

*Full Length Research Paper*

# Isolation of a nitrate-reducing bacteria strain from oil field brine and the inhibition of sulfate-reducing bacteria

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**A nitrate-reducing bacteria (NRB) strain with vigorous growth, strong nitrate reduction ability, strain B9 2-1, was isolated from Suizhong36-1 oilfield, its routine identification and analysis of 16S rRNA and also the competitive inhibition experiments with the enrichment of sulfate-reducing bacteria (SRB) were carried out. The results showed that only the dosing of nitrate, nitrite as electron acceptors, the activation of nitrate-reducing bacteria, as well as the inhibition of sulfide production resulted from a limited capacity, while addition of NRB isolated from the produced fluid, growth and sulfide production activity of sulfate reducing bacteria produced a significant inhibition and antibacterial effects of nitrite, which was better than nitrate. At the same time, the small amount of molybdate dosing showed better results, which will be of significance when applied to shipping and state-defending industries.**

**Key words:** Nitrate-reducing bacteria, sulfate-reducing bacteria, restriction fragment length polymorphism, nitrates, nitrite, oil field, competitive inhibition.

## INTRODUCTION

With years of groundwater or surface water injection, oil dissolved solids are gradually diluted and many oil fields water also contains sulphide ( $H_2S$  and  $HS^-$ ), which are related to sulfate-reducing bacteria (SRB) or other bacteria (Jenneman et al., 1996; Tan et al., 2007; Dong et al., 2008;). Sulfide in the oil production process is harmful, because they are toxic, corrosive, produces insoluble iron compounds of sulfur, which will lead to lower permeability oil fields (McInerney and Sublette, 1997). Representation of the SRB control biocide is chlorine, bromine, aldehydes, amines and seasonal phosphorus salt (Jack and Westlake, 1995; Okabe et al., 1994). These chemicals are toxic, expensive and inefficient (Jack and Westlake, 1995; Telang et al., 1998)

At present, the use of biological competition methods to suppress SRB is increasingly becoming the focus of

research and the use of nitrate-reducing microorganisms is one of the most effective competitive inhibition techniques. The main approach of the technology is importing low concentration of nitrate/nitrite components to the formation, which is easier to become more active electron acceptor than sulfate. The proliferation of nitrate-reducing bacteria (NRB) naturally present in the reservoir was facilitated, while competing with the SRB for living spaces and matrixes. NRB have priority to use the reservoir matrix to prevent SRB to obtain the required nutrients and so the metabolic activities of SRB are suppressed. The technology can be applied to a wide range of upstream operations: reservoirs, production wells and injection wells, pipelines, gas storage reservoirs, ground equipments of mining water and so on. Nitrate control of  $H_2S$  in sewers and other waste water has been known for many years and still has commercial value (Allen, 1949; Bentzen et al., 1995). Jenneman et al. (1986) and Jack et al. (1985) demonstrated that adding

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**Table 1.** Quantity of different treatment bacteria and materials amended.

| Parameter               | SRB blank | NRB blank | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  |
|-------------------------|-----------|-----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| SRB (%)                 | 5         | -         | 5   | 5   | 5   | 5   | 5   | 5   | 5   | 5   | 5   | 5   |
| NRB (%)                 | -         | 5         | 5   | -   | 5   | -   | 5   | -   | 5   | -   | 5   | -   |
| KNO <sub>3</sub> (g/l)  | -         | 1.0       | 0.8 | 0.8 | -   | -   | 0.4 | 0.4 | -   | -   | -   | -   |
| NaNO <sub>2</sub> (g/l) | -         | -         | -   | -   | 0.8 | 0.8 | -   | -   | 0.4 | 0.4 | 0.2 | 0.2 |
| Sodium molybdate (g/l)  | -         | -         | -   | -   | -   | -   | -   | -   | -   | -   | 0.1 | 0.1 |

nitrate can also remove the sulfide in sulfide-rich oil fields, with thanks to the origin of NRB. Reinsel et al. (1996) added water obtained from the North Sea oil field to a sandstone column, which contains origin bacteria, while injecting low concentrations of nitrate or nitrite (0.57 to 0.71 mM) and sulfide concentrations were observed to reduce.

In this experiment, SRB and NRB were isolated from SZ 36-1 oilfield produced water, symbiosis and competitive inhibition relationships between them and influence of the variety and density of added nutrients on the amount of SRB and the activity of the production of H<sub>2</sub>S were examined. These provided a theoretical basis on oil pollution-free, low-cost, long-term prevention and control technology SRB for oil fields.

## MATERIALS AND METHODS

### Basic separation medium

The following medium was used in the initial separation of nitrate-reducing bacteria (g/l): NaCl, 2.5; K<sub>2</sub>HPO<sub>4</sub>, 0.5; NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, 0.5; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>·7H<sub>2</sub>O, 0.5; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.1; KNO<sub>3</sub>, 1.5. The medium pH was adjusted to 8.0.

### CSB medium

For the enrichment culture for the separation and purification of NRB, the medium contained (g/l): NaCl (7.0); KH<sub>2</sub>PO<sub>4</sub> (0.027); MgSO<sub>4</sub>·7H<sub>2</sub>O (0.68); CaCl<sub>2</sub>·6H<sub>2</sub>O (0.24); NH<sub>4</sub>Cl (0.02); NaC<sub>2</sub>H<sub>3</sub>O<sub>2</sub>·3H<sub>2</sub>O (0.68); KNO<sub>3</sub> (0.1). The medium also contained ND trace metals (50 ml/L). The pH of the medium was adjusted to between 7.0 and 7.5. (Gevertz et al., 2000).

### SRB medium

The medium contained (g/l): KH<sub>2</sub>PO<sub>4</sub> (0.5); NH<sub>4</sub>Cl (1); CaCl<sub>2</sub>·6H<sub>2</sub>O (0.06); MgSO<sub>4</sub>·7H<sub>2</sub>O (0.06); sodium lactate (6 ml); yeast extract (1); citrate (0.3). 0.1 g iron filings per 20 ml system was also added.

