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# Screening and Identification of Nitrate-Reducing, Sulfide-Oxidizing Bacteria and their Characteristics

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***Abstract*—To develop microorganism technology for removing SO2 and NOx from flue gas, this study sieved out a strain of nitrate-reducing, sulfide-oxidizing Paracoccus sp. ZGL1 from activated sludge and analyzed its phylogenetic characteristics. The optimal initial pH for the growth and removal of substrate of this strain is 7.0; the optimal temperature is 30 ℃; the optimal rotating table speed is 50 r/min; the optimal substrate concentration is 10 mmol/L; and the best concentration of carbon source is 10 mmol/L. A preliminary analysis on the law of transforming thiosulfate and nitrate-nitrogen in systems under aerobic and anaerobic conditions was conducted.**

***Keywords-nitrate-reducing, sulfide-oxidizing bacteria; screening and identification; characteristics of microorganism***

## I. INTRODUCTION

Controlling $SO_2$ and NOx emissions from fuel combustion effectively has become the focus of world energy and environmental research since air pollution has worsened. Currently, $SO_2$ and NOx are treated separately, resulting in a large work area requirement, the need for complicated equipment, and high investment and operation costs. The simultaneous desulfurization-denitrification microorganism technology, which is characterized by its simplicity, lower operation costs, availability of byproducts, and good technology performance, is currently one of the flue gas treatment technologies being developed [1].

Simultaneous biological removal of $SO_2$ and NOx from flue gas is carried out using sulfate-reducing bacteria (SRB) and nitrate-reducing, sulfide-oxidizing bacteria (NR-SOB). SRB transforms $SO_2$ to sulfide. NR-SOB uses sulfide as the electron donor, whereas it uses NO as the electron acceptor. While sulfide is oxidized as sulfate, NO is reduced to $N_2$. The desulfurization reaction products can be controlled as elemental sulfur by adjusting the reaction conditions to reduce pollution and to obtain economic benefits [2]. Dasu et al. [3] used SRB with *Thiobacillus denitrificans* in a reactor for 24 h to oxidize $SO_2$ in flue gas to sulfate. $SO_2$ and NOx are then removed separately in two and single reactors using *Desulfovibrio desulfuricans* and *T. denitrificans*, respectively. The study found that *D. desulfuricans* is damaged by $O_2$ in flue gas, and $SO_2$ removal is inhibited by NO [4]. Because $O_2$ in flue gas limits the growth of SRB and inhibits NO reduction by NR-SOB, so $SO_2$ and NO removal from flue gas directly through biological processes is unlikely. $SO_2$ and NO in gaseous phase should be dissolved into the solution for further biological treatment. In wastewater disposal research, more studies on sulfate removal by SRB [5] and simultaneous denitrification (i.e., nitrate and nitrite) and desulfurization (i.e., sulfide, elemental sulfur, and thiosulfate) by NR-SOB have been conducted [5, 6]. These studies established a good foundation for research in simultaneous desulfurization-denitrification of industrial waste gas using microorganism.

This study sieved out and identified a strain of NR-SOB. The optimal condition for its growth and substrate removal was obtained using single factor experiment. The transformation law of thiosulfate and nitrate-nitrogen in a system under aerobic and anaerobic conditions was analyzed. This study provides an opportunity to develop simultaneous desulfurization-denitrification microorganism technology.

## II. MATERIALS AND METHODS

### A. Growth medium and condition of culture

The culture of NR-SOB in this study was based on the available literature [7]. The objective of this study is to analyze sodium thiosulfate as the source of sulfur for the bacteria because thiosulfate is more stable and easier to analyze than sulfide. In general, NR-SOB are facultative anaerobes. Aerobic culture method was used to screen and identify the strain and study the bacterial characteristics. Aerobic and anaerobic methods were conducted to observe the desulfurization-denitrification law of the strain.

