African Journal of Biotechnology Vol. 10(56), pp. 11966-11971, 26 September, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.799
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Isolation of a sulfate reducing bacterium and its application in sulfate removal from tannery wastewater

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Accepted 11 August, 2011

In order to remove sulfate in tannery wastewater efficiently, a sulfate reducing bacterium (SRB) was isolated in tannery anaerobic activated sludge. With biochemical and genetics method, the isolated bacterium was identified as *Citrobacter freundii*. Then, the isolate was inoculated to tubes containing sulfate in simulated tannery wastewater to optimize the process. The results show that the effect of *C. freundii* in removing sulfate was best when the temperature was 32°C, pH was 7.0, COD/SO_4^{2-} was 5.0 and the initial SO_4^{2-} concentration was 1500 mg/L. Also, the SRB was inoculated onto an up-flow anaerobic sludge bed (UASB) to remove sulfate in actual tannery wastewater. It was found that the removal rate of sulfate in actual tannery wastewater reached 89.66% which was 12.13% higher than the treatment without inoculating the isolated SRB when the initial SO_4^{2-} concentration was 1069 mg/L. The experiment demonstrates that *C. freundii* could be selected as a new biomaterial to remove sulfate in tannery wastewater.

Key words: Tannery wastewater, sulfate, *Citrobacter freundii*.

INTRODUCTION

Sulfate (SO_4^{2-}) usually pollutes the surface and the ground water because it can reduce the pH of water. When the sulfate is more than 100 mg/L, the drinking water will be bitter and people are easily infected with diarrhea after drinking the water (Li, 2005). Furthermore, the quality of crops is reduced because the sulfate pollutes soil and infield which thus directly has a bad influence on the health of people. Besides, the abundance of sulfate will disturb the anaerobic biologic treatment for wastewater (Li, 2005). Therefore, the sulfate in wastewater has received great attention with the increase of awareness for environmental protection and health.

In leather making industry, a great deal of wastewater containing high concentrations of sulfate is discharged which comes from many processes such as liming, deliming, bating, pickling and chrome tanning (Li, 2005). At present, the sulfate with high concentrations is not treated especially in tannery, but discharged directly into the integrated wastewater. Thus, it has gone over the top of the survival for the environment. In order for the durative development of the leather-making industry, to materialize, the sulfate is required to be removed with new effective methods urgently.

Usually, microorganisms are preferred because of their cheap cost. When the SO_4^{2-} in the solution is removed by the microorganisms, sulfate reducing bacteria (SRB) firstly deoxidize SO_4^{2-} into sulfide including H_2S, S^{2-} and HS^- under anaerobic condition; after which the sulfide may then be oxidized into elemental sulphur (S^0) by sulfide oxidation bacteria (SOB) under aerobic and specific condition (Jiang et al., 2009).

In order to advance the ability to remove sulfate in the intrinsic treatment system, investigators have isolated and selected many efficient pure SRB to treat sulfate (Kjeldsen et al., 2009; Jiang et al., 2009). Until now, there have been some reports about the removal of sulfate in tannery wastewater by SRB (Boshoff et al., 2004) and a

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Competition between SRB and methanogens with anaerobic treatment for tannery wastewater (Shin et al., 1996; Tadesse et al., 2003). However, there has not been any report about removing sulfate in tannery wastewater with isolated predominant SRB strains.

In the experiments, an efficient SRB was first enriched, isolated, screened and identified, after which the isolated efficient SRB was then detected for its optimal conditions through removing sulfate from the simulated tannery wastewater. Subsequently, the isolate was inoculated into an up-flow anaerobic sludge bed (UASB) containing anaerobic activated sludge in order to remove the sulfate from the actual tannery wastewater. After treatment, the removal effect of the sulfate was detected, with the treatment which was without an inoculation of the isolate as control.

MATERIALS AND METHODS

The tannery anaerobic activated sludge and the actual tannery wastewater containing sulfate in the experiments were collected from the anaerobic wastewater treatment system in Sichuan Maoliang Leather Co. Ltd. (China). The Black wattle extract was purchased from Guangxi Baise Forest Chemistry Co. (China); the Black 157 dyestuff was from Shanghai Hanxiong Dyestuff Chemical Industry Co. (China); and the sulfited lanolin fatliquoring agent was from Sichuan Decision leather Chemical Industry Co. (China). In addition, yeast extract, peptone, L-cysteine and gelatin were of biochemical level, while other reagents were of research grade.