### Modified CSB medium

The medium was amended on the basis of CSB; the medium for the antibacterial test.

### Isolation of oil field NRB

#### Cell morphology analysis

NRB strain was classified based on colony morphology (including

single colony size, shape, color, etc.), Gram stain results, on the environment and the strains of the total DNA analysis and RFLP analysis of the total environmental DNA and 16SrRNA gene analysis of the isolated NRB strain. Wizard genomic DNA purification kit was used for the DNA extraction and purification. Amplification of 16S rRNA genes by PCR was done using the bacterial universal primers (Casey and Voordouw, 2007), 8F: 5'-AGR GTT TGA TCC TGG CTC AG -3' and 1492R: 5'-CGG CTA CCT TGT TAC GAC TT -3' (Synthesized by Shanghai Biotechnology Co., Ltd.).

#### Preliminary identification of NRB strains

Restriction enzymes Hha I and Msp I were used for the restriction enzyme sub-type of 16S rRNA gene fragments amplified products of NRB strains (enzyme reaction carried out according to kit instructions). 16S rRNA gene fragment nucleotide sequences were sequenced by the National Human Genome Research Center. Sequences with high homology to the sequences of the new isolates, as well as other sequences of interest, were retrieved from the GenBank database following BLAST searches. Sequence alignment, manual refinement of the alignment and phylogenetic tree reconstruction were performed using the ARB software package. Maximum-likelihood trees were generated using FastDNAML software and distance trees were generated using neighbor-joining algorithms. Bootstrap analysis with 1,000 replicates was performed for the neighbor-joining tree.

#### Reversal test

#### Experimental design

Pure NRB strain was inoculated into the culture medium and enriched SRB was inoculated in PGC culture medium and after culture for five days, inoculated in the culture bottles containing 100 ml of modified CSB medium according to the proportion in Table 1, each dealing with three parallels, added 0.5 g iron filings per system, experimental temperature is 40°C.

#### Analysis of sulfate concentrations

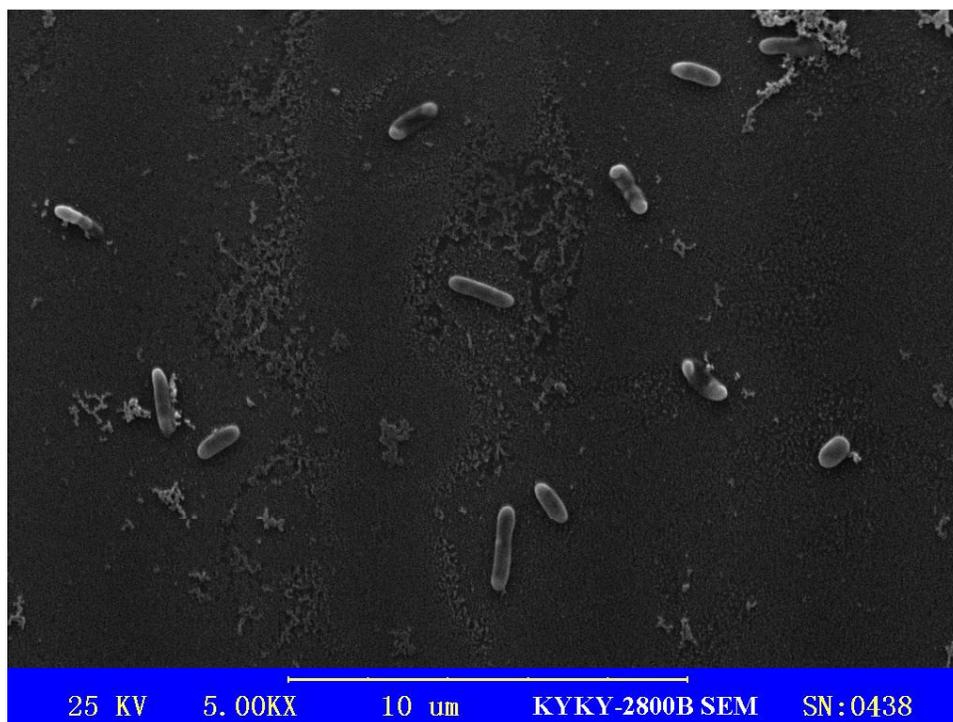
Sulfate concentrations in the test samples were monitored using United States Diana DX-120 ion chromatography.

#### Bacteria concentration (OD600) determination

Optical absorption method, measured at a wavelength of 600 nm absorbance was used.

#### Bacterial counts

SRB number count was determined by American Petroleum Institute



**Figure 1.** SEM micrograph of strain B9 2-1.

**Table 2.** B9 well bacterial diversity statistics.

| Category                 | Total |
|--------------------------|-------|
| $\beta$ -Proteobacteria  | 28    |
| Burkholderiaceae         | 13    |
| Pseudomonadaceae         | 8     |
| $\gamma$ -Proteobacteria | 35    |
| Others                   | 12    |
| Total                    | 96    |

Institute recommended "three parallel extinction dilution method" calculations; the MPN method. SRB was cultured for 7 days at 37°C and counted according to the color change of various tubes. NRB number count was determined by the improved MPN method.

## RESULTS AND DISCUSSION

### Purification culture of NRB

Single colonies were picked from basic separation medium and inoculated into CSB liquid culture medium and colonies with the ability to reduce nitrate were then crossed on solid CSB medium. After three times, pure strains of NRB were obtained.

### Strain identification and molecular biological analysis

A nitrate-reducing bacteria (NRB) strain with vigorous

growth, strong nitrate reduction ability (strain B92-1) was obtained and its routine identification was carried out. Colonies with round, milky white, about 1 mm in diameter, were identified as Gram-negative; the electron microscope and colonies pictures are shown in Figures 1 and 2.

