Isolation, characterization and S\(^2\)-oxidation metabolic pathway of a sulfur-oxidizing strain from a black-odor river in Beijing

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**ABSTRACT**

A single strain capable of efficient S\(^2\)-oxidizing was isolated from a black-odor river in Beijing. The single strain was identified as *Stenotrophomonas* through the physiology and biochemical characteristics as well as the 16S rRNA sequencing experiment. This strain was named as *Stenotrophomonas* sp.sp3 (strain sp3). The experimental results showed that for the strain sp3 growth and S\(^2\) oxidation, the optimal conditions were as follows: 25°C of temperature, initial pH 7, 2.5 g/L of initial glucose concentration and 1.00 g/L of initial cell concentration. It was found that there were 31 kinds of sulfur oxidation related genes in the strain sp3 through the whole genomic analysis. The results of the transcriptome analysis suggested that the main metabolic pathway of S\(^2\) to SO\(_4^{2-}\) was the paracoccus sulfur oxidation process. The bioconversion processes of S\(^2\) to S\(^0\), S\(^2\) to SO\(_3^{2-}\), S\(_2\)O\(_3^{2-}\) to S\(^0\) and SO\(_3^{2-}\) to SO\(_4^{2-}\) were controlled by *hdrA*, *cysIJ*, *tst* and *sox* gene, respectively.

**Key words:** black-odor river, metabolic pathway, oxidation characteristics, S\(^2\)-oxidizing bacteria, Sulfide

**HIGHLIGHTS**

- This study found a novel SOB i.e. *Stenotrophomonas* sp. sp3 that was capable of sulfur oxidation.
- It was proved that S\(^2\) could be converted into SO\(_4^{2-}\) via the paracoccus sulfur oxidation process.

**GRAPHICAL ABSTRACT**

**INTRODUCTION**

Recently, the black-odor water problem has become a very urgent issue in China (Song et al. 2017). Black-odor water is defined as a heavily polluted river or lake that presents with black or green colors and simultaneously gives off an unpleasant smell (Cao et al. 2020). Black-odor pollution not only led to a serious imbalance of water eco-environment but also has a negative impact on the surrounding residential environment (Zhang et al. 2019). Previous research indicated that sulfide (S\(^2\)-) is the key blacking pollutant is because S\(^2\)- can react with some metal ions (such as Fe\(^{2+}\), Mn\(^{2+}\), Cu\(^{2+}\)) and generate metal sulfides. Metal sulfides are suspended in overlying water and cause river to become black (Song et al. 2017). Oxidizing...
S\textsuperscript{2–} into SO\textsubscript{4}\textsuperscript{2–} was proved as effective processes to alleviate even eliminate water blacking (Gagol et al. 2019). This process is mainly performed by sulfur-oxidizing bacteria (SOB) in water and sediment. However, the rate of S\textsuperscript{2–}-oxidizing is very slow under the action of native SOB in the water body, especially in black-odor water (Cai et al. 2019). To improve S\textsuperscript{2–}-oxidizing efficiency, one of the most promising and feasible approaches is to add the SOB into the water body, which can obviously accelerate the bioconversion of S\textsuperscript{2–} to SO\textsubscript{4}\textsuperscript{2–}.

SOB has been discovered worldwide. Many strains have been isolated and characterized, and their application in water pollution treatment have been reported. A bioreactor based on SOB application was used to detect the heavy metals of wastewater (Eom et al. 2019). Several technologies using SOB as the biofilm in a moving bed reactor have been reported. For example, SOB biofilm could remove S\textsubscript{2}O\textsubscript{3}\textsuperscript{2–} and NO\textsubscript{3}\textsuperscript{–} in the wastewater (Khanongnuch et al. 2019). SOB biofilm is also used to remove hydrogen sulfide which is the by-product of the anaerobic digestion process of organic wastewater (Lestari et al. 2016). However, there are few studies applying and evaluating SOB in the black-odor water treatment. It was found that the optimum temperature and pH of each kind of SOB are of great difference (Pokorna & Zabranksa 2015). These differences indicated that there might be different metabolic pathways among different SOB (Ghosh & Dam 2009).

This study aimed to find a SOB that has the ability of oxidizing S\textsuperscript{2–} into SO\textsubscript{4}\textsuperscript{2–}, and attempt to explore its S\textsuperscript{2–}-oxidizing pathway. Firstly, a single strain with S\textsuperscript{2–}-oxidizing ability was isolated from a black-odor river located in Beijing of China, and this strain was identified by the physiology and biochemical characteristics as well as the 16S rRNA sequence. Secondly, the optimal temperature, pH, nutrient condition and initial cell concentration for the growth and S\textsuperscript{2–}-oxidizing of the isolated strain were investigated, respectively. Subsequently, the growth curve and S\textsuperscript{2–}-oxidizing curve of this isolated strain were then analyzed, and S\textsuperscript{2–}-oxidizing process was also investigated by detecting each inorganic sulfur compound in the whole process. Finally, S\textsuperscript{2–}-oxidizing metabolic pathway of this strain was explored using both of genome and transcriptome analysis.

