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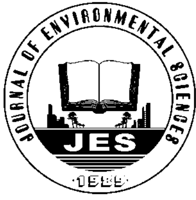

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Enrichment, isolation and identification of sulfur-oxidizing bacteria from sulfide removing bioreactor

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Abstract

Sulfur-oxidizing bacteria (SOB) are the main microorganisms that participate in the natural sulfur cycle. To obtain SOB with high sulfur-oxidizing ability under aerobic or anaerobic conditions, aerobic and anaerobic enrichments were carried out. Denaturing gradient gel electrophoresis (DGGE) profiles showed that the microbial community changed according to the thiosulfate utilization during enrichments, and *Rhodopseudomonas* and *Halothiobacillus* were the predominant bacteria in anaerobic enrichment and aerobic enrichment, respectively, which mainly contributed to the thiosulfate oxidization in the enrichments. Based on the enriched cultures, six isolates were isolated from the aerobic enrichment and four isolates were obtained from the anaerobic enrichment. Phylogenetic analysis suggested the 16S rRNA gene of isolates belonged to the genus *Acinetobacter, Rhodopseudomonas*, *Pseudomonas, Halothiobacillus, Ochrobactrum, Paracoccus, Thiobacillus*, and *Alcaligenes*, respectively. The tests suggested isolates related to *Halothiobacillus* and *Rhodopseudomonas* had the highest thiosulfate oxidizing ability under aerobic or anaerobic conditions, respectively; *Paracoccus* and *Alcaligenes* could aerobically and anaerobically oxidize thiosulfate. Based on the DGGE and thiosulfate oxidizing ability analysis, *Rhodopseudomonas* and *Halothiobacillus* were found to be the main SOB in the sulfide-removing reactor, and were responsible for the sulfur-oxidizing in the treatment system.

Key words: sulfur-oxidizing bacteria (SOB); thiosulfate-oxidizing; microbial community; enrichment; isolation

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Introduction

Biological oxidation of sulfur is an important metabolic process in the natural sulfur cycle. The oxidation reactions are performed mainly by sulfur-oxidizing bacteria (SOB) from the classes Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Epsilonproteobacteria, Chlorobia, and Chloroflexi (Friedrich et al., 2001, 2005). The electrons derived from sulfide oxidation are used by aerobic chemotrophic colorless SOB (i.e., *Thiobacillus, Beggiatoa, Halothiobacillus*, etc.) for energy transformation of the respiratory chain and for autotrophic carbon dioxide reduction; anaerobic phototrophic SOB (green sulfur bacteria, i.e., *Chlorobium*, etc.; purple sulfur bacteria, i.e., *Allohromatium, Halochromatium, Thiocapsa*, etc.) use light energy to transfer electrons from sulfur or other sources for autotrophic carbon dioxide reduction (Friedrich et al., 2005; Ghosh and Dam, 2009). Meanwhile, many heterotrophic SOB were found to participate in the sulfur-oxidizing process under aerobic and anaerobic conditions (Kantachote et al., 2008; Mahmood et al., 2009; Potivichayanon, 2005).

Knowledge regarding the ecology of the SOB communities and abundance in natural or manmade environments is of great importance for the knowledge of microorganisms participating in the sulfur cycle, and the design of specific methods and media to obtain the main SOB with high sulfur-oxidizing ability. In the present work, to reveal the SOB community in a sulfide-removing bioreactor and obtain the main SOB for the further optimization of the wastewater treatment system, we carried out aerobic and anaerobic enrichments and analyzed the SOB community changes during the enrichments, and developed two methods to isolate aerobic and anaerobic SOB from the sulfide-removing bioreactor.

1 Materials and methods

1.1 Sample preparation

Activated sludge samples were collected from a sulfide-removing bioreactor (Lin et al., 2010). Samples from

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five different sites were obtained in sterile polyethylene bottles (500 mL volume), and mixed together before being frozen in an ice cube box (TaKaRa, Dalian, China). After transport to the laboratory, 100 mL of the sludge samples were suspended in sterile phosphate buffer solution (PBS, pH 8.0) with the dilution ratio of 1:10 (W/V) and mixed by shaking with sterile glass beads. After shaking for 30 min at 25°, the suspension was collected after allowing mud particles, stones and sand to settle for 2 min. The suspension was then transferred to clean tubes, and centrifuged at 6000 ×g for 5 min. The pellets were resuspended twice in 100 mL of PBS (pH 8.0) and stored at 4°C until use for enrichments.

