Phylogenetic Characterization and Function Verification of an Anaerobic Strain SN9 with Simultaneous Capabilities of Denitrification and Sulfate Reduction

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Anaerobic bacterium (strain SN9) with simultaneous capabilities of denitrification and sulfate reduction was enriched and isolated in a specially designed medium by anaerobic Hungate technology. The characterization of SN9 was conducted from the morphological, physiological and phylogenetic aspects. It was a rod-shaped, gram-negative, 0.2-0.8 µm in width and 2.0-5.0 µm in length. Most of the fatty acids of SN9 distributed among C12:0~C19-CYC-FAME and the main fatty acids consisted of C14:0 FAME, C16:0-FAME, C18:1c-FAME, C18:0-FAME, C19-CYC-FAME. Based on the morphological and physiological characteristics as well as the phylogenetic analysis, SN9 was identified as Bacillus coagulans since its 16S rDNA beared 99% resemblance to that of Paenibacillus lautus SN9 (Accession No. DQ450463). The removal efficiencies of NO3- and SO42- were up to 80.78% and 96.22 % when the initial concentrations of NO3- and SO42- were 3812.59 mg/L and 552.52 mg/L, respectively. The characterization of the present isolate had important ecological implications in treating organic wastewater containing high concentration of sulfate and nitrogen.

Key words: Strain with simultaneous denitrifying and sulfate reducing capabilities, Characterization, Identification of fatty acids, Function verification.
system including a unique tetraheme c-type cytochrome, NapM (Marietou et al., 2005). Liu et al. detected the hexaheme nitrite reductase for the first time in nitrate-respiring Desulfovibrio desulfuricans ATCC 27774 (Liu et al., 1994). It demonstrated that the nitrate reductase isolated from Desulfovibrio desulfuricans ATCC 27774 was a periplasmic enzyme and seemed to belong to a class of monomeric enzymes (Moura, 1997). K. Mori isolated a novel type of moderate thermophilic autotrophic SRB from a hot spring, which could grow with nitrate in place of sulfate (Mori et al., 2003). Medium applied for the isolation of SRB contained NaNO₃ as nitrogen source, which indicated that the isolated strain could utilize nitrate (Hong et al., 2010).

In an anaerobic baffled reactor (ABR) process operated in our lab for the inhibition of SRB with the dosage of nitrate, the presence of bacteria with simultaneous capabilities of denitrification and sulfate reduction was proposed. In the present study, a special medium for the isolation of strains with simultaneous denitrifying and sulfate-reducing capabilities was designed. An anaerobic strain with simultaneous denitrifying and sulfate-reducing capabilities, named as SN9, was isolated. Morphological observation, physiological tests, fatty acids analysis, phylogenetic analysis of 16S rDNA. The characterization of the present isolate had important ecological implications in treating organic wastewater containing high concentration sulfate and nitrogen generating from light chemical engineering industries, food processing and pharmaceutical factories.

MATERIALS AND METHODS

Strain source
Sample for strain isolation was the activated sludge collected from a denitrification-based SRB inhibition bioreactor. The reactor was fed with a synthetic wastewater, which contained 600 mg/L SO₄²⁻, 4000 mg/L NO₃⁻, 2350 mg/L COD (pH, 8.0).

Medium and isolation
Techniques of Hungate, the most probable number (MPN) and the roll tube were applied for the isolation of the bacterial strain. A special medium was designed for the isolation of strains with simultaneous denitrifying and sulfate-reducing capabilities. The liquid medium was composed of 1750 mL distilled water and the following salts: Na₂SO₄ 4 g, KNO₃ 2.0 g, NaNO₃ 2.0 g, MgSO₄·7H₂O 1 g, K₂HPO₄ 0.5 g, KNaC₄H₄O₆·4H₂O 5 g, KH₂PO₄ 1.0 g, CaCl₂·2H₂O 0.2 g (final pH, 7.5). Resazurin (0.2%, wt/vol) was added as redox indicator to the medium. Then the medium was boiled for complete dissolution and 0.5 g L-cysteine was added. After that, high purity nitrogen was introduced to drive away oxygen for 30 min. The medium was autoclaved for 20 min at 121°C. The sterilized medium was cooled and 0.1 mL 3% FeSO₄·(NH₄)₂SO₄ was added. Powdered agar (1.5%, wt/vol) was added when solid medium was prepared before sterilization.

Characterization of the isolated strain
Gram-stained strains were observed by electron microscope (CX31, Olympus, Japan). Morphological observations of the cells were also carried out through atomic force microscope (AFM) (Di BioScope, Veeco, USA). The physiochemical tests of the isolate were conducted according to the guidance of “Bergey’s Manual of Determinative Bacteriology”. The microbial fatty acids of the isolated strain were identified using the Sherlock MIS (MIDI Sherlock, MIDI, USA) equipped with the gas chromatograph (6890N, Agilent, USA).