### B. Screening and culture of bacterial strain growth

A deflocculation agent was added into a moderately activated sludge from a sewage treatment plant. The sludge suspension was obtained and inoculated into a conical bottle with culture medium. Inoculation was repeated, and culture accumulation was conducted to remove thiosulfate effectively. The cultures were alternated four times to dilute the spreading on the solid culture medium. A single colony was selected based on the characteristics of the colonies to be cultured continuously. A strain of purified NR-SOB was obtained after repeated cultures.

### C. Strain identification

The morphological, physiological, and biochemical characteristics of the bacteria were identified [8]. Gene extraction and sequencing were made by the Takara Biotechnology (Dalian) Co., Ltd. The known nucleotide sequences of 16S rRNA in NCBI and GenBank were identified using Blast analysis to search for homologous sequences. The multiple sequence alignment was constructed using ClustalX 2.0, and the phylogenetic tree was created using Mega4.1.

### D. Study on the characteristics of microorganism

For aerobic culture, 100 mL of culture medium prepared on an air-bath thermostat table was added into a 250 mL conical bottle and sampled to analyze OD and thiosulfate concentration in logarithmic phases.

### E. Simultaneous desulfurization-denitrification experi-ment of NR-SOB

For anaerobic culture, 100 mL of culture medium was added into a serum bottle, inoculated with high-purity nitrogen statically in a thermostat incubator, and sampled to analyze the nitrate-nitrogen and thiosulfate concentrations in logarithmic phases. For aerobic culture, refer to the section D.

### F. Analytical method

Cell concentration measurement: OD value was measured when NR-SOB were located at 420 nm (JASCO, V560 UV Spectrophotometer)

Measurement of thiosulfate: potassium dichromate method [9]

Measurement of nitrate-nitrogen: ultraviolet spectro-photometry [10]

## III. Results and Discussion

### A. Screening identification of strain

This research sieved out a strain of NR-SOB from activated sludge in sewage treatment plant. Under the optical microscope, it was found that the thallus are short, rod-shaped, arranged in singles or pairs, actively moving, and gram-negative. On the solid culture medium, the thallus are pinpoint-like in size, circular, transparent, damp, and flat with smooth edges during the early stages of development. Upon maturity, its diameter becomes ~1 mm.

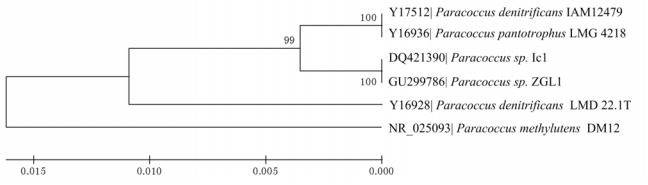


Figure 1. Phylogenetic tree of Paracoccus sp. ZGL1 based on the 16S rRNA gene order homology

The length of 16S rRNA of ZGL1 is 1294 bp (GenBank serial number GU299786). Blast analysis determined that the homeology between ZGL1 and *Paracoccus sp.* is the highest. The strain is identified as *Paracoccus denitrificans*. Fig. 1 shows the phylogenetic dendrogram of ZGL1.

### B. Effect of environmental factor on the growth of P. denitrificans and its desulfurizing ability

The objective of this study is to observe the effect of temperature, initial pH value, and rotating table speed on the growth of the strain and its desulfurizing ability using single factor experiment (Fig. 2).

Temperature has a certain effect on the growth of the strain and its desulfurizing ability. The optimal growing temperature for the strain is ~ 30–35 ℃, at which point it grows very well and its OD value is maximum. However, the growth of the strain is inhibited under different temperatures. Temperature effect on the metabolic processes of the strain refers to the effect of temperature on the characteristics of the enzyme excreted from the endo- and extra-thallus. Different temperatures are required at different reaction processes of the enzyme. An unfavorable temperature will affect the activity of the enzyme directly, thus leading to metabolic processing of the bacteria [11, 12]. The thiosulfate removal efficiency of the strain is up to 80% at 30 °C, whereas the utility ratio of the strain for the substrate is small.