### 16S rDNA and RFLP analysis

Bacterium diversity analysis (Table 2 and Figure 4) of the oil field water sample in B9 well was carried out and strain B9 2-1 was identified by 16S rDNA analysis. RFLP analysis showed that in B9 well, 29% of the 16S rRNA gene clones were similar to the bacteria in lower temperatures in a Canadian oil field production well. Another 14% of the clones were similar to bacteria in mineral water without organic carbon sources in a certain place of Germany; belong to Burkholderiaceae. Both



**Figure 2.** Colonies of strain B9 2-1 growth on CSB solid medium.

belonged to  $\beta$ -Proteobacteria. 8% of the clones belong to Pseudomonadaceae; they were similar to clones in a 28,000-year-old asphalt samples in Los Angeles. 36% of the clones belonged to  $\gamma$ -Proteobacteria, and were similar to the bacteria found in south-west Pacific deep-sea sediment. Another 13% clones was represented by B3 and B75. According to the sequence comparison and the combination of online documents, B3 was closer to  $\beta$ -Proteobacteria class in the phylogenetic tree (Figure 3) and B75 was closer to the  $\gamma$ -Proteobacteria, but they could not be classified into various categories. This may be due to the relatively large sequence differences and there is no article more accurate reporting them.

B9 2-1 sequence analysis (Figure 5) of the strains found in water with natural minerals separated from uncultured *Limnobacter* sp. clone D-15 and I-1 were highly homologous (Figure 5). D-15 and I-1 are sulfur-oxidizing bacteria; D -15 and I-1 were of the similarity of 99% and considered to belong to Burkholderia bacteria branch (Burkholderiaceae). Strain B9 2-1 sequence analysis showed that it was highly homologous with the uncultured *Limnobacter* sp. clone D-15 and I-1 which were separated from natural mineral water.

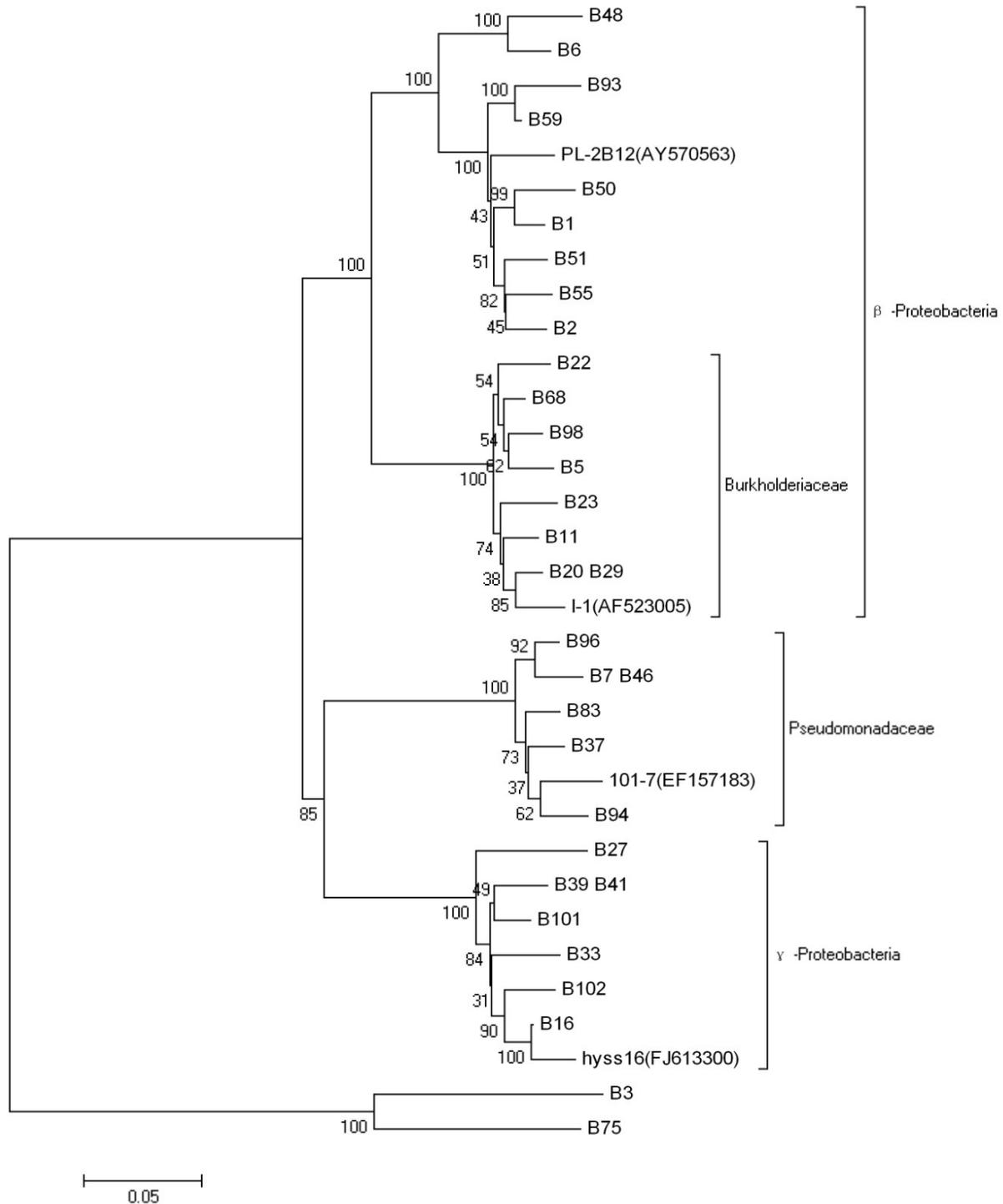
### Reversal test

#### Changes of sulfate concentration in blank experiments

It is shown from Figures 6 and 7 that in the experimental bottles where only the medium and SRB were added, growth and metabolism of SRB's were rapid because of the absence of any material which could inhibit SRB metabolism. After 7 days, sulfate concentration decreased from 2.5 to 0.5 g/l. Sulfate concentration in the NRB blank experiment changed a little, indicating that this strain had non-sulfate-reducing activity and was suitable for the suppression of SRB bacteria.