Enrichment culture medium for SRB

The liquid enrichment culture medium for SRB was prepared with: 0.3 g KCl, 3.0 g $MgSO_4 \cdot 7H_2O$, 2.5 g $MgCl_2 \cdot 6H_2O$, 0.5 g NH_4Cl, 1.0 g NaCl, 0.6 g KH_2PO_4, 20 g sodium lactate, 0.5 g ascorbic acid, 0.5 g L-cysteine, 2.0 g yeast extract, 2.0 g peptone, 2.0 g $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$, and 1000 ml water (Jiang et al., 2009). The pH of the culture medium was adjusted to 6.0~6.5, while the solid medium for SRB was formed with additional 2% (w/w) agar. Before usage, the liquid and solid media except for ascorbic acid, L-cysteine and $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$ were sterilized at 121℃ for 20 min, while ascorbic acid, L-cysteine and $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$ were separately sterilized with a bacteria filter membrane (0.22 μm of aperture) that was added just before culture.

Simulated tannery wastewater containing sulfate (Ma, 2008)

First, 2.5 g CaO and 30 g $Na_2S \cdot 9H_2O$ were added into a beaker and dissolved with 1000 ml water. Then, 50 g scalper hair and 2 g gelatin were added before it was hydrolyzed at 60℃. After the hair was dissolved completely, 1 g NaCOOH, 1 g $(NH_4)_2SO_4$, 0.1 g Black wattle extract, 0.075 g Black 157 dyestuff, 0.365 g sulfited lanolin fatliquoring agent, 0.5 g chromium powder and 1 g NaCl were in turn added to the beaker. When the mixture was dissolved, the wastewater was formed and adjusted to 8~10 of pH, 600~3500 times of color, 60~100 mg/L of trivalent chromium, 50~100 mg/L of sulfide and 3000~4000 mg/L of COD_{Cr} according to the average water quality of actual tannery wastewater. Thus, the simulated tannery wastewater containing sulfate was obtained.

Enrichment of SRB

The tannery anaerobic activated sludge containing SRB was domesticated in the UASB (PJK03, Shanghai Jiangke Experiment Equipment Co., China) by adding the liquid enrichment culture medium of SRB at 32℃ for 72 h as a cycle for a month.

Isolation and purification of SRB

The enriched tannery anaerobic activated sludge containing SRB was dispersed in sterile water to prepare germ suspension. After cells in the germ suspension were singled out, 10^{-1}-10^{-7} dilutions of the germ suspension were made and 0.1 ml of the appropriate dilution was transferred into various culture dishes. Then, 15 ml of the sterilized solid medium, containing SRB cooled to 55℃, was poured into the culture dishes with appropriate dilutions (10^{-3} to 10^{-7}). After the germ and medium were mixed homogeneously and solidified, the culture dishes were placed in a vacuum desiccator and vacuumized to keep no more than 0.055 MPa. Afterwards, the desiccator was incubated at 32℃ and maintained in the vacuum. After growth, the SRB in the medium appeared as a form of black spherical colonies which arose from FeS produced with S^{2-} in colonies and Fe^{2+} in medium. Subsequently, the single black SRB colonies were picked and inoculated to the liquid enrichment culture medium for SRB. When incubated for 24 h, the isolated SRB were purified again and the purified isolates were obtained by repeated streaking of the single colony on the fresh agar plates. After it was incubated at 32℃, each pure culture was maintained in the test tubes of the liquid enrichment culture medium at 4℃ in vacuum and transferred to a fresh test tube every two weeks.

Reselection of the SRB

First, 15 ml of the liquid enrichment culture medium for SRB was added to the test tubes (15 ×150 mm). After it was sterilized, the tubes containing the medium were inoculated with the pure SRB at an initial concentration of 1 to 5 × 10^{10} CFU/ml (colonies formed units per milliliter). Then, the tubes were placed in a vacuum desiccator and vacuumized for not more than 0.055 MPa, after which the desiccator was incubated at 32℃ and maintained in vacuum. After 72 h, the concentration of sulfide in the medium was measured through standard ISO 10530:1992, namely: Water quality - determination of dissolved sulfide - photometric method, using methylene blue (ISO 10530:1992). Thus, the efficient SRB was selected.