**MATERIALS AND METHODS**

Source of the isolated strain

A single strain was isolated from the mixture of sludge and water that were obtained from the Dongsha river (a heavily polluted black-odor river) located in Beijing, China. The water was collected from surface layer water of the Dongsha river. The sludge was collected from a depth of 0.5–1.0 m underwater using a sediment sampler. All samples were collected from the Dongsha river at a temperature of 25 °C in September. There were some cyanobacteria floating on the surface of the black-odor water body, no fish, and the water body was stagnant. The mean values of S\textsuperscript{2–}, TP, NH\textsubscript{3}-N, COD and DO in the water samples were 11.7, 1.3, 3.6, 47 and 2.0 mg/L.

Culture medium

In order to enrich the initial culture, the composition of enrichment medium was as follows: 0.162 g/L Na\textsubscript{2}S·9H\textsubscript{2}O, 0.1 g/L KNO\textsubscript{3}, 0.05 g/L K\textsubscript{2}HPO\textsubscript{4}, 0.05 g/L NaCl, 0.1 g/L MgSO\textsubscript{4}·7H\textsubscript{2}O, 0.001 g/L FeSO\textsubscript{4}, 2 g/L soluble starch and 20 g/L agar. In order to screen the strain, the isolation medium was prepared, and the following composition was used: 0.162 g/L Na\textsubscript{2}S·9H\textsubscript{2}O, 0.05 g/L NaCl, 0.1 g/L KNO\textsubscript{3}, 0.05 g/L MgSO\textsubscript{4}·7H\textsubscript{2}O, 0.002 g/L FeSO\textsubscript{4}·7H\textsubscript{2}O, 0.05 g/L K\textsubscript{2}HPO\textsubscript{4}, 20 g/L glucose, 10 g/L peptone, 5 g/L yeast extract and 20 g/L agar. The liquid growth medium was prepared to incubate the isolated strain, and its composition was as follows: 20 g/L glucose, 10 g/L peptone and 10 g/L yeast extract. The pH values of culture mediums including the enrichment, isolation and liquid growth medium were all adjusted to 7.0 by 1 M HCl or 1 M NaOH. Sterilization was subsequently performed by autoclaving at 121 °C for 20 min.

Artificial S\textsuperscript{2–}-containing wastewater

The composition of artificial S\textsuperscript{2–}-containing wastewater was as follows: 2.50 g/L glucose, 0.40 g/L K\textsubscript{2}HPO\textsubscript{4}, 1.00 g/L NH\textsubscript{4}Cl and 0.162 g/L Na\textsubscript{2}S·9H\textsubscript{2}O (Zhuang et al. 2017). The pH value was adjusted to 7.0 by 1 M HCl or 1 M NaOH. Sterilization of the artificial wastewater was then carried out at 121 °C for 20 min.

Enrichment and isolation of single strain

The suspension of the mixture sample of sludge and water was performed by adding 5 mL of the sample into 500 mL asepsis NaCl solution with mass concentration of 5%. A small drop of the suspension was spread on the enrichment medium and incubated at 25 °C for 2–3 days. Then the colonies on the enrichment medium were inoculated on the isolation medium and incubated at 25 °C for 2–3 days. The strain was purified by repeating single colony re-isolation on the isolation medium.
Preparation of microbial inoculum

The isolated strain was incubated at 25 °C for 48 hr to obtain the microbial inoculum. After 48 hr of cultivation, the cell concentration of the microbial inoculum reached $2.5 \times 10^8$ cfu/mL. More microbial inoculum was obtained by transferring the original microbial inoculum to other 250 mL erlenmeyer flask with fresh 100 mL liquid growth medium at a volume percent of 5%, and was incubated at 25 °C on a rotary shaker at 120 rpm for 48 hr.

Experiment of different $S^2^-$-oxidizing conditions

Effects of temperature, initial pH, glucose concentration and initial cell concentration on the growth and $S^2^-$-oxidizing of the strain were investigated using the artificial $S^2^-$-containing wastewater. For the temperature experiment, the microbial inoculum was transferred to a 500 mL erlenmeyer flask with 200 mL artificial wastewater at a volume percent of 5% and was incubated at different temperatures (5, 15, 20, 25, 30 and 35 °C) on a rotary shaker at 120 rpm for 48 hr. For the initial pH experiment, the same experimental conditions were adopted except that the initial pHs of the artificial wastewater were respectively adjusted to 4, 5, 6, 7 and 8, and the temperature was kept at 25 °C. The glucose at different concentrations (0.5, 1.0, 2.5, 5.0 and 10 g/L) and initial cell of microbial inoculum at different concentrations (0.01, 0.10, 1.00, 2.00 and 5.00 g/L) were designed, and the experiment was conducted, respectively. Both the strain growth and the $S^2^-$ oxidation rate were measured after bio-reaction.

Experiment of $S^2^-$-oxidizing process

The experiment of the strain growth curve and $S^2^-$-oxidizing curve was performed by transferring the microbial inoculum to a 500 mL erlenmeyer flask with 200 mL artificial wastewater at a volume percent of 5%, and was incubated at 25 °C and 120 rpm for 72 hr. The strain growth and the concentration of $S^2^-$ were measured regularly.