#### Changes of sulfate concentration in the experiments to which NRB and high concentrations of nitrate or nitrite were added

According to Figure 8, each treatment had inhibitory effect on SRB sulfate reduction. Treatment 3 (dosing NRB and 0.8 g/l nitrite) had the longest inhibition time



**Figure 3.** Bacterial phylogenetic tree of water sample in B9 well.

and most obvious effect and sulfate concentration remained at about 2.0 g/l at the 10th day, showing that the input of NRB and sodium nitrite played a very good inhibitory effect on the activity of SRB. Treatment 4 (dosing 0.8 g/l nitrite) and treatment 1 (dosing NRB and 0.8 g/l nitrate) had significant inhibitory effect in 72 h;

however, the effect began to decline after 72 h. Treatment 4 showed that nitrite have the effect of directly inhibiting the growth of SRB. The cultivation test of Nemati et al. (2001) showed that only 4 mmol/l of nitrite can inhibit SRB producing  $H_2S$  obviously, which is consistent with the conclusions of the tests. Treatment 1

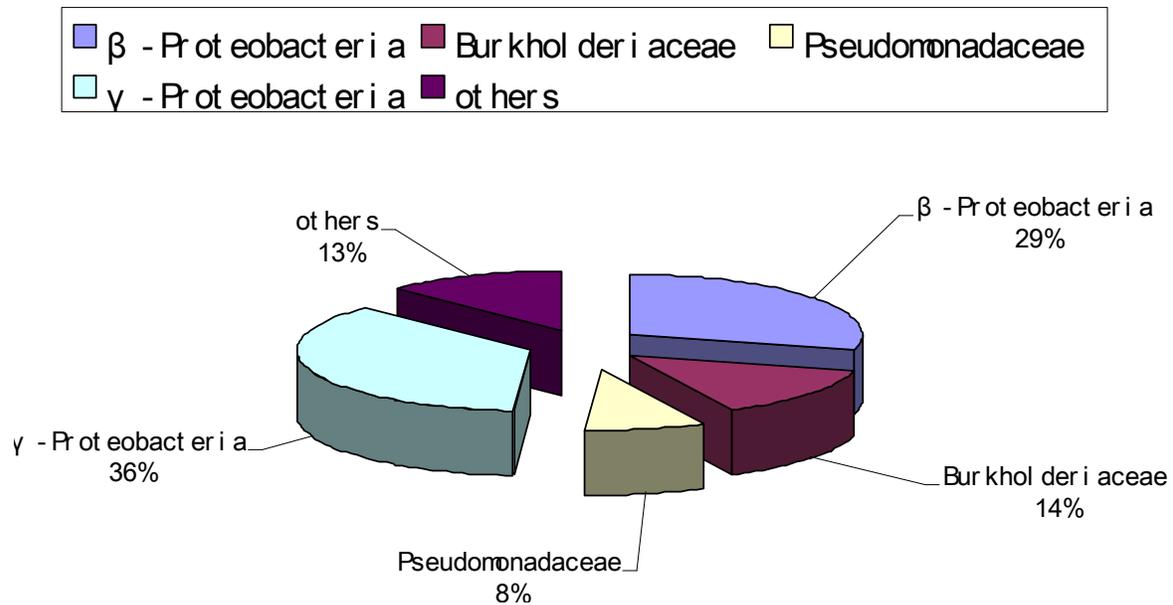


Figure 4. Scale map of bacterial diversity statistics in B9 well.

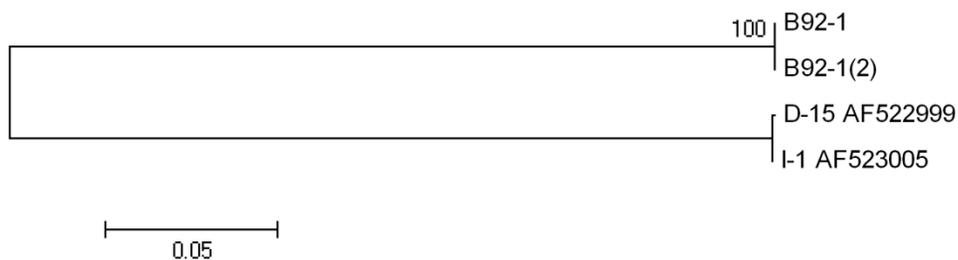


Figure 5. Bacterial phylogenetic tree of strain B9 2-1.