Identification of the selected SRB

Morphology

The screened SRB15# strain was primarily identified based on Gram reaction, colony morphology and microscopic examination with SEM (JSM-5900LV, Philips Co., Netherlands).

Physiological and biochemical characters

The SRB15# was further identified through glycolysis test, productivity H_2S test, indole test, methyl red test (M. R test), acetylmethyl carbinol test (V. P test), phenotypical characteristics, contact enzyme test and gelatin liquification test (Ma et al., 2002).

Genetic identification

According to the manufacturer's instructions, the extraction of total genomic DNA was performed using DNA extraction kit (Promega, USA), and PCR amplification was performed. A portion of the

Table 1. Factors and levels of the orthogonal test for sulfate removal by *Citrobacter freundii* CZ1001.

Factor	Level		
	1	2	3
A (Initial SO_4^{2-} concentration; mg/L)	1500	2000	2500
B (COD/SO_4^{2-})	1.0	3.0	5.0
C (pH)	6.0	7.0	8.0
D (Temperature; ℃)	28	32	35

bacterial 16S-rRNA gene was amplified using the primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-TACGGCTACCTTGTTACGACTT-3'). The reaction mixture was set up on ice as follows: 5 µl 10 × Taq buffer (with Mg^{2+}), 3.5 µl DNTP, forward primer and reverse primer (each 1.5 µL), 0.5 µl Taq DNA polymerase, 2 µl template DNA, with the addition of ddH_2O up to 36 µl as the final volume. The PCR program was 94℃ denaturizing for 5 min, 94℃ denaturizing for 30 s, 57℃ annealing for 40 s, 72℃ extension for 90 s; these steps cycling a total of 32, and a final extension at 72℃ for 10 min. Subsequently, the PCR product was separated by 1% agar-gel electrophoresis, and the band of expected size was cut off and purified with purification kit. The expected bands were sequenced by Huada Genomic Company (Beijing, China).

Sequence identification was initially estimated by using the BLAST facility of the National Center for Biotechnology Information. All available subsets of 16S-rRNA gene sequences were selected, analyzed and aligned with CLUSTALX 1.8.

Optimal conditions to remove sulfate with *Citrobacter freundii* CZ1001

Preparation of inoculum's suspension

The reselected *C. freundii* CZ1001 was first inoculated into a tube which contained the enrichment culture medium for SRB, and then incubated in a vacuum desiccator at 32℃ with not more than 0.055 MPa for two days. Subsequently, a sterile transfer loop was used to scrape a few loops of pure inoculums. The inoculums were well dispersed in 50 ml physiological saline solution (0.85%, w/w) after been shaken in a vibrator. Through the physiological saline solution, the concentration of inoculums was adjusted as 10^6 to 10^7 cfu/ml (colonies formed units per milliliter).

Sulfate removal with C. freundii at different conditions

C. freundii was inoculated into tubes containing the simulated tannery wastewater at different conditions of the orthogonal test (Table 1). After incubating it for 48 h, the effect of sulfate removal was detected with the removal rate of sulfate as the guide line. The detection of SO_4^{2-} was done according to the method of ISO 22743:2006 (ISO 22743:2006).

Removal of sulfate from the actual tannery wastewater through inoculation of the isolate

C. freundii CZ1001 was inoculated into 3000 ml of the liquid enrichment culture medium for SRB. After 36 h, the SRB grown in the medium was pumped into the bottom of the UASB containing anaerobic activated sludge which was domesticated for several months and the sulfate was steadily removed. When *C. freundii* CZ1001 and the sludge were mixed adequately, the redundant liquid medium was discharged. Then, the actual tannery wastewater with 1069 mg/L of initial SO_4^{2-} concentration was added and the culture condition was adjusted to the optimal conditions for *C. freundii* to remove sulfate which meant pH 7.0, 32℃ and 5.0 of COD/SO_4^{2-}. After 48 h, the concentration of SO_4^{2-} in the UASB was analyzed. Before inoculating the SRB, the anaerobic activated sludge was used to remove sulfate from the actual tannery wastewater with the same conditions as the inoculated activated sludge. The effect of sulfate removal between the anaerobic activated sludge inoculating the SRB and that without inoculation of the SRB was thus compared.

