Comparison of the capacity of biological desulfurization of *Thiobacillus ferrooxidans* from different sulfur-containing substrates with or without additional ferrous iron

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Abstract: *Thiobacillus ferrooxidans*, abbreviated as *T. ferrooxidans* is one of the important microorganisms in the field of biological desulfurization. Effects of ferrous iron and sulfur-containing substrates on biological desulfurization of *T. ferrooxidans* were studied. Results show that in the absence of Fe^{2+}, *T. ferrooxidans* can utilize three kinds of sulfur-containing substrates of $Na_2S_2O_3$, elemental S and Na_2SO_3 for growth and metabolism. For utilization complexity, $Na_2S_2O_3$ was easiest to use, next was elemental S, and Na_2SO_3 was the worst for use. During the utilization of ferrous iron and sulfur-containing substrates by *T. ferrooxidans*, the iron oxidation system was first started. With the decrease of the Fe^{2+} concentration, the sulfur oxidation system was started, and then the two systems synergistically acted. The presence of three sulfur-containing substrates had different effects on Fe^{2+} oxidation, and elemental S did not inhibit the oxidation of Fe^{2+}, while $Na_2S_2O_3$ and Na_2SO_3 had some inhibition on the oxidation of Fe^{2+}, especially the inhibition of Na_2SO_3 was significant, and complete oxidation of ferrous iron needed more time. The isolated *T. ferrooxidans* is applied to the removal of H_2S gas, aiming to provide a new technological approach for biological removal of H_2S.

Keywords: *Thiobacillus ferrooxidans*, ferrous iron, sulfur-containing substrate, biological desulfurization
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1 Introduction

With the rapid development of modern agriculture, the production of organic solid waste such as straw and livestock manure has been increasing year by year. Improper treatment and disposal can cause serious environmental pollution problems, endanger human health, and also result in serious resource waste. Anaerobic fermentation is a process in which insoluble organic matter is gradually decomposed into water-soluble small molecule organic matter through the metabolic activity of facultative and anaerobic microorganisms in an anaerobic environment, ultimately producing biogas mainly composed of methane. It is an effective way to achieve the resource utilization and energy recovery of agricultural waste such as straw and livestock manure. The biogas produced by anaerobic fermentation contains H_2S, which has extremely strong toxicity[1-3]. When using biogas, H_2S removal treatment must be carried out first[4-6]. Recently, as a new desulfurization technology, H_2S removal by *T. ferrooxidans* has gained much attention[7-9]. *T. ferrooxidans* is a kind of obligate aerobic and eosinophilic chemoautotrophic bacterium[10-12]. It exists by oxidizing Fe^{2+} into Fe^{3+} and by oxidizing low valent sulfur into sulfate, and is gram negative[13,14]. The removal of H_2S by *T. ferrooxidans* can utilize its indirect oxidation[15-17]. The desulfurization mechanism is[18]:

$$H_2S + Fe_2(SO_4)_3 \rightarrow 2FeSO_4 + H_2SO_4 + S\downarrow \quad (1)$$

$$2FeSO_4 + 0.5O_2 + H_2SO_4 \xrightarrow{T.ferrooxidans} H_2O + Fe_2(SO_4)_3 \quad (2)$$

The rate of Fe^{2+} oxidation by *T. ferrooxidans* is at least 200 000 times faster than the chemical oxidation rate alone[19], but with the increase of Fe^{3+}, a certain amount of ferro-vanadium precipitation will be produced in the medium[20,21]. It can also be used to oxidize H_2S to elemental S and H_2SO_4 by its direct oxidation[22,23]. The reaction formula is:

$$2H_2S + O_2 \rightarrow 2S + 2H_2O \quad (3)$$

$$2S + 3O_2 + 2H_2O \rightarrow 2H_2SO_4 \quad (4)$$

With comparison, *T. ferrooxidans* tends to use ferrous iron more, and most researches on *T. ferrooxidans* use ferrous iron as the energy source[24-26]. In order to avoid the precipitation during the indirect oxidation process of *T. ferrooxidans*, improve its direct oxidation of low valent sulfur[27-29], and realize better application for H_2S removal, this paper studied the utilization of sulfur-containing substrates by *T. ferrooxidans* in the presence and absence of Fe^{2+}, providing the theoretical basis for its industrialization application in biological desulfurization.

2 Materials and methods

2.1 Materials

2.1.1 Microbial strain and medium

T. ferrooxidans was isolated from acid mine water by dilution

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and coating method, and was identified by colony morphology, microscopic observation, staining treatment, culture characteristics and 16S rRNA gene sequence analysis.

1) Isolation of strains

(1) Method 1: Serial dilution method

100 μL culture medium was inoculated in 1 mL 9K liquid medium (15 mL test tube), and 10 tubes of gradient dilution (10-1) were cultured at 30°C, 170 r/min, and the color change of test tubes was observed day by day for 10 days. In addition, single colony isolation was performed on 9K solid medium.