1.2 Media

The aerobic culture medium (ACM) used for enrichment and isolation of aerobic SOB contained the following components at the specified concentrations (in g/L): $Na_2S_2O_3$, 6.00; NaH_2PO_4, 1.22; Na_2HPO_4, 1.39; NH_4Cl, 1.00; $MgCl_2$, 0.10; $FeCl_3$, 0.03; $CaCl_2$, 0.03; $MnCl_2$, 0.03; KNO_3, 0.50; CH_3COONa, 1.00; $NaHCO_3$, 2.00. The anaerobic culture medium (ANCM) used for enrichment and isolation of anaerobic phototrophic SOB was (g/L): $Na_2S_2O_3$, 10.00; NaH_2PO_4, 2.45; Na_2HPO_4, 2.78; NH_4Cl, 1.00; $MgCl_2$, 0.50; CH_3COONa, 1.00; sodium succinate, 1.00; $NaHCO_3$, 3.00; KNO_3, 0.50; NaCl, 1.00; yeast extract, 0.50; plus 1 mL of trace element solution which included (g/L): $FeSO_4·4H_2O$, 1.80; $CoCl_2·6H_2O$, 0.25; $CuCl_2·2H_2O$, 0.01; $NiCl·6H_2O$, 0.01; $MnCl_2·4H_2O$, 0.70; $ZnCl_2$, 0.10; H_3BO_3, 0.50; $(NH_4)_6Mo_7O_{24}·4H_2O$, 0.10; EDTA, 2.50; in 1 L distilled water. Because S^{2-} will convert into H_2S and be lost as a gas from aerobic enrichment, $Na_2S_2O_3$ instead of Na_2S was used as the sulfur substance. $Na_2S_2O_3$ was added aseptically from a sterile stock solution of 50 g/L $Na_2S_2O_3$ to the sterile media. The initial pH of media was adjusted to 6.8. Agar (2%) was added as a solidifying agent.

1.3 Enrichment cultures and isolation of pure cultures from enrichments

A 10-mL of prepared sample was inoculated into Erlenmeyer flasks containing 100 mL ACM medium and ampoule bottles containing 100 mL ANCM medium, respectively. The enrichment for aerobic SOB was cultivated at 30°C and rotation speed of 180 r/min. Successive enrichments of aerobic SOB were prepared with 5% (V/V) culture fluid used as the inoculums for each subculture. After four successive cultures, 0.1 mL of the enrichment and diluted samples (10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) were spread onto agar ACM plates and incubated at 30°C for 2–3 days. Colonies were picked from plates and inoculated in fresh fluid ACM to determine the sulfur-oxidizing ability.

The enrichment of anaerobic and phototrophic SOB was cultured in an illumination incubator (BSG-300, Boxun, China) with light intensity of 2000 lux and temperature of 30°C. The ANCM culture medium was flushed with N_2 gas for 2 min to create an anaerobic condition. After enrichment, 0.1 mL of the culture sample and diluted samples (10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) were spread onto the agar ANCM plates and incubated under 30°C and 2000 lux in an AnaeroPouch^{TM}-Anaero system (MITSUBISHI Gas Chemical Co., Inc., Japan) for 3–4 days. Colonies were picked from plates and inoculated in flesh fluid ANCM to determine the sulfur-oxidizing ability.

SOB was re-spread onto the agar ACM plates or agar ANCM plates, and was re-streaked several times on fresh agar to obtain purified isolates.