RESULTS AND DISCUSSION

Isolation of SRB

According to isolation and purification, 50 strains of SRB were obtained, which were named SRB1# to SRB50#. After growth in the solid medium, these strains produced black FeS deposition which was made with S^{2-} in the grown colonies and Fe^{2+} in the medium.

Reselection of SRB

After the isolated 50 strains of SRB grew in the liquid enrichment culture medium for three days, they were all in a certain concentration of the S^{2-} that was produced. In the process of deoxidizing SO_4^{2-} in the medium, the produced S^{2-} for SRB15# was 682.42 mg/L, which was higher than the other 49 strains evidently (not shown in Tables or Figures). It indicates that the ability to transfer SO_4^{2-} to S^{2-} was the best for SRB15#. Thus, we hope that SRB15# will be applied to the treatment of sulfate removal in tannery wastewater in order to advance the removal ability of sulfate. Therefore, SRB15# needs to be further discussed.

Morphological characteristics of the isolates

The SRB15# was gram-negative (G^-) from the gram reaction, and the cells were short rod shaped with the size of about 0.4 × 0.9 µm as shown in Figure 1. In vacuum conditions, the colonies that are 1 mm in diameter

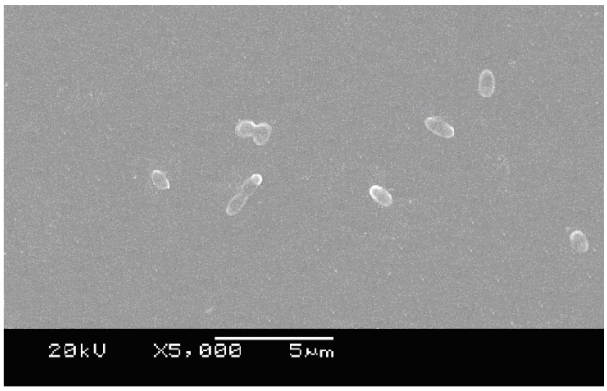

Figure 1. SEM image of the SRB15# (× 5000 times).

Table 2. Physiological and biochemical characters of the strain SRB15#.

Physiological and biochemical character	Reaction
Glycolysis test	
Saccharose	+↑
Dextrose	+↑
Chorogi alcohol	+↑
H_2S production test	+
Indole test	+
Methyl red test (M. R test)	+
Acetylmethyl carbinol test (V. P test)	-
Phenotypical characteristics	-
Contact enzyme test	+
Gelatin liquification test	+

"+".positive reaction; "-", negative reaction; "↑", produced gas.

were round and had a raised dark center. Also, they had an orderly slick edge when the strain grew for 36 h on the solid medium.

Physiological and biochemical characters

The physiological and biochemical characters of the SRB15# are shown in Table 2. As can be seen from the glycolysis test, the SRB15# could utilize saccharose, dextrose and chorogi alcohol as carbon and energy sources. Through the H_2S production test, it was found that the isolate could decompose sulf-organic compound and produce H_2S. From the indole test, the SRB15# contained tryptophan enzymes and could decompose tryptophan in protein. In the methyl red test, it fermented dextrose to produce acid in the process of the glycolytic metabolism. Through the contact enzyme test, it catalysed and decomposed H_2O_2 to H_2O and O_2 which indicate that the SRB15# was not strictly anaerobic, but could grow in aerobic conditions. Besides, the isolate had the ability to decompose protein in the gelatin liquification test. However, the isolate could not ferment dextrose to produce acetylmethyl carbinol from the V. P test and could not produce amylase from the phenotypical characteristics. Integrating all the physiological and biochemical characters, the SRB15# was *Citrobacter* referring to the Bergey's Manual (Buchanan and Gibbons, 1984).