9K liquid medium: $(NH_4)_2SO_4$ 3.00 g, K_2HPO_4 0.50 g, KCL 0.10 g, $MgSO_4 \cdot 7H_2O$ 0.50 g, Ca($NO_3)_2$ 0.01 g, $FeSO_4 \cdot 7H_2O$ 44.30 g, distilled water 1000 mL, pH 2.0.

9K solid medium: adding 2% agarose to 9K liquid medium.

(2) Method 2: Coating method

Since many bacteria that can oxidize ferrous ions also have sulfur oxide function, it is speculated that the bacteria in the culture medium may also oxidize low-price sulfur. 200 μL culture medium was directly coated on SO (sulfur oxidization medium) solid medium and cultured at 30°C for 2 d. Individual colonies were selected and striated on the same medium for purification.

SO liquid medium: $CH_3COCOONa$ 1.98 g, Na_2S 0.21 g, yeast extract 0.3 g, distilled water 300 mL, pH 7.0.

SO solid medium: adding 2% agar powder to SO liquid medium.

2) Identification of strains

According to the colony morphology, microscopic observation, staining treatment, culture characteristics and 16S rRNA sequence analysis, the strains were determined. The primers used for 16S rRNA gene sequence analysis were 8f (5'-AGAGTTTGATCMTGGC-2') and 1542r (5'-AAAGGAGGTGATCCA-2'). PCR reaction conditions were as follows: denaturation temperature 94°C, 2 min; annealing temperature 55°C, 1 min; extended temperature 72°C, 1 min, total 25 cycles.

2.1.2 Reagent

$(NH_4)_2SO_4$, AR, Guangdong Guanghua Chemical Co., Ltd; K_2HPO_4, AR, Nanjing Chemical Reagent Factory; KCl, AR, Nanjing Chemical Reagent Factory; $MgSO_4 \cdot 7H_2O$, AR, Shanghai Tongya Fine Chemical Factory; $Ca(NO_3)_2$, AR, Wenzhou Chemical Factory; $FeSO_4 \cdot 7H_2O$, AR, Sinopharm Chemical Reagent Co., Ltd.; $CH_3COCOONa$, AR, Sinopharm Chemical Reagent Co., Ltd.; Na_2S, AR, Sinopharm Chemical Reagent Co., Ltd.; Yeast extract, AR, Shanghai Shenggong Biotechnology Co., Ltd. S, AR, Nanjing Ningshi Chemical Reagent Co., Ltd.; $Na_2S_2O_3$, AR, R&D center of Guangdong Chemical Reagent Engineering Technology; Na_2SO_3, AR, Nanjing Ningshi Chemical Reagent Co., Ltd.

2.1.3 Apparatus

Air oscillating shaker (THZ-C constant-temperature oscillator, Taicang Jiangsu); pH meter (6171C type microcomputer pH/mV/temperature, Shanghai Jenco Electronic Co., Ltd.); scales (Sartorius electronic scales, Beijing Sartorius scale Co., Ltd.).

2.2 Methods

9K liquid medium containing no Fe^{2+} was prepared, and elemental S, $Na_2S_2O_3$ and Na_2SO_3 were added separately. Measured as per S, concentration of S added was 1 g, which was 0.03 mol/L, cultured at 30°C and 120 r/min. Due to that sulfuric acid generation by oxidation of sulfur compounds is an acid-producing process, and the degree of pH reduction of the solution can be used to indicate the amount of sulfur compounds oxidized by the bacteria. Therefore, the pH and SO_4^{2-} concentration in the solution were measured at regular intervals, and utilization of sulfur compounds by *T. ferrooxidans* was analyzed.

In order to investigate the utilization of two energy sources by *T. ferrooxidans* in the simultaneous presence of ferrous and sulfur-containing substrates, 9K liquid medium containing Fe^{2+} was prepared, and elemental S, $Na_2S_2O_3$ and Na_2SO_3 were added separately. Measured as per S, concentration of S added was 1 g/L, which was 0.03 mol/L, cultured at 30°C and 120 r/min. PH, Fe^{2+} and SO_4^{2-} concentration in the solution were measured at regular intervals, and effects of ferrous iron and sulfur-containing substrates on biological oxidation process of *T. ferrooxidans* was analyzed.

The pH value was determined by pH meter; Fe^{2+} determined by O-phenanthroline spectrophotometry[30]; SO_4^{2-} concentration determined by barium chromate spectrophotometry[31].