1.4 DNA preparation and 16S rRNA gene amplification

The aerobic and anaerobic enrichment culture samples were pre-treated with 0.5 mg/mL Propidium Monoazide (Biotium, Inc., Hayward, USA) before community DNA extraction; the total DNA extraction and the PCR amplification protocol to prepare PCR products for DGGE analysis were carried out according to a method previously described by Luo et al. (2010). 16S rRNA genes of SOB pure cultures were PCR amplified directly with primer pair 27F/1492R (27F: 5'-AGA GTT TGA TCC TGG CTC AG-3'; 1492R: 5'-TAC CTT GTT ACG ACT T-3').

1.5 Denaturing gradient gel electrophoresis (DGGE)-16S rRNA gene analysis

DGGE was performed on a 10% (W/V) polyacrylamide gel with a denaturing gradient of 30% to 60% denaturants (Muyzer et al., 1993). Electrophoresis was carried out in 1×TAE (Tris-Acetate-EDTA) buffer at 60°C and at a constant voltage of 80 V for 12 hr using a Bio-Rad DCode system (DCode, Bio-Rad, USA). The gel staining was performed by a modified method previously described by Zakaria et al. (2007). In brief, gels were washed for 15 min in a fixing solution (10% (V/V) ethanol and 1% (V/V) acetic acid), stained for 20 min in a staining solution (0.2% (W/V) $AgNO_3$), washed thrice in deionized water, and visualized in a developing solution (2% (W/V) NaOH and 0.5% (W/V) formaldehyde). The DNA in DGGE gel was extracted and the V3 region DNA was re-amplified according to the method as described by Lin et al. (2010). The PCR products amplified by the primer pair with GC clamp were run again on DGGE gel to verify their position and purity, and the PCR products amplified by the primer pair without GC clamp were sequenced with primer 341F by Beijing Genomics Institute, China.

1.6 Phylogenetic analysis

16S rRNA gene sequences from DGGE bands and SOB isolates were analyzed using the Nucleotide-nucleotide BLAST (Blastn) database (http://www.ncbi.nlm.nih.gov/BLAST) and the Seqmatch program and CHIMERA_CHECK program of the Ribosomal Database Project (http://rdp.cme.msu.edu/).

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Sequences and their closest matches retrieved from the database were aligned using the software Clustal X version 1.8 (Thompson et al., 1997), and phylogenetic trees were constructed by the neighbor-joining method with the software MEGA 3 (Kumar et al., 2004). Evolutionary distances of nucleotide sequences were calculated with Kimura's 2-parameter model; bootstrap values were obtained with 1000 re-samplings.

1.7 Analytical methods

The sulfate was determined by a modified Barium Chromate method according to Foster (1936). In brief, the water sample was acidified with 1 mol/L HCl, and boiled for 5 min to eliminate CO_3^{2-} and HCO_3^-. Barium chromate suspension was added in the sample and boiling was maintained for 5 min. After cooling, the excess barium chromate was precipitated by ammonium hydroxide; the color of the fluid changed from orange yellow to lemon yellow after adding 3 drops of ammonium hydroxide solution. The sample was centrifuged at 4000 r/min for 3 min, and the supernatant was collected. The absorbance of the supernatant was determined at 420 nm using a spectrophotometer (Unico UV-2802S, Unico, China). The concentration of thiosulfate was determined by an iodometric method (Kelly and Wood, 1994) after precipitating the solid and cells by centrifugation.

1.8 Nucleotide sequences accession numbers

The sequences reported in this article have been deposited in the GenBank database with the accession numbers: HQ693538 to HQ693547 for DGGE bands and HQ693548 to HQ693555 for isolates.

2 Results

2.1 Thiosulfate utilization and sulfate production in enrichments

During the enrichment for anaerobic and phototrophic SOB, the concentration of thoisulfate remained steady for the first 55 days, and decreased quickly in the next week, then decreased slowly in the last 20 days. The concentration of sulfate fluctuated at 1.50 g/L for the first 55 days, and increased quickly in the next week, then became steady in the last 10 days (**Fig. 1a**), changing according to the change in thiosulfate concentration.