The experiment of $S^2^-$-oxidizing process was carried out in a biochemical reactor with 5 L artificial wastewater at the cell concentration of 1 g/L, and a biochemical reactor without the microbial inoculum was set as a blank control. The incubation was performed at 25 °C and 120 rpm for 60 hr and the concentrations of $S^2^-$, $S^0$, $S_2O_3^{2-}$, $S_4O_6^{2-}$, $SO_3^{2-}$ and $SO_4^{2-}$ were measured regularly.

Identification of strain

DNA extraction and 16S rRNA sequencing

The total DNA of the strain was extracted using an ezup column type bacterial genomic DNA extraction kit SK8255 (Sangon Biotech., Shanghai, China). The extraction processes and steps were followed the manufacturer’s instructions of this kit. The concentration of total DNA was measured using a microfluorometer (TBS-380, Turner BioSystems, USA); while the purity of total DNA was analyzed by a UV microspectrophotometer (NanoDrop2000, Thermo Fisher, USA). The experiment of 16S rRNA sequencing was performed by Sangon Biotech. (Shanghai, China). For PCR, primers specific for 16S rRNA 27F (AGTTTGATCMTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) were used. The 16S rRNA of the strain was compared with other sequences in GenBank using the blast program of national center for biotechnology information (NCBI). Alignment with a representative set of related 16S rRNA gene sequences was carried out with the clustal-w program implemented in the phylogenetic analysis package mega (version 7.0).

Morphological, physiology and biochemical tests

For morphological characteristics (colony characteristics, shape, motility and gram’s reaction), the pure strain was cultured in the same media used for isolation at 25 °C for 2 d. The physiology and biochemical tests for strain identification were carried out following the standard method described by Dong & Cai (2002).

Genome sequencing and analysis

DNA extraction and sequencing

Genomic DNA was isolated using E.Z.N.A.® DNA kit (Omega Bio-Tek, USA). The isolated DNA was fragmented using a high performance sample processing system (Covaris M220, Thermo Fisher Scientific, USA). The PE library was constructed using TruSeq™ DNA sample prep kit (Illumina, USA). For bridge PCR, the hiseq PE cluster kit v4-cbot (Illumina, USA) was used. The genome was sequenced using the Illumina hiseq program.
Assembly and annotation of genomic sequence
The assembly of quality filtered reads was performed using IDBA-UD program. Automated annotation was performed using Glimmer 3.02 program. The annotated genes were compared with database Nr, Genes, String and Go.

RNA extraction and sequencing
The samples for RNA extraction were respectively collected in the experiment of $\text{S}_2\text{O}_3^{2-}$-oxidizing process at 1, 18, 30 and 60 hr. Total RNA from the collected samples was extracted using a trizol reagent (Invitrogen) kit (Thermo Fisher Scientific, USA). RNA purification was performed with deoxyribonuclease I (Thermo Fisher Scientific, USA) and ribo-zero™ rRNA removal kit (Thermo Fisher Scientific, USA). The mRNA was extracted using oligo (dT) 18 magnetic substance (Thermo Fisher Scientific, USA). The cDNA library was prepared using reverse transcription PCR method and the first-strand cDNA was synthesized using random hexamers and fragmented mRNA. For successful sequencing, the quality and size of the cDNA libraries were checked by an Agilent 2100 system. The cDNA library was sequenced using Illumina hiseq program.

Analytical method
The cell concentration during the growth of the strain was evaluated at 600 nm (OD600). According to the methylene blue spectrophotometric method, $\text{S}_2\text{O}_3^{2-}$ was measured at 665 nm by a spectrophotometer (Chinese National Environmental Protection Agency 1996); while $\text{S}_0^0$ was examined spectrophotometrically at 350 nm using the previous method (Miura et al. 1995). The concentrations of $\text{S}_2\text{O}_3^{2-}$, $\text{SO}_3^{2-}$ and $\text{SO}_4^{2-}$ were measured using an ion chromatograph (CIC-D120, Qingdao Shenghan Chromatography Technology, China). The concentration of $\text{S}_4\text{O}_6^{2-}$ was determined using a high performance liquid chromatograph (LC-10AD, Shimadzu, Shanghai, China).

RESULT AND DISCUSSION
Identification of the isolated strain
The physiology and biochemical characteristics of the isolated strain are shown in Table 1. The isolated strain formed round-shaped opaque yellow colony (0.5–1 mm in diameter) with ammonia smell; the edge of the colony was smooth and partial irregular. The center of the colony was bulge; the cell was motile rods and gram-stain negative. The phylogenetic tree of the isolated strain is shown in Figure 1. The results of 16S rRNA gene identification revealed that the strain was 99% homologous with genus Stenotrophomonas. According to the results of morphological observation, physiology and biochemical characteristics and 16S rRNA analysis, the isolated strain was identified as Stenotrophomonas sp. and named as Stenotrophomonas sp.sp3 (strain sp3). The sequence of the strain sp3 was uploaded to NCBI and the GenBank accession number was MH181796.

$\text{S}_2^{2-}$-oxidizing characteristics of the isolated strain
The effects of temperature, initial pH, initial glucose concentration and initial cell concentration on the growth of the strain sp3 and the $\text{S}_2^{2-}$-oxidizing rate were analyzed, as shown in Figure 2.

