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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 $C_{12:0}$~$C_{19\text{-CYC-FAME}}$ and the main fatty acids consisted of $C_{14:0\ FAME}$, $C_{16:0\text{-}FAME}$, $C_{18:1c\text{-}FAME}$,$C_{18:0\text{-}FAME}$,$C_{19\text{-}CYC\text{-}FAME}$. 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 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, 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.

The sulfate reducing bacteria (SRB) were characterized by their ability to reduce sulfate to sulfide with the simultaneous oxidation of the organic substrates (Jenneman *et al*.,1986). Sulfide production is a major concern to the petroleum industry since it is toxic and corrosive and causes plugging due to the formation of insoluble iron sulfides (Reinsel *et al*.,1996; Li Wei *et al*.,2011). Inhibition of SRB by nitrate (NO_3^-) injection had been widely investigated. Sulfide production was temporarily inhibited due to the preferential use of nitrate as an electron acceptor. The prolonged decrease of SRB as a result of prolonged exposure to an oxidzing environment was due to the buildup of N_2O or NO or both by nitrate reduction. Similarly, other studies(Senez *et al*.,1998) also concluded that the production of intermediate metabolites (N_2O and NO) by denitrification led to the raise of redox potential and thus long-term inhibition sulfate reduction was realized.

It was reported that the genera of *Desulfovibrio*, *Desulfobulbus* and *Desulfomonas* could utilize nitrate as electron acceptor and obtain energy for their growth. Successful dissimilatory nitrate reduction to ammonia was achieved by a strain of *Desulfovibrio desulfuricans* (a strict anaerobic SRB), which confirmed that dissimilatory nitrate reduction to ammonia was not confined to facultatively anaerobic bacteria (Keith *et al*.,1983). A. Marietou *et al*. found that the nitrate reduction by strain *Desulfovibrio desulfuricans* ATCC 27774 was catalyzed by a periplasmic nitrate reductase

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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 $NaNO_3$ 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. A 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 SO_4^{2-}, 4000 mg/L NO_3^-, 2350 mg/L COD (pH, 8.0)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: Na_2SO_4 4 g, KNO_3 2.0 g, $NaNO_3$ 2.0 g, $MgSO_4 \cdot 7H_2O$ 1g, K_2HPO_4 0.5 g, $KNaC_4H_4O_6 \cdot 4H_2O$ 5 g, KH_2PO_4 1.0 g, $CaCl_2 \cdot 2H_2O$ 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 0.5g 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% $FeSO_4 \cdot (NH_4)_2SO_4$ 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 37.5µL deionized water. The thermal cycling included: 94°C for 5 min followed by 30 cycles of denaturation at 94 °C for 30s, annealing at58 °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 NO_3^-, NO_2^- and SO_4^- 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 H_2S production tests confirmed that strain SN9 had denitrification and sulfate reduction capabilities. Besides, the analysis of fatty acids analysis indicated that most of the fatty acids distributed around $C_{12:0}$~$C_{19\text{-CYC-FAME}}$. The main fatty acids consisted of $C_{14:0\ FAME}$, $C_{16:0\text{-FAME}}$, $C_{18:1c\text{-FAME}}$, $C_{18:0\text{-FAME}}$ and $C_{19\text{-CYC-FAME}}$, which accounted for 87.63% of the total fatty acids with 8.68%, 53.29%, 8.51%,9.61% and 7.54%, respectively.

Table 1. The results of the physiochemical tests

Items	Results
Methyl red test	bþ
Indole test	bþ
Fructose	-
Citrate utilization	bþ
Glucose fermentation	Acid production through fermentation
Starch hydrolysis	-
Grease hydrolysis	bþ
Lactose	-
Sucrose	-
Ethanol	-
Urea hydrolysis	cþ
Gelatin hydrolysis	cþ
Nitrate reduction	bþ
H_2S production	bþ
Catalase test	-
Oxygen demand	Anaerobic
Gas production from glucose	-
Vogers-Proskauer test	+
Ammonia production	-
Litmus milk test	Organic acid production and solidification

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

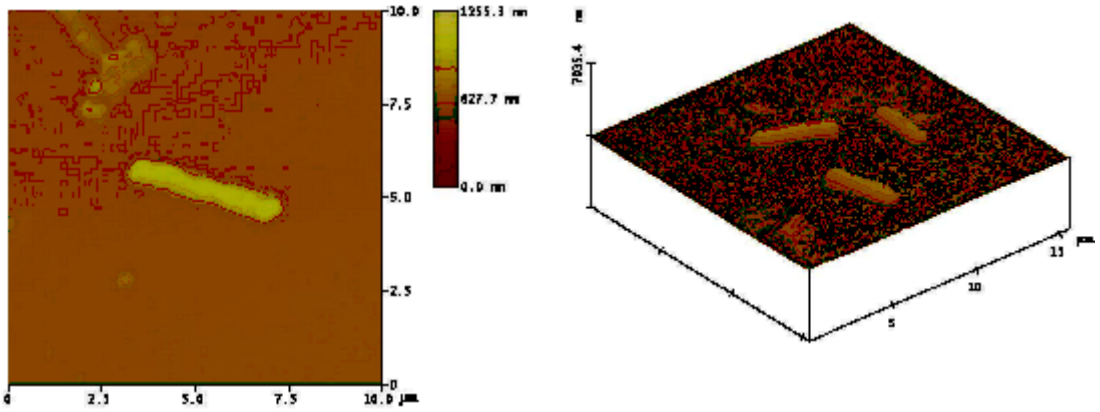

Fig. 1. AFM images of Strain SN9

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 4th day and the highest was 97.76%. The greatest concentration of NO_2^- was detected on the 4th day (997.61 mg/L), which was produced as the intermediate product when the concentration of NO_3^- dropped dramatically.