In aerobic enrichment, subculturing was carried out four times (**Fig. 1b**). The concentration of thiosulfate decreased from 6.07 to 2.60 g/L in the first cultivation, 12.30 to 5.45 g/L in the first subculture, 6.58 to 2.09 g/L in the second subculture, 29.29 to 12.52 g/L in the third subculture, and from 8.15 to 0.74 g/L in the fourth subculture. The concentration of sulfate changed according to the concentration change of thiosulfate.

2.2 DGGE profile analysis of the enrichments

The microbial community structures and dynamic changes during both enrichments were tracked by PCR-DGGE analysis (**Fig. 2**). At the beginning of the enrichment, the microbial community structure was complex, so that more than 20 DNA bands were presented in the lane of DGGE profiles. During the anaerobic enrichment, the microbial community remained relatively steady during the first 41 days, but then changed substantially after day 48; at the end of the enrichment, only six DNA bands were visible in the lane (**Fig. 2a**). During the enrichment, four DNA bands (AN2, AN4, AN5, and AN6) were visible in the lanes almost all of the time, but two DNA bands (AN1 and AN3) were present from day 35 and 60, respectively, and the band density of AN1 became more and more strong.

During the aerobic enrichment, the microbial community changed substantially from the beginning to day 24, and remained relatively steady after this initial transition period; at the end of the enrichment, only seven DNA bands were visible in the lane (**Fig. 2b**). During the enrichment, five DNA bands (A1, A2, A3, A4, and A7) were visible in the lanes almost all of the time, but two DNA bands (A5 and A6) were visible from day 28.

To better understand the microbial community after two enrichments, six bands from the anaerobic enrich-

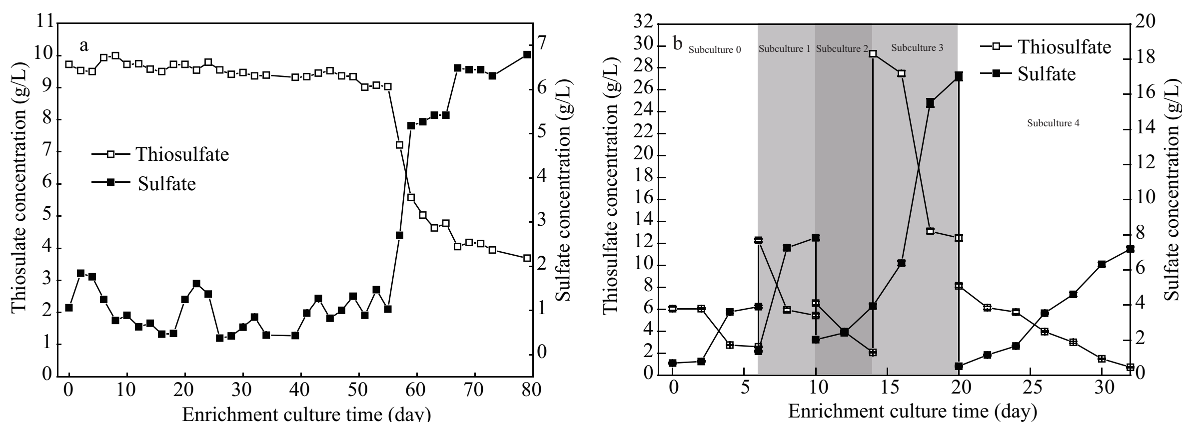

Fig. 1 Changes of substrate (thiosulfate) concentration and product (sulfate) concentration in anaerobic phototrophic enrichment culture (a) and aerobic enrichment culture (b).