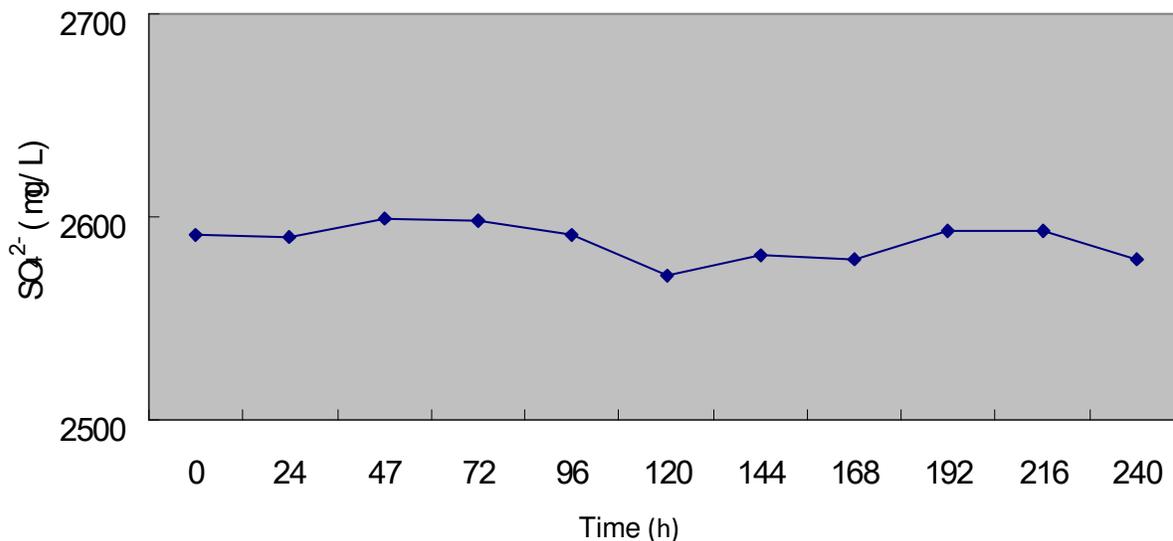
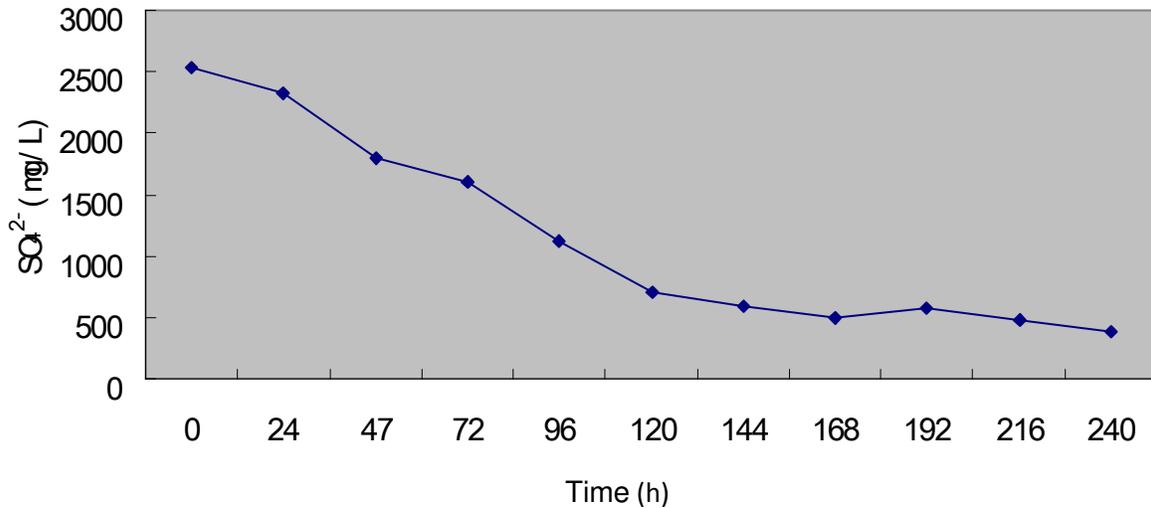
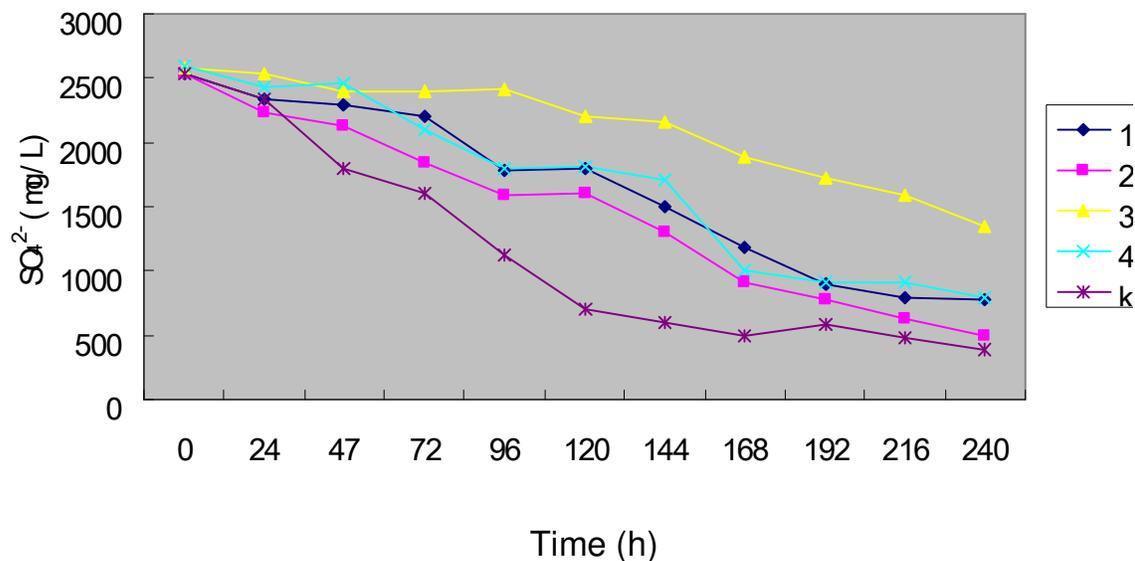


Figure 6. Curve of changes of sulfate concentration in NRB blank experiment.



