “T” points out type strains and microorganisms’ names are followed by accession numbers.

Figure 2. COD change and removal rate of the tested strain to WHPSM.

3.3. Characteristic of the effective degrading bacteria

3.3.1. Growth characteristics. The range of growth temperature and initial pH of SQ8-1, SQ6-1 and SQ17 were determined (Table 2). The results showed that SQ8-1 and SQ6-1 could grow at 10 - 40 °C, and the optimum growth temperature was 35 °C. However, SQ17 could grow from 20 - 40 °C, and the optimum growth temperature was 30 °C. When incubated at 40 °C, all the effective degrading bacteria could grow, while SQ6-1 and SQ8-1 grew faster than SQ17.
The optimum growth pH of the effective degrading bacteria was pH 7.0. The initial growth pH range of strain SQ8-1 and SQ6-1 was pH 6.0-10.0, while that of SQ17 was pH 6.0-9.0. All the strains were tolerant to pH 9.0, but they couldn’t grow under pH 4.0. SQ8-1 and SQ6-1 had good tolerance to high pH environment, and they grew slowly at pH 10.0 medium condition after inoculated 48 h.

Table 2. Growth of efficient degrading bacteria at different temperatures and pH.

| Strain | Growth temperature (°C) | Original growth pH |
|--------|-------------------------|--------------------|
|        | 10  | 20  | 25  | 30  | 40  | 4   | 6   | 7   | 9   | 10  |
| SQ17   | -   | +   | ++  | +++ | +   | -   | +++ | +++ | ++  | -   |
| SQ6-1  | +   | ++  | ++  | +++ | +++ | +++ | +   | ++  | +   |    |
| SQ8-1  | +   | +   | ++  | +++ | +++ | -   | ++  | +++ | ++  | +   |

*Notes: -, no growth; +, grow sparse; ++, grow well; ++++, grow very dense

3.4. Degradation characteristic

The variation of total bacteria quantity of medium inoculated single effective degrading bacteria and the bacterial mixture during the degradation process was analyzed (Table 3). The results indicated that the bacterial quantity increased significantly after incubated for 5 d, and SQ6-1 had the highest quantity (75.33 × 10^7 cfu/ml). In addition, the number of SQ6-1 was higher than that of the other strains at the different incubation time. However, the treatment inoculated the bacterial mixture had the lowest number, which was 14.67 × 10^7 cfu/ml.

Table 3. Quantity of high efficient degrading bacteria in different periods(10^7 cfu/ml).

| Strain | Incubation time(d) | 0 d     | 1 d     | 3 d     | 5 d     |
|--------|---------------------|---------|---------|---------|---------|
|        |                     | 0.31±0.017a | 1.49±0.062b | 18.57±2.49c | 22.1±2.29c |
| SQ17   |                     | 0.38±0.020 a | 3.30±0.22 b  | 49.33±2.49c | 52.33±1.70c |
| SQ8-1  |                     | 0.37±0.020 a | 8.13±0.25 b  | 57.33±3.68c | 75.33±3.30d |
| SQ6-1  |                     | 0.32±0.020 a | 3.53±0.25 b  | 3.3±0. 17c  | 14.67±0.53d |
| Mixture|                     | 0.32±0.020 a | 3.53±0.25 b  | 3.3±0. 17c  | 14.67±0.53d |

*Notes: different letters in the same peer mean significant difference (P<0.05), the same letter means no significant difference (P>0.05)

Figure 3. COD removal and degradation rate of tested strains.

The COD degradation and removal rate of inoculated single strain and mixture was analyzed (Figure 3). The results showed that the COD decreased significantly, and had the similar variation
trends along with incubation time. On the 5th day, SQ6-1 showed the best removal effect. The COD reduced from 476.1 mg/L to 295.70 mg/L, and the COD degradation rate was 37.89%. In addition, the COD degradation rate of SQ17 and SQ8-1 was 33.95% and 32.16%, while the bacterial mixture had the lowest COD removal rate (27.48%).

The fitting curve of effective degrading bacteria quantity and COD degradation rate was constructed (Figure 4). The results showed that the COD degradation rate is proportional to the number of effective degrading bacteria, and the correlation coefficient R² was 0.87, which indicated that the equation had good fitting effect, and there was a good linear positive correlation between the quantity of degrading bacteria and the rate of COD degradation.

Figure 4. The fitting curve of bacteria quantity and COD degradation rate.

4. Discussion

In this paper, the bacterial isolates showed very diverse in population, and distributed into genera Bacillus, Fictibacillus and Arthrobacter. Bacillus were the predominant one, with 6 species in total, accounting for 75%. Fu revealed that the abundance of Bacillus was the highest in oxidized soil, with 35.47% [17]. Tang had isolated 5 strains, 3 of which belonged to genus Bacillus, occupying dominant status [18], and strains in these genera had good ability to degrade organic pollutants [19]. The 16S rDNA sequence, physiological and growth characteristics of SQ17, SQ6-1 and SQ8-1 were similar with strains existed in deep groundwater or deep sea [20-22]. Though 16S rRNA gene sequences between SQ8-1 and SQ8-2 were more than 99% similarity, the degradation ability was different. The degradation ability of SQ8-1 was significantly higher than SQ8-2, but was lower than SQ6-1, indicating the metabolic diversity was existed among the tested strains.

The adaptation and tolerance of microorganisms determine the response to the natural environment [23]. SQ8-1 and SQ6-1 had wide growth temperature and growth pH value, which may help them grow well in summer season and high pH soil environment. Yan has indicated that Bacillus coagulans screened from pickle could reached up to 94% survival rate under gastric acid treatment for 120 min [24]. Furthermore, Bacillus subtilis and Bacillus licheniformis could adapt to the environment of pH 3.0-10.0 and tolerate high temperatures below 90 °C, which indicated the strong resistance to acid, alkali and high temperature [25].

Previous researches suggested that the increase of biomass was the main factor affecting the removal efficiency of organic pollutants [26-27]. In this study, the tested strains had good metabolic ability to WHPSM, and there was significantly positive correlation between COD degradation rate and the amount. Interestingly, inoculation of single effective strain showed better degradation effect on WHPSM than mixed strains in this research, which was different from previous studies [28-29]. Hua isolated and screened 4 degrading bacteria from oil sludge, the removal rate of oil by the mixed bacteria was nearly 20% higher than single strain [30].

Relevant studies presented that the competition in mixed strains intensified due to the fight for nutrients and space or some substances inhibiting the growth of bacteria were not conducive to the
growth of strains and the degradation of organic matter [31]. Furthermore, mixed strains with different inoculum ratios also lead to different degradation ability [32]. There may be synergistic or antagonistic effects among different strains, and the mixing principle of simple equal ratio of mixed strains is hard to achieve the optimal effect, hence, how to obtain and utilize degrading bacterial community efficiently still needs to be further studied.

5. Conclusions
In this paper, eight WHPSM degrading bacteria were isolated and purified, and *Bacillus* were the predominant. Three effective bacteria with strong COD degradation ability were screened. These bacteria could utilize WHPSM as the sole carbon source, and utilize other organic pollutants in fluid as energy source. The COD degradation rate was significant positive correlation to bacterial quantity in the process of degrading. These effective degrading bacteria were good candidates for treating high performance synthetic-based drilling fluids, and had good application potential for bioremediation of shale gas drilling.

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