pH has an effect on the growth of the microorganism. The interaction between the hydrogen ions and the enzyme on the membrane of the cytoplasm and cytoderm will result in enzyme inactivation, thus affecting the growth of the enzyme [13]. The favorable initial pH value for the growth of the strain is from 5.0 to 8.0 (Fig. 2). The optimal initial pH value is 7.0，indicating that a neutral environment is fit for the growth and reproduction of the strain. The thiosulfate removal efficiency is different in different pH values. At pH 7.0, thiosulfate removal efficiency is the highest at 83%. OD is higher if acidity is higher (pH = 4.0) because the suspension is formed from the white precipitate from the culture medium.

Oxygen, as an electron acceptor under bacterial aerobic respiration, is essential when microorganisms decompose organic substances to obtain energy and nutrition [11]. Conventional rotating table supplies air to the liquid culture medium continuously during aerobic respiration to keep the concentration of dissolved oxygen. The optimal rotating table speed is 50 r/min. OD drops when the rotating table speed is not 50 r/min. The thiosulfate removal efficiency is consistent with OD.

Therefore, the optimal environmental conditions for the growth and substrate removal of *P. denitrificans* are as follows: 30 ℃, initial pH = 7.0, and rotating table speed 50 r/min.

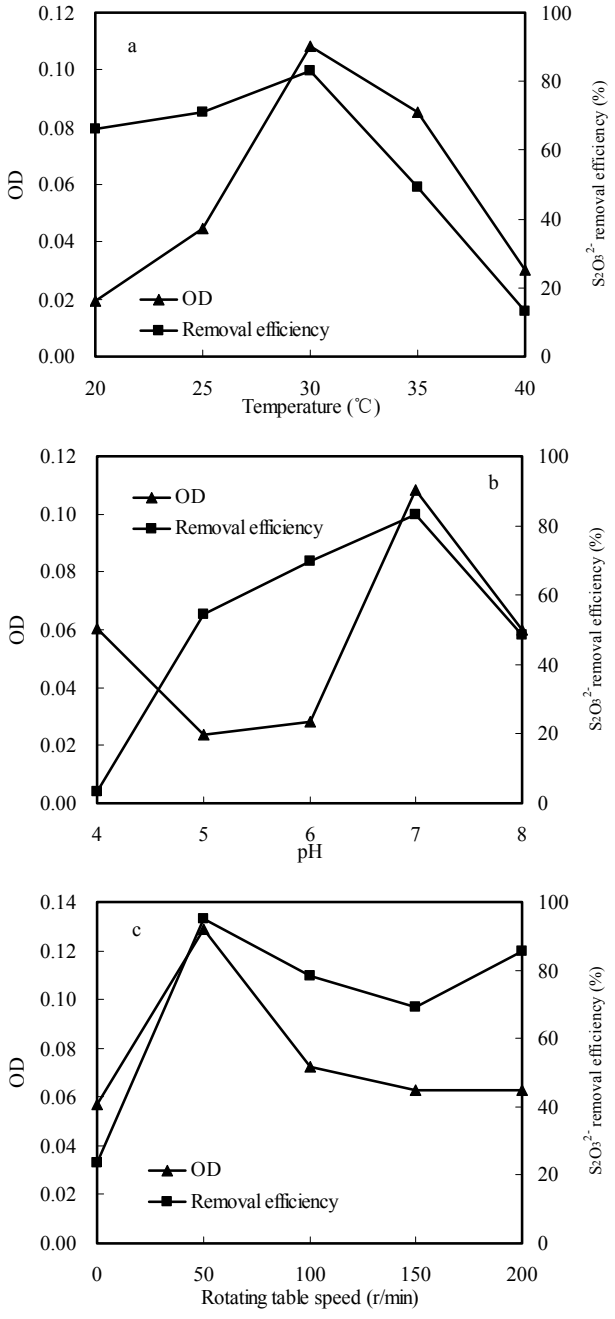


Figure 2. Effect of temperature, initial pH, and rotating table speed on the growth of the strain and its desulfurizing ability (a: aerobic, initial pH=7.0, rotating table speed=150 r/min; b: aerobic, T=30 ℃, rotating table speed=150 r/min; c: aerobic, T=30 ℃, initial pH=7.0)