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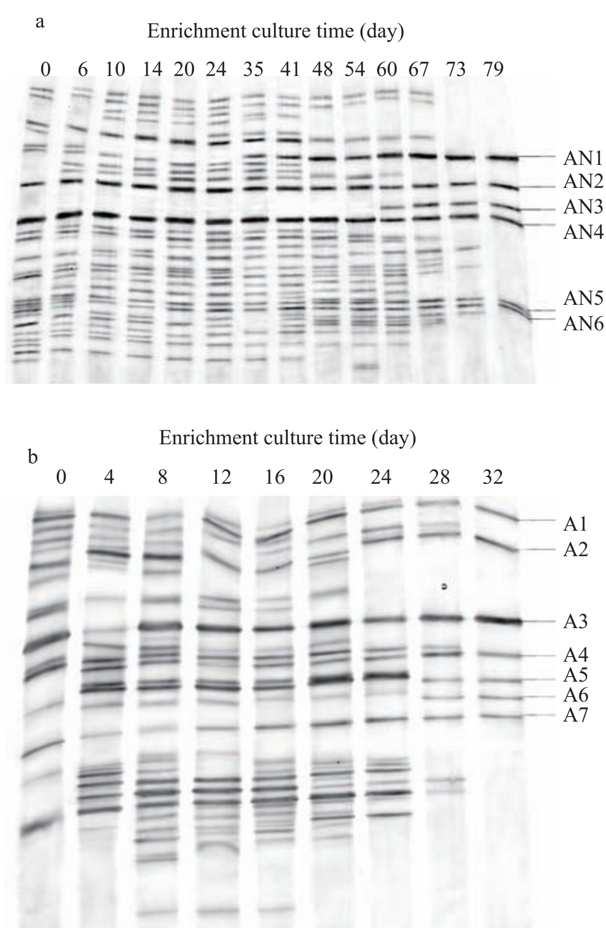

Fig. 2 DGGE profiles of microbial communities in anaerobic phototrophic enrichment culture (a) and aerobic enrichment culture (b).

ment DGGE gel and seven bands from the aerobic enrichment DGGE gel were excised, purified, and characterized by 16S rRNA gene sequence analysis (**Fig. 2**). Five known bacteria were closely related to *Acinetobacter, Rhodopseudomonas, Paracoccus, Alcaligenes*, and *Clostridium*, respectively, and one bacterium closely related to unknown *Enterobacteria* were identified in the bands from the anaerobic enrichment; and seven known bacteria closely related to *Pseudomonas, Halothiobacillus, Ochrrobactrum, Paracoccus, Thiobacillus*, and *Alcaligenes,* respectively, were identified in the bands from the aerobic enrichment (**Fig. 3**). The most abundant DNA bands AN1 in anaerobic enrichment and A3 in aerobic enrichment were closely related to the genus *Rhodopseudomonas* and *Halothiobacillus*, respectively.

2.3 Isolation, identification and characterization of the isolates

After enrichments, serial dilutions of enrichment cultures were plated on agar ACM plates or agar ANCM plates. Representative colonies of different morphotypes from plates were selected for further thiosulfate utilization tests. Finally, six isolates were obtained from the aerobic enrichment and four isolates were obtained from the anaerobic enrichment, respectively. Based on the 16S rRNA gene sequence analysis, the sequences of isolates were matched by BLAST to the genus *Acinetobacter, Rhodopseudomonas, Pseudomonas, Halothiobacillus, Ochrobactrum, Paracoccus, Thiobacillus*, and *Alcaligenes*, respectively (**Fig. 3**). The sequence similarities of all the isolates were more than 98% (data not shown).

Sequences of isolates showed high homology to the DGGE band sequences from the same enrichments (**Fig. 3**). After the phylogenetic analysis, all sequences were grouped into 4 clusters (Alpha-, Beta-, and Gammaproteobacteria, and Clostridia). The Alphaproteobacteria were dominated by Bradyrhizobiaceae, Brucellaceae, and Rhodobacteraceae; the Betaproteobacteria were dominated by Hydrogenophilaceae and Alcaligenaceae; the Gammaproteobacteria were dominated by Pseudomonadaceae, Enterobacteriaceae, Halothiobacillaceae, and Moraxellaceae; and the Clostridia was dominated only by Clostridiaceae.

After the identification, all isolates were tested for their thiosulfate utilization (**Fig. 4**). In the aerobic test, isolate JFA2 closely related to the genus *Halothiobacillus* showed the highest thiosulfate oxidizing ability, using up the thiosulfate in 8 days (**Fig. 4a, b**). Meanwhile in the anaerobic test, isolate JFANg related to the genus *Rhodopseudomonas* showed the highest thiosulfate oxidizing ability (**Fig. 4c, d**).