As shown in Figure 2(a), $\text{S}_2^{2-}$-oxidizing rate increased with the increase of incubation temperature from 5 °C to 25 °C. The $\text{S}_2^{2-}$-oxidizing rate by the strain sp3 under 5 °C was only 21% and the strain sp3 showed the maximum $\text{S}_2^{2-}$-oxidizing rate under 25 °C. The maximum $\text{S}_2^{2-}$-oxidizing rate was 85.2%. However, the $\text{S}_2^{2-}$-oxidizing rate decreased with the increase of incubation temperature from 25 °C to 35 °C, indicated that 25 °C was the most optimum incubation temperature of $\text{S}_2^{2-}$ oxidation. The

Table 1 | Physiology and biochemical characteristics of the isolated strain

| Items                              | Result |
|------------------------------------|--------|
| Gram stain                         | −      |
| Lysine decarboxylase test           | +      |
| Maltase test                        | +      |
| Nitrate reductase enzymes test      | +      |
| Esculin hydrolysis test             | +      |
| Gelatin liquefaction test           | +      |
Figure 1 | Phylogenetic tree of the isolated strain.

Figure 2 | Effects of (a) temperature, (b) initial pH, (c) initial glucose concentration and (d) initial cell concentration broth on the growth of strain sp3 and S²⁻-oxidizing rate.
growth curve of the strain sp3 at various temperatures showed the same tendency: increased with the increase of temperature from 5 °C to 25 °C and decreased with the increase of temperature from 5 °C to 25 °C. Thus, 25 °C was the optimal temperature for strain growth and \( S^{2^-}\)-oxidizing.

The strain growth and \( S^{2^-}\)-oxidizing varied significantly with the change of initial pH, as shown in Figure 2(b). It was found that the \( S^{2^-}\)-oxidizing rate increased with the increase of pH from 4 to 7, and then decreased with the increase of pH from 7 to 8. The maximum \( S^{2^-}\)-oxidizing rate was achieved at the initial pH of 7, and it reached 85% after 48 hr of bio-reaction. Variation of the growth of the strain sp3 at various initial pH was similar to \( S^{2^-}\)-oxidizing, indicated the initial pH 7 was the optimal for strain growth and \( S^{2^-}\)-oxidizing.

The effect of initial glucose concentration on the strain growth and \( S^{2^-}\)-oxidizing was shown in Figure 2(c). It was found that the \( S^{2^-}\)-oxidizing rate increased with the increase of initial glucose concentration from 0.5 to 2.5 g/L and decreased with the increase of initial glucose concentration from 2.5 to 10.0 g/L. The strain sp3 showed the maximum \( S^{2^-}\)-oxidizing activity at initial glucose concentration of 2.5 g/L and the maximum \( S^{2^-}\)-oxidizing rate was 83%. The strain growth curve showed that the growth of the strain sp3 increased with the increase of initial glucose concentration from 0.5 to 2.5 g/L, and then increased slowly when initial glucose concentration increased from 2.5 to 10.0 g/L. The above results indicated that glucose was a kind of efficient carbon resource for the strain sp3 and was able to accelerate strain growth and \( S^{2^-}\)-oxidizing, when the initial glucose concentration increased from 0.5 to 2.5 g/L. It was showed that 2.5 g/L was the optimal initial glucose concentration for strain growth and \( S^{2^-}\)-oxidizing.

As shown in Figure 2(d), variation of strain growth and \( S^{2^-}\)-oxidizing were different with the increase of initial cell concentration of the microbial inoculum. It was found that the \( S^{2^-}\)-oxidizing rate increased with the increase of initial cell concentration from 0.01 to 1.00 g/L, and then maintained stability. The strain sp3 showed the maximum \( S^{2^-}\)-oxidizing when the initial cell concentration was 1.00 g/L, and it reached 81.3%. The strain growth curve showed that the growth of the strain sp3 kept increasing with the increase of initial cell concentration of the microbial inoculum from 0.01 to 5.00 g/L. This result indicated that increasing the initial cell concentration of the microbial inoculum was efficient for \( S^{2^-}\)-oxidization. Thus, 1.00 g/L was the optimal initial cell concentration of the microbial inoculum for \( S^{2^-}\)-oxidizing efficiency.

Based on the above results, the optimal conditions including temperature, initial pH, initial glucose concentration and initial cell concentration were 25 °C, 7, 2.5 g/L and 1.00 g/L, respectively.

**Growth curve and \( S^{2^-}\)-oxidizing curve of strain sp3**

Growth curve and \( S^{2^-}\)-oxidizing curve of the strain sp3 were shown in Figure 3. In the first of 16 hr, the OD\(_{600}\) value of the strain sp3 increased slowly, indicated the first 16 hr was the lag phase of the strain growth. During the lag phase of the strain growth, the concentration of \( S^{2^-}\) decreased significantly, showing that the lag phase was the main phase for \( S^{2^-}\) oxidation. After 16 hr, the OD\(_{600}\) value of the strain increased significantly until the 35 hr, indicating that the logarithmic phase of the strain growth was appeared in the range of 16 to 35 hr. During the logarithmic phase, the concentration of \( S^{2^-}\) kept
decreasing and reached the minimum value at 35 hr. The minimum value of S\textsuperscript{2−} concentration was 2.9 mg/L and the S\textsuperscript{2−} oxidation rate reached 86.6%. The OD\textsubscript{600} value of the strain maintained stability from 35 to 60 hr indicated that the stationary phase of the strain growth was in the range of 35 to 60 hr. In the last 12 hr, the OD\textsubscript{600} value of the strain sp3 decreased rapidly, indicated this period was the decline phase of the strain growth. During the stationary phase and the decline phase of the strain growth, the concentration of S\textsuperscript{2−} maintained stability.