### C. *Effect of concentration of thiosulfate and inorganic carbon sources on the growth of P. denitrificans and its desulfurizing ability*

This study analyzed the effect of different thiosulfate concentrations on the growth of *P. denitrificans*. The OD and the thiosulfate removal efficiency are the highest when the

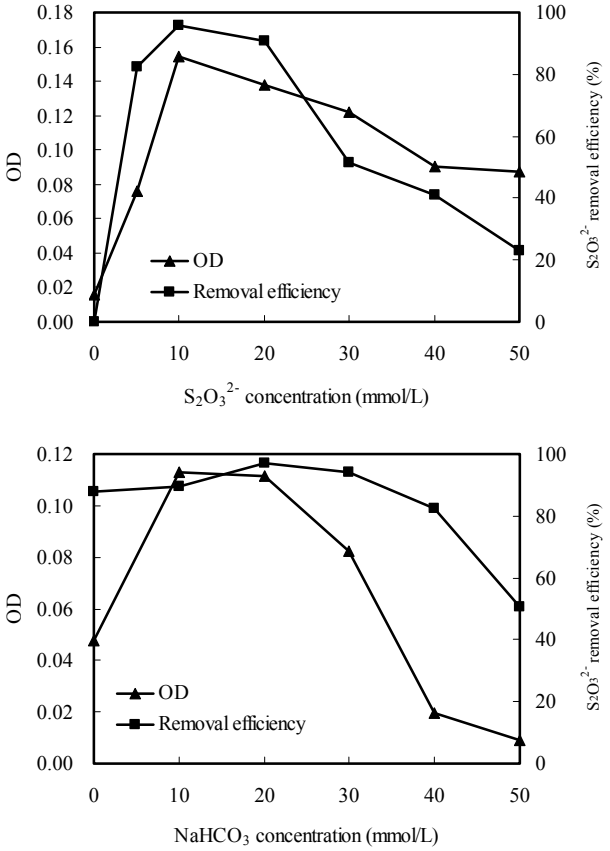


Figure 3. Effect of initial concentration of S2O32- and NaHCO3 on the growth of the strain and its desulfurizing ability (aerobic, T=30 ℃, initial pH=7.0, rotating table speed=50 r/min)

concentration of the substrate is 10 mmol/L (Fig. 3). The thiosulfate removal efficiency of *P. denitrificans* is higher when the concentration of the substrate is from 5–20 mmol/L. When the concentration of the substrate is 5 mmol/L, the breeding of the bacteria is limited due to the lesser supply of energy. There is a slip between OD and the removal efficiency when the concentration of the substrate is too high (> 30 mmol/L).

*P. denitrificans* is an autotroph, and it can synthesize its cell components using $CO_2$ and $HCO_3^-$ as its carbon source. The experiment was carried out on the same air-bath table with the same $CO_2$ content. The objective of this study is to analyze the effect of different concentrations of $HCO_3^-$ on the growth of the strain and its desulfurizing ability by adjusting $HCO_3^-$ concentration. The growth condition of the strain is not proportional to the quantity of the carbon source. When the $NaHCO_3$ concentration is less than or equal 30 mmol/L, the thiosulfate removal efficiency of *P. denitrificans* are the same. The OD is lower when $NaHCO_3$ concentration is 0 or greater than 30 mmol/L. There is no direct relationship between thiosulfate removal efficiency and carbon source contents at the same carbon source concentration. Thiosulfate removal efficiency is highest when sodium bicarbonate concentration is 20 mmol/L.

However, too much sodium bicarbonate concentration inhibits the growth of the strain. In addition, the dosage of a small quantity of carbon source is inexpensive.