**Figure 7.** Curve of changes of sulfate concentration in SRB blank experiment.



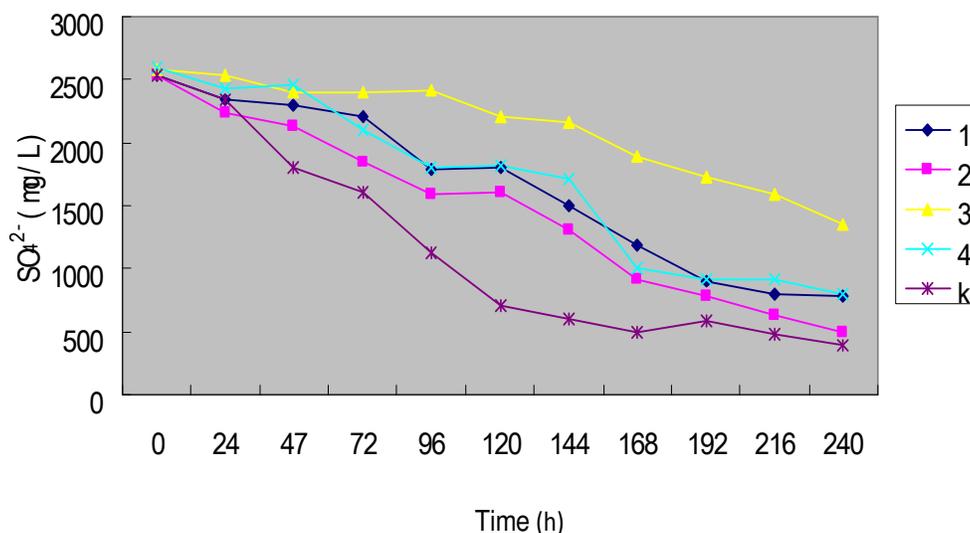
**Figure 8.** Effects of treatments 1 to 4 (see Table 1) on sulfate-reducing capacity of SRB.

showed that dosing NRB with a certain degree of nitrate at the same time had a certain inhibitory effect on the growth of SRB and sulfate reduction; this was just the same effect with adding the same concentration of nitrite. Treatment 2 (dose of 0.8 g/l nitrate) had a certain inhibitory effect early, due to its increased pH value of the medium and changed the acidic environment of the SRB growth. So, the growth of SRB was suppressed. But with the acidification of the medium, the environment changed from alkaline to acidic which was suitable for SRB growth; the growth of SRB was accelerated, so the

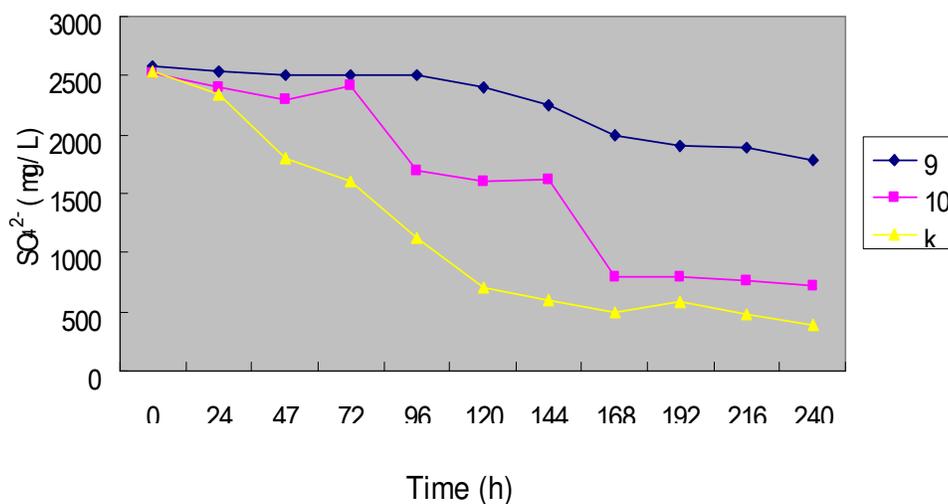
inhibitory effect gradually disappeared.

#### **Changes of sulfate concentration in the experiments to which NRB and low concentrations of nitrate or nitrite were added**

According to Figure 9, in the process of adding low concentrations of nitrate and nitrite, the changing trend of sulfate concentration was similar to its corresponding high concentration formulations and bacteriostasis from



**Figure 9.** Effects of treatments 1 to 4 (see Table 1) on sulfate-reducing capacity of NRB.



**Figure 10.** Effects of treatments 9 and 10 (see Table 1) on sulfate-reducing capacity of SRB.

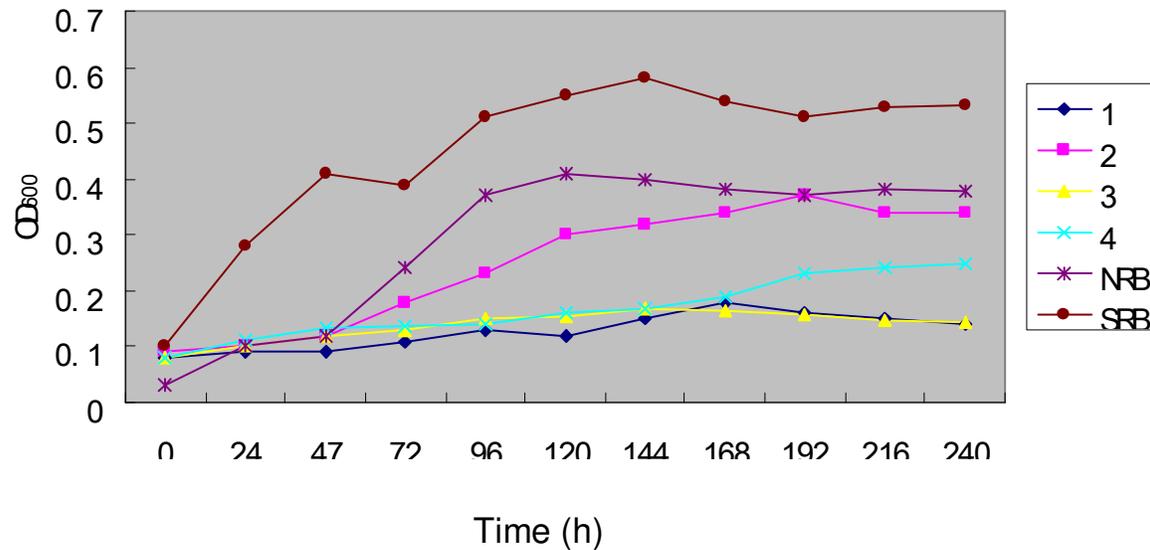
strong to weak. This was observed in treatment 7 (dosing NRB and 0.4 g/l sodium nitrite), followed by treatment 8 (dosing 0.4 g/l sodium nitrite), treatment 5 (dosing NRB and 0.4 g/l sodium nitrate) and treatment 6 (dosing 0.4 g/l sodium nitrate). The rate of decline of trend line in each treatment was faster than their corresponding high concentration formulations; furthermore, sulfate concentration was much lower at the 10th day.