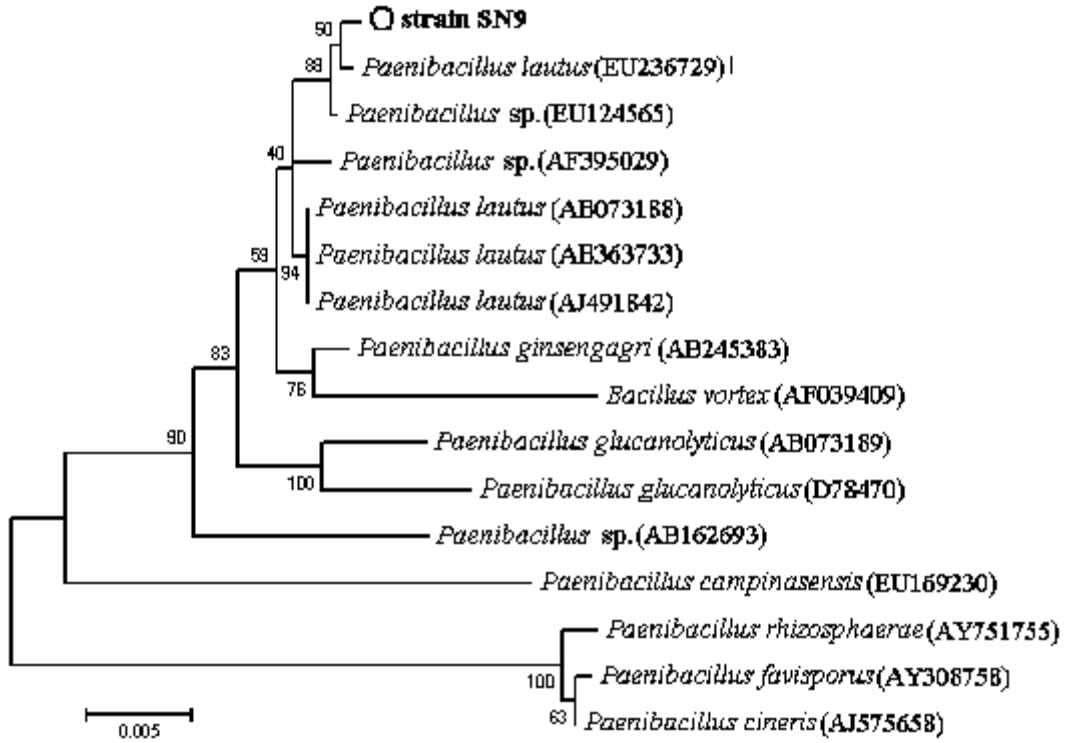

Fig. 2. Phylogenetic tree based on the 16S rDNA gene sequences of strain SN9 and the selected species from GenBank Database by using the neighbour-joining method

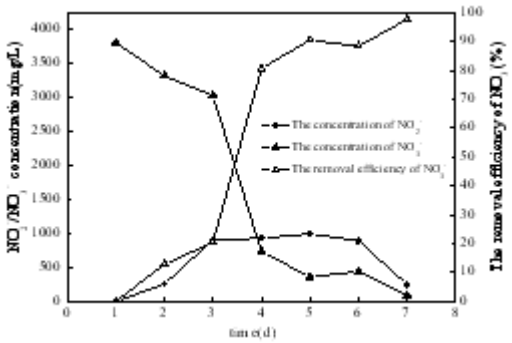

Fig. 3. The daily variations of NO_3^- and NO_2^- concentrations and the removal efficiency of NO_3^-

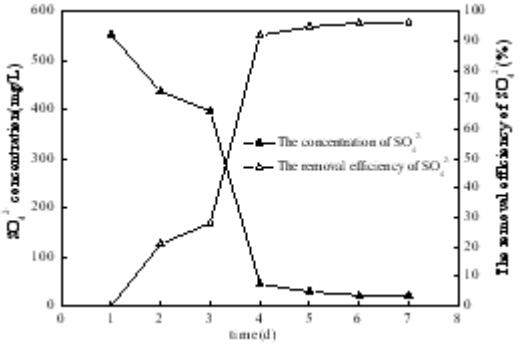

Fig. 4. The daily variations of SO_4^{2-} concentrations and the removal efficiency of SO_4^{2-}

Along with the cultivation of strain SN9, the NO_2^- 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

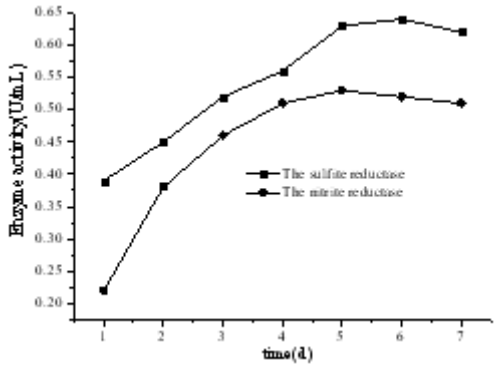

Fig. 5. The nitrite and sulfite reductase activities of SN9

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 $C_{12:0}$~$C_{19\text{-CYC-FAME}}$ and the main fatty acids consisted of $C_{14:0\ FAME}$, $C_{16:0\text{-FAME}}$, $C_{18:1c\text{-FAME}}$, $C_{18:0\text{-FAME}}$ and $C_{19\text{-CYC-FAME.}}$ 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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