**S\textsuperscript{2−}-oxidizing process of strain sp3**

Each inorganic sulfur compound including S\textsuperscript{2−}, S\textsuperscript{0}, S\textsubscript{2}O\textsubscript{3}\textsuperscript{2−}, S\textsubscript{4}O\textsubscript{6}\textsuperscript{2−}, SO\textsubscript{3}\textsuperscript{2−} and SO\textsubscript{4}\textsuperscript{2−} in the whole process of S\textsuperscript{2−} oxidation were detected, and the results were showed in Figure 4. Four inorganic sulfur compounds (S\textsuperscript{0}, S\textsubscript{2}O\textsubscript{3}\textsuperscript{2−}, SO\textsubscript{3}\textsuperscript{2−} and SO\textsubscript{4}\textsuperscript{2−}) were formed in the S\textsuperscript{2−}-oxidizing process and their concentrations varied significantly at different phase of the S\textsuperscript{2−}-oxidizing process. As shown in Figure 4(a), as the main electron donor in the S\textsuperscript{2−}-oxidizing process, the concentration of S\textsuperscript{2−} decreased significantly in the first 10 hr. Then the decreasing rate of S\textsuperscript{2−} concentration slowed in the range of 10 to 30 hr. At 30 hr, the concentration of S\textsuperscript{2−} reached the minimum value and maintained stability. The minimum value of S\textsuperscript{2−} concentration was 3.0 mg/L and the S\textsuperscript{2−} oxidation rate reached 86.1%. Variation of the concentrations of S\textsuperscript{0} and SO\textsubscript{3}\textsuperscript{2−} showed a wave-type tendency during the whole S\textsuperscript{2−}-oxidizing process, indicated the dynamic synthesis and decomposition of the two compounds in the S\textsuperscript{2−}-oxidizing process. The maximum value of S\textsuperscript{0} concentration was 1.75 mg/L at 18 hr, and the maximum value of SO\textsubscript{3}\textsuperscript{2−} concentration was 1.78 mg/L at 35 hr. The concentration of S\textsubscript{2}O\textsubscript{3}\textsuperscript{2−} increased in the first 8 hr and then turned to decrease, indicated that the synthesis rate of S\textsubscript{2}O\textsubscript{3}\textsuperscript{2−} was higher than the decomposition rate in the first 8 hr and became lower than the decomposition rate in the period of 8 to 60 hr. The maximum value of S\textsubscript{2}O\textsubscript{3}\textsuperscript{2−} concentration was 11.23 mg/L. The concentration of SO\textsubscript{3}\textsuperscript{2−} kept increasing in the S\textsuperscript{2−}-oxidizing process and reached the maximum value at 60 hr, and its maximum value was 9.2 mg/L. The concentration of S\textsubscript{4}O\textsubscript{6}\textsuperscript{2−} was not detected in the whole process, indicated that this compound was not produced during S\textsuperscript{2−} oxidation. As shown in Figure 4(b), the concentration curves of the inorganic sulfur compounds were of great difference in the blank control. The concentration of S\textsuperscript{2−} decreased at a slow rate, and the concentrations of S\textsuperscript{0}, SO\textsubscript{3}\textsuperscript{2−} and S\textsubscript{4}O\textsubscript{6}\textsuperscript{2−} were not detected and the concentrations of S\textsubscript{2}O\textsubscript{3}\textsuperscript{2−} and SO\textsubscript{4}\textsuperscript{2−} were low. These results indicated that the oxygen in the air has little effect on the S\textsuperscript{2−}-oxidizing process in the artificial water.

The above results show that the addition of the strain sp3 accelerated the S\textsuperscript{2−}-oxidizing process. S\textsuperscript{2−} oxidation was a complex process involving in the production and bioconversion of S\textsuperscript{0}, S\textsubscript{2}O\textsubscript{3}\textsuperscript{2−}, and SO\textsubscript{3}\textsuperscript{2−}, and finally the unstable S\textsuperscript{2−} was oxidized to stable SO\textsubscript{4}\textsuperscript{2−}. Pervious research also indicated that the addition of SOB can accelerate the oxidation process of S\textsuperscript{2−} to SO\textsubscript{4}\textsuperscript{2−} (Zhuang et al. 2017).

**Analysis on S\textsuperscript{2−}-oxidizing metabolic pathway of strain sp3**

Some previous research on the metabolic pathway of SOB show that there are two major pathways for oxidation of sulfur compounds including paracoccus sulfur oxidation (PSO) pathway and S4 intermediate (S4I) pathway (Kelly et al. 1997). Part of the oxidation of sulfur compounds exist in both of PSO pathway and S4I pathway, including these bioconversion pathways. The above results show that the addition of the strain sp3 accelerated the S\textsuperscript{2−}-oxidizing process. S\textsuperscript{2−} oxidation was a complex process involving in the production and bioconversion of S\textsuperscript{0}, S\textsubscript{2}O\textsubscript{3}\textsuperscript{2−}, and SO\textsubscript{3}\textsuperscript{2−}, and finally the unstable S\textsuperscript{2−} was oxidized to stable SO\textsubscript{4}\textsuperscript{2−}. Pervious research also indicated that the addition of SOB can accelerate the oxidation process of S\textsuperscript{2−} to SO\textsubscript{4}\textsuperscript{2−} (Zhuang et al. 2017).