The studies of Jayaraman et al. (1997) showed that for some SRB, nitrite has effects on variable acid suppression and H<sub>2</sub>S removal in the bioreactor, while nitrate are not valid in these areas. Therefore, nitrite is

more effective in preventing some high-temperature oil field from acidizing, because of its direct reaction with the sulfide and is proved to be an effective inhibitor of sulfate-reducing long-term.

#### **Changes of sulfate concentration in the experiments to which NRB, nitrite and molybdate were added**

Figure 10 shows that treatment 9 (dosing NRB, 0.2 g/l of sodium nitrite and 0.1 g/l sodium molybdate at the same time) had a very significant inhibitory effect on SRB



**Figure 11.** Changes of bacteria concentrations in treatments 1 to 4 (see Table 1).

sulfate reduction and sulfate concentration remained at about 2.0 g/l at the 10th day. Therefore, even a small amount of sodium molybdate, which in collaboration with the NBR and sodium nitrite, had a significant inhibitory effect. The cultivation test of Nematı et al. (2001) showed that only a small amount of sodium molybdate which may have the effect to directly kill or inhibit the growth of SRB can also play a role in inhibiting SRB sulfate-reducing. Treatment 10 (only dosing 0.2 g/l nitrite and 0.1 g/l sodium molybdate) had significant inhibitory effect at 72 h; however, the effect began to decline after 72 h, which means there was lack of antagonism of the NRB. Although, SRB activity can be inhibited under the influence of pharmaceutical in a short period of time, alkalinity changed to the acidic environment which was suitable for the growth of SRB with the acidification of SRB growth medium, thus, the growth of SRB was sped up and the inhibition disappeared gradually. Taking the economic cost into consideration for dosing nitrate or nitrite, dosing NRB and a small amount of sodium molybdate at the same time, can play a good role in suppression of SRB activity.

#### Changes of bacteria concentrations in different treatments

Combining Figures 11 and 13, it can be observed that the concentrations of each antibacterial formula bacteria were lower than that of the SRB and NRB blank, which showed that the growth of SRB was inhibited. In Figure 11, the concentration of bacteria in treatment 2 (dosing 0.8 g/l sodium nitrate) was higher than that of treatment 1 (dosing NRB and 0.8 g/L sodium nitrate), which proved that NRB and SRB occurred in antagonism to reduce the

concentration of bacteria in treatment 1. The comparison between treatment 4 (dosing 0.8 g/l sodium nitrite) and treatment 3 (dosing NRB and 0.8 g/l sodium nitrite) was similar to that of treatments 1 and 2. The concentrations of the bacteria in treatments 3 and 4 were lower than that of treatments 1 and 2, which proved that the growth of bacteria was easily inhibited in the case of nitrite than nitrate.

The trend lines of the concentrations of low-concentration formulation of various bacteria in Figure 12 are similar to the corresponding trend lines of the concentrations of high-concentration formulation of the various bacteria. The difference is that the concentrations of bacteria in low-concentration formulation were generally higher than that in the corresponding high-concentration formulation, which proved that the use of high concentrations of antimicrobial agents on the inhibition of SRB was more obvious and was consistent with the sulfate concentration trends.

#### Effects of different treatments on number of SRB

As observed in Table 3, the growth of SRB in the medium was well inhibited in the 10th day by the addition of a concentration of 0.8 g/l of nitrate or nitrite. Compared with the blank treatment, the number of SRB significantly reduced, which differed in six orders of magnitude at the maximum. Furthermore, the effect was more obvious by adding NRB. The effect of nitrite was more effective than the nitrate; it differed in four orders of magnitude until the 10th day. Adding a small amount of molybdate had a significant role in inhibiting the number of SRB and NRB. Although the antibacterial formula reduced the number of SRB, it could not completely kill the SRB, but had

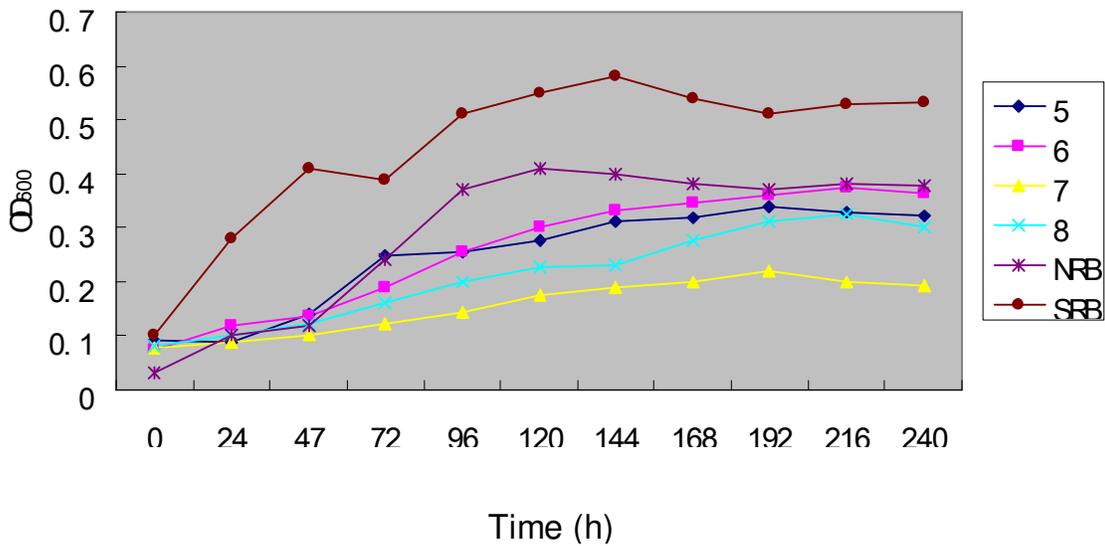


Figure 12. Changes of bacteria concentrations in treatments 5 to 8 (see Table 1).