3 Discussion

The DGGE analysis suggested that the microbial community changed according to the thiosulfate utilization during enrichments, and the predominant strain in aerobic enrichment was most closely related to the genus *Halothiobacillus*, while the predominant strain in anaerobic enrichment was most closely related to the genus *Rhodopseudomonas*. Concerning the abundance, these two kinds of strains were suggested to be the main SOB that contributed to the oxidation of thiosulfate in enrichment, and might be the main SOB for sulfur-oxidizing in the wastewater treatment system. These deductions were further supported by the thiosulfate utilization tests after SOB isolates were obtained. In the tests, isolate JFA2 closely related to *Halothiobacillus* and isolate JFANr closely related to *Rhodopseudomonas* showed the highest thiosulfate-oxidizing ability in aerobic and anaerobic conditions, respectively. Then, based on the abundance and thiosulfate-oxidizing ability analysis, we concluded the SOB closely related to the genus *Rhodopseudomonas* and *Halothiobacillus* were the main SOB in the sulfide-removing reactor, and responsible for the sulfur-oxidizing in the treatment system.

Strains from genus *Halothiobacillus* are mesophilic, obligately chemolithoautotrophic, and strictly aerobic SOB (Ito et al., 2005; Wood et al., 2005), and usually found to be

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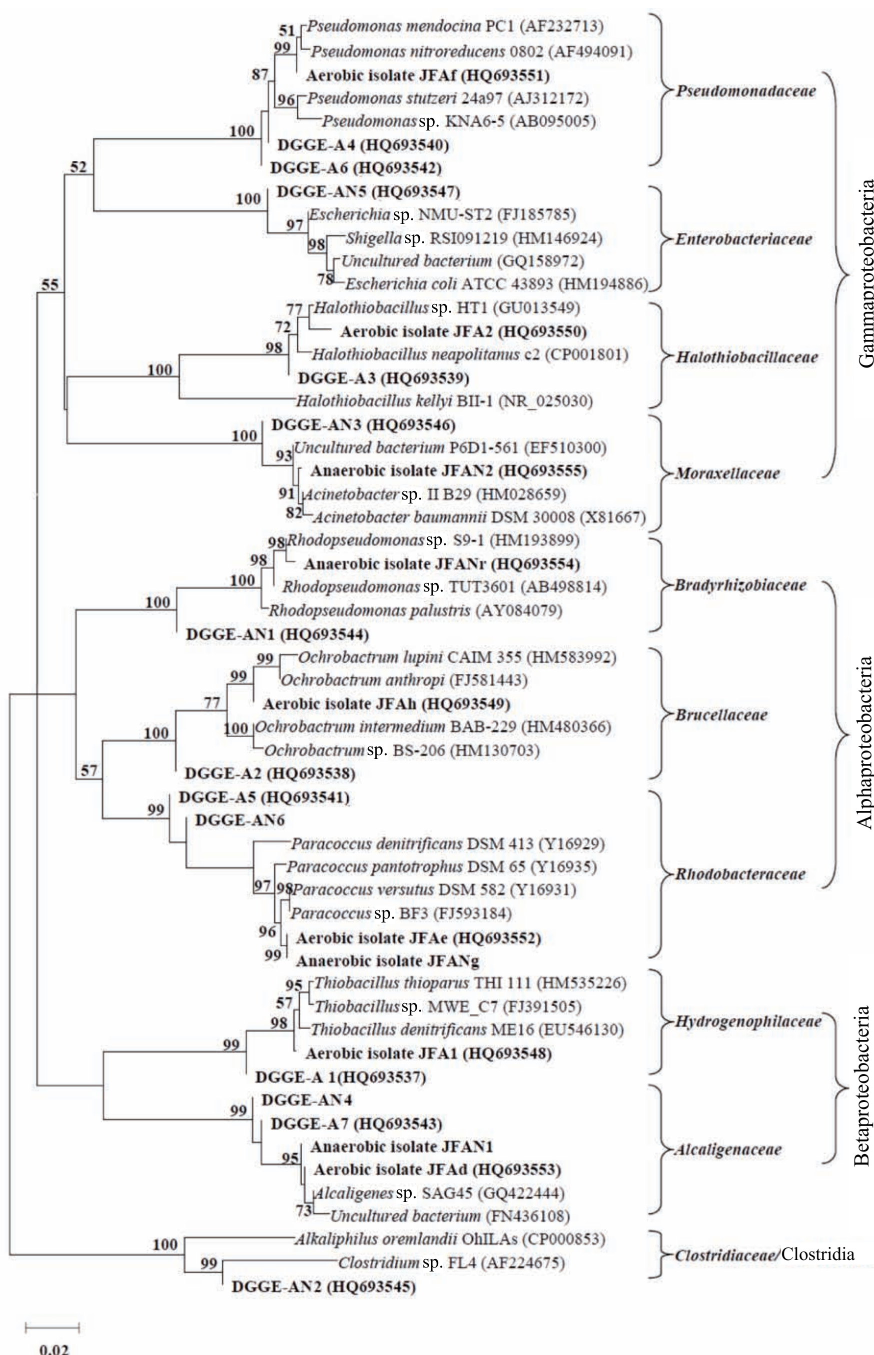