![Figure 4](https://example.com/figure4.png) **Figure 4** | Variation of each inorganic sulfur compound concentration: (a) experiment, (b) control.
processes of $S^{2-}$ to $S^0$, $S^{2-}$ to $SO_2^-$, $S^0$ to $SO_2^-$, $S_2O_3^{2-}$ to $S^0$ and $SO_3^{2-}$ along with $SO_2^{3-}$ to $SO_2^{4-}$. The process of $S^{2-}$ converted to $S^0$ was controlled by enzyme sulfide quinone oxidoreductase (SQO) or flavocytochrome c (FCC). The enzyme SQO was encoded by the sqr gene and the enzyme FCC was encoded by the fcc gene (Kelly et al. 1997). The process of $S^{2-}$ converted to $SO_2^-$ was controlled by enzyme sulfite reductase (SRN, reduced form of nicotinamide adenine dinucleotide) that was encoded by the cysIJ gene (Tan et al. 2013). The process of $S^0$ converted to $SO_2^-$ was controlled by grx gene and the enzyme hetero disulfide reductases (HDR) that was encoded by hdrBC gene (Quatrini et al. 2009). The process of $S_2O_3^{2-}$ converted to $S^0$ and $SO_2^-$ was controlled by the enzyme thiosulfate sulfurtransferase (TST) that was encoded by tst gene (Rohwerder & Sand 2007). The process of $SO_3^{2-}$ converted to $SO_2^-$ could occur in two different ways: direct and indirect oxidation. The direct oxidation process of $SO_3^{2-}$ converted to $SO_2^-$ was controlled by the enzyme sulfite oxidase (SO) that was encoded by sox gene (Toghrøl & Southerland 1983; Bruser et al. 2000). The indirect oxidation process was controlled through the enzyme phosphoadenosine phosphosulfate reductase (PAPS) that was encoded by paps gene (Bruser et al. 2000). Apart from the common part of the two pathways, some oxidation of the sulfur compounds including the process of $S_2O_3^{2-}$ converted to $S_2O_2^{3-}$ and the process of $S_4O_6^{2-}$ converted to $SO_3^{2-}$ doesn’t exist in the PSO pathway but the S4I pathway (Rohwerder & Sand 2007; Quatrini et al. 2009; Sakurai et al. 2010). The process of $S_2O_3^{2-}$ converted to $S_2O_2^{3-}$ was controlled by the enzyme thiosulfate quinone oxidoreductase (TQO) that was encoded by soxYV gene (Holden et al. 2004; Moller & Hederstedt 2008). The process of $S_2O_2^{3-}$ converted to $SO_3^{2-}$ was controlled by the enzyme tetrathionate hydrolase (THH) that was encoded by tetH gene (Rohwerder & Sand 2007; Sakurai et al. 2010). Thus, it could be inferred that there was no existence of tetH gene in PSO pathway. Moreover, $S_2O_2^{3-}$ would be not detected in the whole of $S^{2-}$-oxidizing process for the PSO pathway.

The whole genome sequence of the strain sp3 was aligned to the public database kyoto encyclopedia of genes and genomes (KEGG) and NCBI. The results indicated that 31 kinds of sulfur oxidation related genes were detected in the strain sp3 (Table 2). Some key genes belonging to the major pathways for oxidation of sulfur compounds were detected in the strain sp3, including cysIJ, grx, tst and sox gene . However, some key genes including sqr, fcc, hdrBC, paps and tetH gene were not detected in the strain sp3. Although sqr gene was not detected in the strain sp3, hdrA gene was found in the strain sp3, which was proved to be able to encode the enzyme SQO (Imhoff & Thiel 2010). The results that both of tetH gene and $S_2O_3^{2-}$ were not detected in the strain sp3 suggested that the S4I pathway was not the metabolic pathway of the strain sp3 and PSO pathway might be the only $S^{2-}$-oxidizing metabolic pathway.

Transcriptome analysis of Stenotrophomonas sp. sp3 was then conducted, and the result indicated that 4,733 protein-encoding genes were detected. Visualization of differences and similarities among these detected protein-encoding genes was performed by cluster analysis. The cluster heatmap of differential expression of 4,733 protein-encoding genes is shown in Figure 5. The gene expression level was of great difference at the $S^{2-}$-oxidizing process of 1, 18 and 30 hr. and subsequently the similarities existed in the samples collected at 30 and 60 hr. This indicated that the microbial metabolisms of the strain sp3 appeared significant difference before the bio-reaction of 30 hr, and then maintained stability in the rest of bio-reaction. This resulted in the obvious variation of the concentration of each inorganic sulfur compound during the first 50 hr of the bio-reaction (Figure 4).