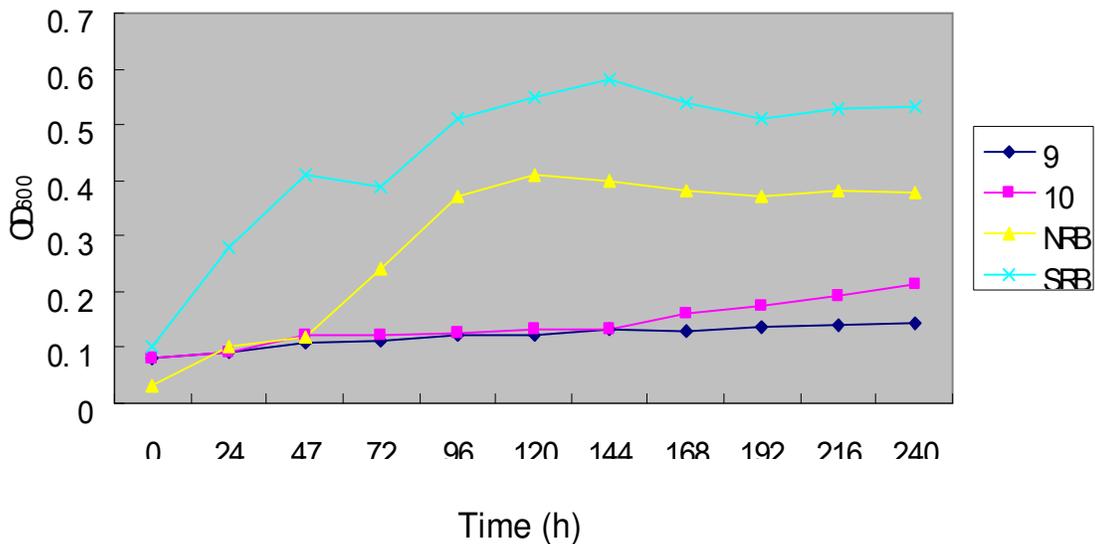


Figure 13. Changes of bacteria concentrations in treatments 9 and 10 (see Table 1).

nutritional source of competition with it or inhibited its metabolism directly by the NRB. Therefore, dosing NRB, nitrate/nitrite and molybdate continuously can inhibit the metabolic activity of SRB completely, inhibit the production of H<sub>2</sub>S and solve the problem of pollution caused by the growth and reproduction of SRB.

**CONCLUSION**

The results of this experiment showed that NRB had a

certain inhibitory effect on the growth of SRB and sulfate reduction. Under the conditions of adding NRB nutrients (such as nitrate and nitrite, etc.), NRB had better inhibitory effect on SRB sulfate reduction. The same concentration of nitrite not only had better inhibitory effect than the role of nitrates, but also was more significant on reducing the number of bacteria SRB.

Dosing NRB with 0.4 to 0.8g/l nitrate inhibited the production of H<sub>2</sub>S by SRB in the medium. Addition of 0.8 g/l of nitrate which can inhibit the production of more than 10 days H<sub>2</sub>S had the most significant inhibitory effect.

**Table 3.** Effects of different treatments on number of SRB.

| Time (day) | SRB-control          | 1                  | 2                 | 3                 | 4                 | 9                 | 10                |
|------------|----------------------|--------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| 3          | $1.4 \times 10^{11}$ | $4.5 \times 10^7$  | $2.0 \times 10^8$ | $9.0 \times 10^6$ | $9.0 \times 10^6$ | $6.0 \times 10^5$ | $7.0 \times 10^5$ |
| 5          | $1.1 \times 10^{11}$ | $1.65 \times 10^6$ | $6.5 \times 10^7$ | $3.0 \times 10^5$ | $2.0 \times 10^6$ | $2.0 \times 10^4$ | $4.5 \times 10^4$ |
| 7          | $9.5 \times 10^9$    | $2.0 \times 10^5$  | $2.0 \times 10^6$ | $9.5 \times 10^4$ | $1.1 \times 10^5$ | $1.1 \times 10^3$ | $2.0 \times 10^4$ |
| 10         | $4.5 \times 10^9$    | $1.5 \times 10^6$  | $4.5 \times 10^6$ | $4.5 \times 10^5$ | $7.5 \times 10^6$ | $1.5 \times 10^4$ | $1.1 \times 10^4$ |

Addition of lower concentration of 0.4 g/l nitrate in 10 days also inhibited the production of H<sub>2</sub>S better. In addition, the experimental results with only the dosing nitrate and without dosing any NRB showed that nitrate had certain inhibition on SRB in the beginning of the trial.

Dosing NRB while adding 0.4 to 0.8 g/l nitrite inhibited the SRB in the medium and each concentrations of nitrite inhibited the production of more than 10 days H<sub>2</sub>S. In addition, the experimental results with only the dosing nitrite and without dosing any NRB showed that nitrite had direct inhibition on SRB.

Only a small amount of molybdate played a role in inhibiting the SRB sulfate reduction, which may have direct role of killing or inhibiting the growth of the SRB. Taking the economic cost into consideration while dosing nitrate or nitrite, dosing NRB and a small amount of sodium molybdate at the same time, can play a good role in the suppression of SRB activity.

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