Fig. 3 Phylogenetic tree based on the 16S rRNA gene of DGGE bands and isolates from aerobic and anaerobic enrichments, and the selected sequences from formally described species. The tree was inferred using the neighbor-joining method, and evolutionary distances were computed using Kimura's 2-parameter model. Bootstrap values (1000 replicates) above 50 are shown at each node.

predominating members in manmade systems for sulfide removal (Okabe et al., 2007; Vannini et al., 2008). As representative members of the Rhodobacteraceae family, *Rhodopseudomonas* are kinds of purple nonsulfur bacteria that are capable of oxidizing sulfide and thiosulfate both under anaerobic conditions in the light and under aerobic conditions in the dark (Hashwa, 1975; Rodova and Pedan, 1980). Besides *Rhodopseudomonas* and *Halothiobacillus*, strains related to the genus *Thiobacillus*, *Paracoccus, Acinetobacter, Pseudomonas, Ochrobactrum,* and *Alcaligenes* also participated in the thiosulfate oxidization in the enrichments, and might be contributors to the sulfur oxidization in the treatment system. Strains from the genus *Thiobacillus* are obligately chemolithoautotrophic, aerobic or facultative anaerobic SOB (Kelly and Wood, 2000; Vlasceanu et al., 1997), which are often found in wastewater treatment systems and with a high richness (Ji et al., 2009; Silva et al., 2010). Strains from the