Identification of the isolate by 16S rDNA-based phylogenetic analysis
The DNA extraction of the isolate was conducted with a bacterial Genomic DNA Extraction Kit (TaKaRa, Dalian, China). The 16S rDNA genes were amplified by using universal primers (TaKaRa, Dalian, China). The 50 µL PCR mixture contained 2 µL template DNA, 0.5 µL rTaq polymerase (TaKaRa, Dalian, China, 5 U/µL), 5 µL 10×PCR buffer, 4 µL of dNTPs (2.5 mM of each dNTP), 1 µL of each primer (50 mM) and 37.5 µL deionized water. The thermal cycling included: 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 45 s and extension at 72°C for 90 s, and a final extension at 72°C for 10 min. PCR product was subjected to Agarose Gel Electrophoresis. Target fragment was purified using an Agarose Gel DNA Purification Kit (TaKaRa, Dalian, China) and ligated to a TA type vector pGEM-T (Promega, Madison, WI, USA). Ligated product was transformed to an
E. coli chemical competent cell TOP10 (Tiangen Biotech, Beijing, China). Transformed E. coli was selected on LB solid culture medium with Amp (50 µg/mL) and X-gal (200 µg/mL). The transformed E. coli was analyzed using the universal primers T7 and SP6 and sequenced by the Shanghai Sangon Biological Engineering Co., Ltd, China. The sequenced 16S rDNA were compared with a non-redundancy nucleotides database by using the Basic Local Alignment Search Tool (BLAST). Multiple sequence alignment was conducted using BioEdit Version 5.06, and a phylogenetic tree was constructed by MEGA Software Version 4.1 with a neighbor-joining method.

Analytical methods

The concentrations of NO₃⁻, NO₂⁻ and SO₄²⁻ were detected by ion chromatography (ICS-3000, Dionex, USA). The activity of nitrite reductase was determined according to the method of Cole et al. (Cole et al., 1974). The determination of sulfite reductase followed the method described by Ostrowski et al. (Ostrowski et al., 1989).

RESULTS AND DISCUSSION

Morphological and physiochemical characterization of the isolated strain SN9

An anaerobic strain, named as SN9, was isolated for its simultaneous denitrification and sulfate-reducing capabilities.

Figure 1 showed that strain SN9 was long rod-shaped, 0.2–0.8 µm in width and 2.5–5.0 µm in length. It was Gram-positive with polar flagella. Its colony on the agar plate was white and round in moderate size with convex surface. The results of physiochemical tests were listed in Table 1. It showed that strain SN9 was mesophilic growing at temperatures ranging from 20 to 40 °C with an optimum growth temperature of 36 °C. Optimal growth occurred at pH 7.5. Growth was observed on the following substrates: glucose, citrate and grease. Strain SN9 was not able to grow using fructose, lactose, sucrose, ethanol, starch, urea and gelatin as electron donors. The nitrate reduction and H₂S production tests confirmed that strain SN9 had denitrification and sulfate reduction capabilities. Besides, the analysis of fatty acids indicated that the major fatty acids distributed around C₁₂:0~C₁₉-CYC-FAME. The main fatty acids consisted of C₁₄:0-FAME, C₁₆:0-FAME and C₁₈:1c-FAME, which accounted for 87.63% of the total fatty acids with 8.68%, 53.29%, 8.51%, 9.61% and 7.54%, respectively.

Phylogenetic analysis based on the 16S rDNA sequences

A 1464 bp sequence of 16S rDNA gene from of SN9 were obtained. The nucleotide sequences of 16S rDNA of SN9 had been deposited in the GenBank database under accession numbers DQ450463. Representative strains had high homology with SN9 were selected and the phylogenetic tree was constructed. As shown in Figure 2, the average genetic distances of the 16 strains was 0.042. The resemblance between strain SN9 and Paenibacillus lautus (Accession No. EU236729) was 99%. Consequently, based on the results of morphological observation, physiological tests, fatty acids and 16S rDNA analysis, strain SN9 was identified as Paenibacillus lautus and named as Paenibacillus lautus SN9.

The denitrification and sulfate-reduction capabilities of strain SN9

The bacterial solution of SN9 was inoculated in the specially designed medium with NO₃⁻, NO₂⁻ and SO₄²⁻ to observe their growth as well as the production of H₂S. The growth of strain SN9 was observed on the following substrates: glucose, citrate and grease. The denitrification and sulfate reduction capabilities were confirmed through the determination of nitrite reductase and sulfite reductase activities. The results showed that strain SN9 was capable of denitrification and sulfate reduction.