The results of differential gene expression analysis are shown in Figure 6. As shown in Figure 6(a), the number of up gene in the range of 1–18 hr was 751, and the number of down gene was 745. With the further bio-reaction up to 30 hr, the number of up gene arrived at 820, while down gene was 784 (Figure 6(b)). As for the $S^{2-}$-oxidization process in the range of 30 to 60 hr, the number of up gene decreased 44, while down gene was also reduced to188 (Figure 6(c)). Moreover, the differential expression of sulfur oxidation-related genes was significant from 1 to 30 hr, and the rate of $S^{2-}$-oxidation was rapid; but there was no significant differential expression of sulfur oxidation-related genes from 30 to 60 hr, and the concentration of $S^{2-}$ decreased to the lowest value and kept relatively stable (Figure 4). These results suggested that most of the microbial metabolism of the strain sp3 happened in the first 30 hr of the bio-reaction. This time range was the main phase for $S^{2-}$-oxidizing, too (Figure 3).

The expression level of sulfur oxidation-related genes detected in the strain sp3 was chosen from the cluster heatmap of differential expression genes. The heatmap of sulfur oxidation genes expression level is shown in Figure 7. The expression level of eleven kinds of genes, especially hdrA, cysIJ and cybB gene, increased significantly during the bio-reaction of 1 to 18 hr. The process of $S^{2-}$ converted to $S^0$ was controlled by hdrA gene through encoding the enzyme SQO and the process of $S^{2-}$ converted to $SO_2^-$ was controlled by cysIJ gene through encoding the enzyme SRN (Tan et al. 2013). Figure 4 shows that the concentrations of $S^0$ and $SO_2^-$ increased in the first 18 hr of the bio-reaction, indicated that the processes of $S^{2-}$-
converted to S\(^0\) and SO\(_3^2\) were performed in this period. The formation progress of other sulfur compounds could be found and concluded in this way. The electron transportation was controlled by \(\text{cybB}\) gene (Chen et al. 2012) and the concentration of the electron donor S\(^2\)/C\(_0\) decreased in first 18 hr of the bio-reaction. The expression level of nine kinds of genes, especially \(\text{tst}\), \(\text{sox}\) and \(\text{sbp}\) gene, increased in the range of 18 to 30 hr. The process of S\(_2\)O\(_3^2\)/C\(_0\) converted to S\(^0\) and SO\(_3^2\)/C\(_0\) was controlled by \(\text{tst}\) gene through encoding the enzyme TST (Rohwerder & Sand 2007) and was performed during the process of 18 to 30 hr. The process of SO\(_3^2\)/C\(_0\) converted to SO\(_4^2\)/C\(_0\) was controlled by \(\text{sox}\) gene through encoding the enzyme SO (Toghrol & Southerland 1983) and \(\text{sbp}\) gene controlled the transportation of SO\(_4^2\)/C\(_0\) (Sirko et al. 1995). After the bio-reaction of 30 hr, the expression level of most genes maintained stability except that the expression level of some genes like \(\text{hdrA}\) and \(\text{sbp}\) gene decreased significantly, and some genes such as \(\text{soxX}\), \(\text{soxW}\) and \(\text{soxV}\) became higher.

According to the above experimental results, the major metabolic pathway of the strain sp3 was the PSO pathway, as shown in Figure 8. Four inorganic sulfur compounds including S\(^0\), S\(_2\)O\(_3^2\), SO\(_3^2\) and SO\(_4^2\) were formed in the process of S\(^2\) oxidization. In the PSO pathway, some S\(^2\) were converted to S\(^0\) under the action of the enzymes SQR that was encoded

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Table 2 | Sulfur oxidation related genes of Stenotrophomonas sp.sp3 genome