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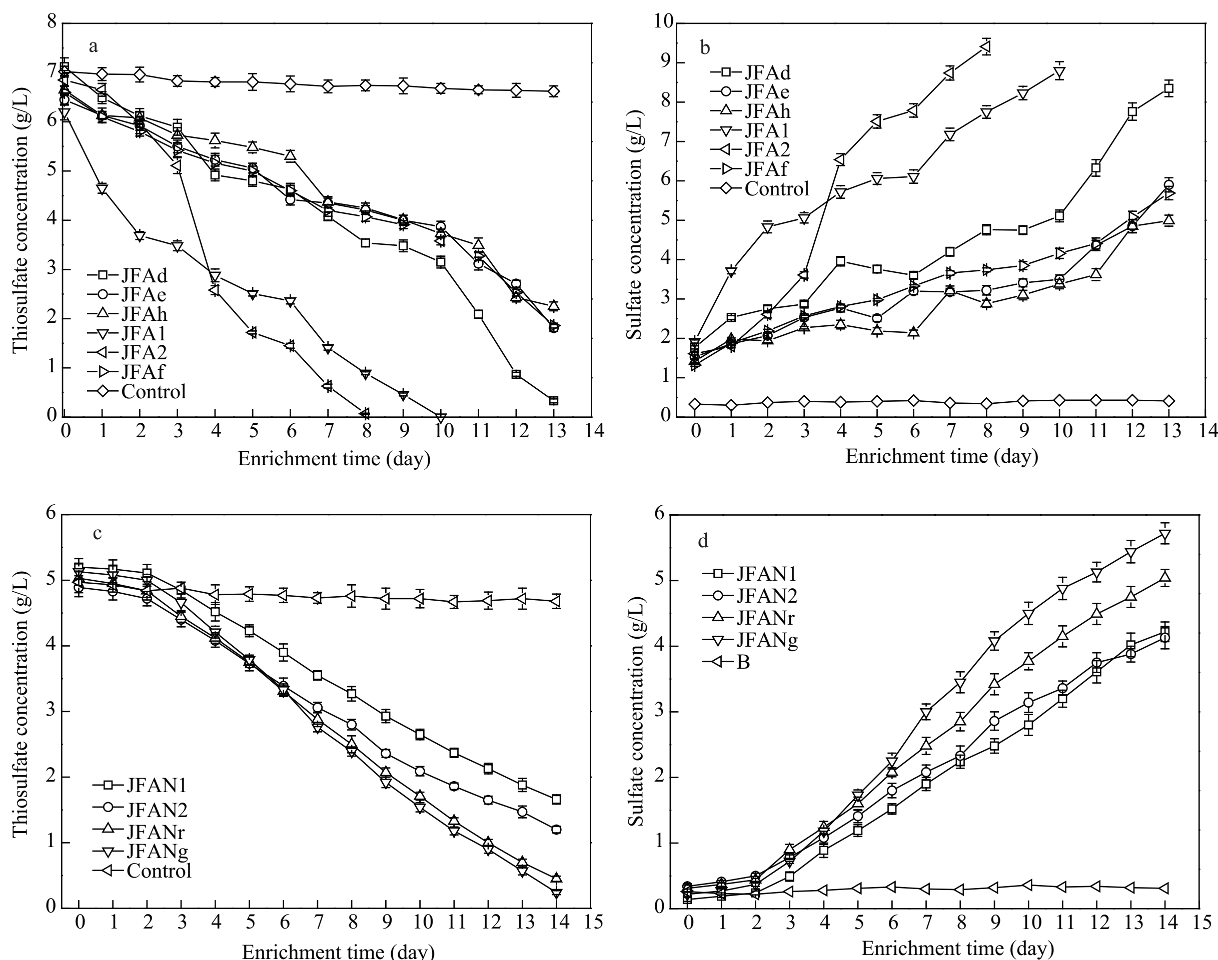

Fig. 4 Utilization of thiosulfate and production of sulfate by isolates isolated from aerobic enrichment (a, b) and anaerobic enrichment (c, d).

genus *Alcaligenes* are kinds of facultatively anaerobic bacteria; some of them have been reported to be colorless sulfur-oxidizing bacteria which could oxidize sulfide under anaerobic conditions (Bharathi, 1997) and aerobic conditions (Kantachote et al., 2008; Potivichayanon, 2005). Members of *Paracoccus* are facultatively aerobic and grow heterotrophically with various carbon sources and chemoautotrophically with thiosulfate and sulfide as electron donors under aerobic conditions (Frierich et al., 2008). The genus *Ochrobactrum* was reported to be able to utilize sulfide and thiosulfate under aerobic and thermophilic alkaline conditions, and use sulfide as a source of electrons to reduce nitrite anaerobically (Mahmood et al., 2009; Zhang et al., 2008). In addition, Aguilar et al. (2008) isolated SOB from an artificial wetland for tannery wastewater treatment and classified these SOB to the genera *Acinetobacter*, *Pseudomonas, Ochrobactrum,* and *Alcaligenes*, which is similar with our results.

The result established that the genus *Rhodopseudomonas* and *Halothiobacillus* were the main SOB in the sulfide-removing reactor, and mainly responsible for the sulfur-oxidizing in the treatment system. To optimize the sulfur-oxidizing ability of the wastewater treatment system in the future, we could apply a microbial augmentation method that inoculates a high concentration of *Rhodopseudomonas* or *Halothiobacillus* into the system according to the aerobic or anaerobic processes, or adds some specific substance for enhancing the growth of *Rhodopseudomonas* or *Halothiobacillus* in the system.

Acknowledgments

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