The growth of the strain is better, and its desulfurizing ability is higher when sodium bicarbonate concentration is 10 mmol/L. The optimal concentration of the substrate is 10 mmol/L of thiosulfate, whereas the optimal concentration of the carbon source of the strain is 10 mmol/L of $NaHCO_3$.

### *D. Simultaneous desulfurization-denitrification experi-ment of P. denitrificans*

To study the simultaneous desulfurization-denitrification process of *P. denitrificans*, the changing nitrate-nitrogen and thiosulfate concentrations under aerobic and anaerobic conditions were observed (Tab. Ⅰ). The reduced quantity of nitrate-nitrogen (19.32 mmol/L) in the culture medium is similar to that of thiosulfate (19.44 mmol/L) under anaerobic conditions after 48 h. The reduced quantity of nitrate-nitrogen (5.10 mmol/L) in the culture medium is lesser than that of thiosulfate (18.32 mmol/L) under aerobic conditions.

*P. denitrificans* uses $O_2$ as the electron acceptor under aerobic conditions, whereas it uses nitrate as the electron acceptor under anaerobic conditions. *P. denitrificans* transfers electrons between nitrate and thiosulfate to obtain energy through a series of enzyme catalytic actions in the bacteria. The simultaneous desulfurization-denitrification is completed while the cell is growing, as shown in (1) [14]:

$$5S_2O_3^{2-}+8NO_3^-+H_2O\rightarrow 4N_2+10SO_4^{2-}+2H^+ \quad (1)$$

Similar to *T. denitrificans*, (2) can supply more energy to bacteria under aerobic conditions, thus the consumption of nitrate-nitrogen and thiosulfate is less in 24 h. After 24 h, concentration of thiosulfate is decreased and small quantities of $NO_3^-$ are used with elemental sulfur generation and the increasing running level of (3). The transformation of thiosulfate is accelerated with the consumption of elemental sulfur [15]:

$$S_2O_3^{2-}+1.5O_2+2H_2O\rightarrow 2SO_4^{2-}+4H^+ \quad \triangle G=-418.7 \text{ kJ/mol} \quad (2)$$

$$5S^0+6NO_3^-+8H_2O\rightarrow 5H_2SO_4+6OH^-+3N_2$$

$$\triangle G=-55.3 \text{ kJ/mol} \quad (3)$$

TABLE I. SIMULTANEOUS DESULFURIZATION-DENITRIFICATION OF P. DENITRIFICANS

| Time (h) | $NO_3^-$N (mmol/L) | | $S_2O_3^{2-}$ (mmol/L) | |
|---|---|---|---|---|
| | *Aerobic* | *Anaerobic* | *Aerobic* | *Anaerobic* |
| 0 | 31.30 | 32.88 | 18.32 | 19.44 |
| 24 | 32.53 | 17.80 | 14.01 | 0.00 |
| 48 | 26.20 | 13.56 | 0.00 | 0.00 |

## IV. CONCLUSIONS

1) This study sieved out a strain of NR-SOB, *Paracoccus sp.* ZGL1 (GenBank serial number: GU299786), from activated sludge. The facultative aerobic bacteria strain is identified as *P. denitrificans*, which can grow in an inorganic salt medium.

2) Using the single factor experiment, the optimal condition for its growth and substrate removal is determined as follows: 30 ℃, pH = 7.0, rotating table speed 50 r/min, substrate concentration 10 mmol/L, and carbon source concentration 10 mmol/L.

3) Under aerobic conditions, *P. denitrificans* uses oxygen as an electron acceptor, and its consumption of nitrate-nitrogen and thiosulfate is less in 24 h. After 24 h, concentration of thiosulfate is decreased and small quantities of $NO_3^-$ are used with elemental sulfur generation. Under anaerobic conditions, *P. denitrificans* uses nitrate as an electron acceptor, and simultaneous desulfurization-denitrification is completed while the cell is growing. The reduced quantity of nitrate-nitrogen is similar to that of thiosulfate.

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