Genetics characteristics of the isolate

Species-specific identification for the SRB isolate could be obtained using 16S-rRNA sequence analysis. An approximately 1450 bp sized-fragment of the 16S-rRNA gene of the isolate was amplified and sequenced. Sequence analysis of the 16S-rRNA gene and BLAST sequence comparison (http://blast.ncbi.nlm.nih.gov/Blast.cgi) identified that the isolated SRB15# was *C. freundii*, which was then named *Citrobacter freundii* CZ1001, as there was a similarity of 99.5% with *Citrobacter freundii* SA-A5-89 (GenBank accession NO. is AF025365.1).

It is known that *C. freundii*, which is a gram-negative,

Table 3. Results of the orthogonal test for *C. freundii* CZ1001

Number	A (Initial SO_4^{2-} concentration)	B (COD/SO_4^{2-} ratio)	C (pH)	D (Temperature)	Removal rate of SO_4^{2-}(%)
1	1	1	1	1	56.12
2	1	2	2	2	80.15
3	1	3	3	3	71.31
4	2	1	2	3	53.33
5	2	2	3	1	54.97
6	2	3	1	2	83.05
7	3	1	3	2	46.67
8	3	2	1	3	51.49
9	3	3	2	1	76.73
I/3	69.19	52.04	63.55	62.61	
II/3	63.78	62.20	70.07	69.96	
III/3	58.30	77.03	57.65	58.71	
range	10.89	24.99	12.42	11.25	
Optimum	A1	B3	C2	D2	

non-strict anaerobic species and which produces H_2S, belongs to the *Citrobacter* of Enterobacteriaceae (Buchanan and Gibbons, 1984). This is accorded with the morphological, physiological and biochemical characters of the SRB15#.

At present, *C. freundii* is not included in the class of SRB because there are few studies about *C. freundii* removal of sulfate from wastewater. Until now, there has been no report about the removal of sulfate with *C. freundii*. Therefore, it will be an innovative method if *C. freundii* is used to remove sulfate in wastewater especially in tannery wastewater.

Optimal conditions for sulfate removal with *C. freundii* CZ1001

As can be seen from Table 3, the optimal conditions used to remove sulfate in the simulated tannery wastewater for *C. freundii* CZ1001 was $A_1B_3C_2D_2$, which meant that the removal effect of sulfate for *C. freundii* CZ1001 reached the best when it was temperature was 32°C, pH was 7.0, COD/SO_4^{2-} was 5.0 and of the initial SO_4^{2-} concentration was 1500 mg/L. Among the factors, the COD/SO_4^{2-} had the biggest influence on the removal of sulfate, and the initial SO_4^{2-} concentration had the least. The optimal conditions used to remove sulfate in the simulated tannery wastewater may also contribute to the sulfate removal of the actual tannery wastewater because the former can simulate the actual tannery wastewater properly.

Removal of sulfate from the actual tannery wastewater through inoculation of the isolate

The removal rate of sulfate reached 89.66% after treatment through inoculation of the *C. freundii* CZ1001 to the anaerobic activated sludge when the initial SO_4^{2-} concentration was 1069 mg/L in the actual tannery wastewater after 48 h. The removal rate of sulfate (89.66%) was 12.13% higher than that of the treated by the anaerobic activated sludge without inoculating the isolate (77.53%), which indicate the advantages of inoculating the *C. freundii* CZ1001. It therefore shows that there was an increase in the removal rate of sulfate from the actual tannery wastewater through inoculation of the isolated *C. freundii*, which thus indicate that the isolate is an efficient SRB used to remove sulfate.

Conclusion

There is an efficient SRB used for removing sulfate from tannery wastewater. As such, *C. freundii* CZ1001 was screened by the anaerobic activated sludge through enrichment, isolation, purification, reselection and identification. Through optimizing its conditions to remove sulfate and applying it to the actual tannery wastewater, it was found that the removal rate of sulfate from actual tannery wastewater was higher after inoculating the isolated SRB. The experiment demonstrates that *C. freundii* could be selected as a new biomaterial to remove sulfate from tannery wastewater.

ACKNOWLEDGEMENTS

This work was financially supported by the Startup Project of the Scientific Research for Sichuan University of Science and Engineering (Item No. 2010XJKRL003) and the Key Project of Chinese Ministry of Education (Item No. 211161).

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