Table 1. The results of the physiochemical tests

<table>
<thead>
<tr>
<th>Items</th>
<th>Results</th>
</tr>
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<tbody>
<tr>
<td>Methyl red test</td>
<td>bþ</td>
</tr>
<tr>
<td>Indole test</td>
<td>bþ</td>
</tr>
<tr>
<td>Fructose</td>
<td>-</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>bþ</td>
</tr>
<tr>
<td>Glucose fermentation</td>
<td>Acid production through fermentation</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>-</td>
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<tr>
<td>Grease hydrolysis</td>
<td>bþ</td>
</tr>
<tr>
<td>Lactose</td>
<td>-</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
</tr>
<tr>
<td>Ethanol</td>
<td>-</td>
</tr>
<tr>
<td>Urea hydrolysis</td>
<td>cþ</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>cþ</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>bþ</td>
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<tr>
<td>H₂S production</td>
<td>bþ</td>
</tr>
<tr>
<td>Catalase test</td>
<td>-</td>
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<tr>
<td>Oxygen demand</td>
<td>Anaerobic</td>
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<tr>
<td>Gas production from glucose</td>
<td>-</td>
</tr>
<tr>
<td>Voges-Proskauer test</td>
<td>+</td>
</tr>
<tr>
<td>Ammonia production</td>
<td>-</td>
</tr>
<tr>
<td>Litmus milk test</td>
<td>Organic acid production and solidification</td>
</tr>
</tbody>
</table>

C₁₈:1c-FAME and C₁₉-CYC-FAME, which accounted for 87.63% of the total fatty acids with 8.68%, 53.29%, 8.51%, 9.61% and 7.54%, respectively.
a 5% volume ratio to the medium. The concentrations of SO$_4^{2-}$, NO$_3^-$ and NO$_2^-$ were detected at daily intervals for the assessment of its denitrification and sulfate-reduction capabilities. As shown in Figure 3, the concentration of NO$_3^-$ declined from 3812.59 mg/L in the beginning to 85.53 mg/L at the end. The removal efficiency of NO$_3^-$ was 80.78% on the 4$^{th}$ day and the highest was 97.76%. The greatest concentration of NO$_2^-$ was detected on the 4$^{th}$ day (997.61 mg/L), which was produced as the intermediate product when the concentration of NO$_3^-$ dropped dramatically.

Fig. 1. AFM images of Strain SN9

Fig. 2. Phylogenetic tree based on the 16S rDNA gene sequences of strain SN9 and the selected species from GenBank Database by using the neighbour-joining method
Along with the cultivation of strain SN9, the NO$_3^-$ concentration was gradually declined without accumulation. Thus, strain SN9 had great denitrification capability. Figure 4 showed that the concentration of SO$_4^{2-}$ dropped from 552.52 mg/L to 20.91 mg/L during the denitrification process of strain SN9. The removal efficiency of SO$_4^{2-}$ on the 4th day was up to 91.85% and the highest was 96.22%. It demonstrated that strain SN9 was efficient for sulfate reduction. Consequently, SN9 was a strain with simultaneous capabilities of denitrification and sulfate reduction.

**The detection of enzyme activities**

Nitrite reductases were a group of enzymes that catalyze the reduction of nitrite. Dissimilatory sulfite reductase was a key enzyme in sulfate reduction that catalyzes the reduction of sulfite to sulfide. Figure 5 showed that the activity of the nitrite reductase enhanced from 0.23 U/mL on the 1st day to 0.64 U/mL on the 6th day. For the sulfite reductase, its activity increased from 0.39 U/mL on the 1st day to 0.62 U/mL on the 7th day. It gave further evidence that SN9 was a strain with denitrification and sulfate reduction capabilities.

Evidence had been presented that SRB could utilize various types of refractory organics (such as polycyclic aromatic hydrocarbons and n-alkenes) as carbon sources (Tsai et al., 2009). The use of SRB for metal and radioactive waste remediation was also widely studied (Zhengji et al., 2010). However, the application of SRB in the treatment of wastewater containing high concentration of sulfate and nitrogen was rare. Generally, the transformations of sulfate and nitrogen were performed in individual reactors or with different technology (Wang et al., 2009). The isolated of SN9 with simultaneous capabilities of denitrification and sulfate-reduction had great potential in the treatment of wastewaters produced from light chemical engineering industries, food processing and pharmaceutical factories. These wastewaters were rich in sulfate and nitrogen and the isolated strain could be applied without the consideration of interspecific competition. Further studies were necessary to explore the feasibility and the characteristics of the isolate in practical wastewater treatment.

**CONCLUSIONS**

An anaerobic strain, named as SN9 with simultaneous denitrifying and sulfate-reducing abilities was isolated. It was a rod-shaped, gram-negative, 0.20–0.80 µm in width and 2.0–5.0 µm in length. Most of the fatty acids of SN9 distributed among C$_{12:0}$~C$_{19}$-CYC-FAME and the main fatty acids consisted of C$_{14:0}$-FAME, C$_{16:0}$-FAME, C$_{18:1c}$-FAME, C$_{18:0}$-FAME and C$_{19}$-CYC-FAME. Strain SN9 was identified as
Bacillus coagulans as its 16S rDNA beared 99% resemblance to that of Paenibacillus lautus (Accession No. EU236729). The removal efficiencies of NO$_3^-$ and SO$_4^{2-}$ were up to 80.78% and 96.22 % when the initial concentrations of NO$_3^-$ and SO$_4^{2-}$ were 3812.59 mg/L and 552.52 mg/L. High activities of nitrite reductase and sulfate reductase were detected in the culture medium of SN9. In conclusion, strain SN9 had simultaneous denitrifying and sulfate reducing capabilities. The characterization of the present isolate had important ecological implications in treating organic wastewater containing high concentrations of sulfate and nitrogen.

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