| Gene   | Gene annotation                        | Reference                          |
|--------|----------------------------------------|------------------------------------|
| sufES  | Cysteine desulfurase                   | Albrecht et al. (2011)             |
| cybB   | Cytochrome b561                        | Chen et al. (2012)                 |
| coxA/B/C/D | Cytochrome c oxidase                | Marks et al. (2010)                |
| nuo    | NAD(P)H-quinone oxidoreductase         | Eickelmann et al. (1994)           |
| ato    | Cytochrome c oxidase subunit III       | Yang & Hekimi (2010)               |
| his    | Phosphoribosyl-AMP cyclohydrolase      | Methé et al. (2003)                |
| stp    | Sulfate ABC transporter permease       | Rohwerder & Sand (2007)            |
| hdrA   | Pyridine nucleotide-disulfide oxidoreductase | Hedderich et al. (1994) |
| psrB   | 4Fe-4S cluster-binding                 | Cai et al. (2012)                  |
| fisR   | Transcriptional regulator              | Hadizadeh et al. (2016)            |
| cysIJ  | Sulphite reductase (NADPH)             | Shigenobu et al. (2000)            |
| thiS   | Sulfur carrier protein ThiS adenyllyltransferase | Wang et al. (2001) |
| St     | S-transferase                          | Carkaci et al. (2016)              |
| sbp    | Sulfate transporter                    | Sirko et al. (1995)                |
| Dsb/soxW | Thiol:disulfide interchange protein    | Holden et al. (2004)               |
| trxB   | Thioredoxin reductase                  | Negri et al. (2010)                |
| trxAC/soxV | Thioredoxin                        | Moller & Hederstedt (2008)         |
| tst    | Thiosulfate sulfurtransferase          | Rohwerder & Sand (2007)            |
| grx    | Glutaredoxin                           | Belda et al. (2016)                |
| gst    | Glutathione S-transferase              | Romine (2011)                      |
| gsr    | Glutathione reductase                  | Bowler et al. (2008)               |
| gshp   | Glutathione peroxidase                 | Romine (2011)                      |
| gsh-dtr | Glutathione-dependent thiol reductase  | Romine (2011)                      |
| cydAB  | Cytochrome d ubiquinol oxidase         | Holden et al. (2004)               |
| cyoABCD | Cytochrome o ubiquinol oxidase         | Friericks et al. (2006)            |
| soxBI  | Cytochrome b6                          | Eisen et al. (2002)                |
| soxBII | Thioesterase                           | Eisen et al. (2002)                |
| soxC   | Molybdenum cofactor                   | Eisen et al. (2002)                |
| soxF   | FAD                                   | Deckert et al. (1998)              |
| soxX   | Cytochrome c                           | Eisen et al. (2002)                |
Figure 5 | Cluster heatmap of differential expression genes (red line represented high expression level genes; blue line represented low expression level genes; each column represented the sample collected at 1 hr, 18 hr, 30 hr and 60 hr of the bio-reaction respectively).
**Figure 6** | Analysis of differential gene expression: (a) Volcano plot of 1~18 hr, (b) Volcano plot of 18~30 hr, (c) Volcano plot of 30~60 hr. Red dot represents up genes; blue dot represents down genes; gray dot represents none-differentially expressed genes.
by *hdrA* gene; while other part of $S^2-$ were converted to $SO_3^{2-}$, which was controlled by *cysIJ* gene through encoding the enzyme SRN. The process of $S^0$ converted to $SO_3^{2-}$ was controlled by *grx* and *hdrBC* gene. However, although *grx* gene was detected in the strain sp3, *hdrBC* gene was not detected in the strain sp3, suggesting that the process of $S^0$ converted to $SO_3^{2-}$ was not available in the strain sp3. Meanwhile, some $SO_3^{2-}$ reacted with $S^0$ spontaneously to form $S_2O_3^{2-}$, and other part of $SO_3^{2-}$ were converted to $SO_4^{2-}$ in the control of *sox* gene through encoding the enzyme SO. $S_2O_3^{2-}$ released $S^0$ and $SO_3^{2-}$ at the same time through the action of the enzyme TST encoded by *tst* gene. The *sox* gene was detected in the strain sp3 while *paps* gene was not detected, indicated that the direct oxidation process was the only metabolic pathway of $SO_3^{2-}$ converted to $SO_4^{2-}$.

The strain sp3 isolated in our study was biologically classified as genus *Stenotrophomonas*. *Stenotrophomonas* has various metabolic functions. In the previous studies, *Stenotrophomonas* was mainly found to have the abilities of antibiotic resistance, organophosphorus degradation, and dichlorodiphenyltrichloroethane degradation (Shen *et al.* 2010; Dubey & Fulekar 2012; Fang *et al.* 2018). In our study, the strain sp3 was isolated from a typical black-odor water body. The experimental results

![Figure 7](http://iwaponline.com/ws/article-pdf/doi/10.2166/ws.2022.011/985113/ws2022011.pdf)
confirmed that this strain was capable of sulfur oxidation, and $S^{2-}$ could be converted into $SO_4^{2-}$ via the PSO pathway. Therefore, a novel SOB (i.e. *Stenotrophomonas* sp.sp3) was found in our study. In the subsequent treatment of black-odor water bodies, three aspects might be selected to proceed: 1) compounding with other organisms which have other sulfur-oxidizing metabolic pathways to improve the treatment efficiency on black-odor water bodies; 2) investigating the removal efficiency of other items (such as TP, NH$_3$-N, COD, etc.) in black-odor water bodies under the action of this compounded microorganisms; 3) A solid bacterial agent which consisted of this compounded microorganisms would be attempt to prepare for treating the black-odor water by direct dosing on the water surface, and this has similar cases in previous studies (Cao et al. 2020).

**CONCLUSION**

For the strain sp3 growth and $S^{2-}$ oxidation, the optimal conditions were as follows: 25 °C of temperature, initial pH 7, 2.5 g/L of initial glucose concentration, and 1.00 g/L of initial cell concentration. After the bio-reaction of 60 hr, $S^{2-}$-oxidization ratio by the strain sp3 arrived at 86.6%. Four kinds of inorganic sulfur compounds including $S^0$, $S_2O_3^{2-}$, $SO_3^{2-}$ and $SO_4^{2-}$ were produced during the $S^{2-}$ oxidation. Combining high-throughput sequencing with transcriptomics analysis, the PSO pathway was the main metabolic process of $S^{2-}$ to $SO_4^{2-}$, and the microbial metabolism of $S^{2-}$ oxidation mainly happened in the first 30 hr of the bio-reaction. $S^{2-}$ was efficiently oxidized by the strain sp3, which possessed significant potential for the black-odor water treatment.

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**DATA AVAILABILITY STATEMENT**

All relevant data are included in the paper or its Supplementary Information.

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