3 Results and discussion

The change of pH in solution is shown in Figure 1 when *T. ferrooxidans* uses three sulfur-containing substrates as energy materials with or without additional ferrous iron, and the change of SO_4^{2-} concentration in six culture systems is shown in Figure 2. It was found that among the three sulfur-containing substrates, $Na_2S_2O_3$ was easiest to use, and next was elemental S, and Na_2SO_3 was worst to use. When there were only sulfur-containing substrates in the medium without Fe^{2+}, *T. ferrooxidans* had a certain lag period at the beginning, with different duration time. During the lag period, decrease of pH of the solution was not significant, and the concentration of SO_4^{2-} in the solution was very low. With the growth of *T. ferrooxidans*, the pH value in the solution decreased, and the concentration of SO_4^{2-} in the solution also gradually increased and then stabilized. Since Na_2SO_3 is a weak acid and strong alkali salt, the pH of the solution during the whole process of *T. ferrooxidans* growth with Na_2SO_3 as substrate is higher than that of elemental S and $Na_2S_2O_3$. Oxidation of sulfur compounds by *T. ferrooxidans* is an acid-producing process, and the pH value in the solution still shows a downward trend.

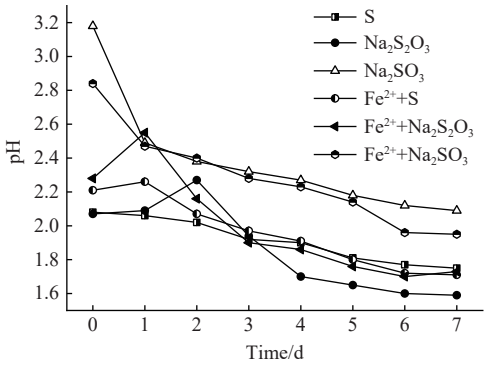

Figure 1 The pH change of the solution with different substrates

Though $Na_2S_2O_3$ would become disproportionate as the SO_3^{2-} and S under acidic conditions, the growth rate of SO_4^{2-} was still faster than that when Na_2SO_3 was used as a substrate. So it can be inferred that the oxidation of $S_2O_3^{2-}$ and SO_3^{2-} by *T. ferrooxidans* is not the effect by the same enzyme, and the utilization rate of $S_2O_3^{2-}$ is faster than that of SO_3^{2-} [32,33]. Since SO_3^{2-} in Na_2SO_3 is of in the ionic state and the air shall oxidize part of the SO_3^{2-}, the concentration of SO_4^{2-} in the solution containing Na_2SO_3 as substrate in the first 2 days thus was higher than that in the solution containing elemental S as substrate. But with the utilization of elemental S by *T. ferrooxidans*, the concentration of SO_4^{2-} in solution containing elemental S shall increase rapidly. At the end of

the experiment, the concentration of SO_4^{2-} in the solution with elemental S as the substrate increased by 105%, the concentration of SO_4^{2-} in the solution with $Na_2S_2O_3$ as the substrate increased by 112%, and the concentration of SO_4^{2-} in the solution with Na_2SO_3 as the substrate increased by 72% with the smallest increase. For *T. ferrooxidans* growth with elemental S as the substrate, thallus was first adsorbed on the surface of elemental S particles, and elemental S passed through the cell wall and entered the interior of the cell to form a polysulfide compound with glutathione, then the first-grade product sulfite was produced. Then SO_3^{2-} was further oxidized into SO_4^{2-} by action of adenosine monophosphate. The whole process involved a series of complex mass transfer processes such as adsorption of *T. ferrooxidans* on the surface of solid particles and diffusion of products through the cell wall. Due to the slow adsorption rate of *T. ferrooxidans* on the surface of elemental S particles, the step of solid-phase interface mass transfer became a rate-limiting step for the utilization process of elemental S[34,35]. Once the sulfur was oxidized to SO_3^{2-}, energy utilization by cells became faster.

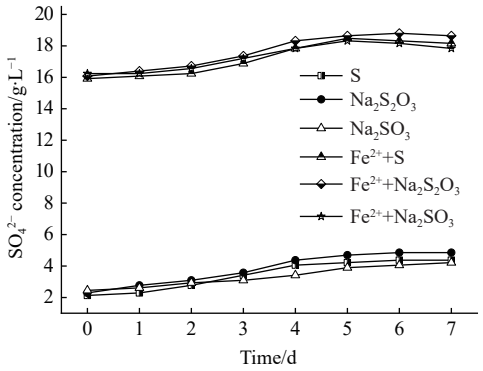

Figure 2 The SO_4^{2-} concentration change of the solution with different substrates

The change of Fe^{2+} concentration in the solution with Fe^{2+} is showed in Figure 3. Since Fe^{2+} oxidation by *T. ferrooxidans* is easier, and elemental S is a poorly soluble solid particle, there was no adaptation period of bacteria during the cultivation process, that is, the utilization of Fe^{2+} was not limited by presence of S, and the Fe^{2+} enzyme system was first activated[36,37]. $Na_2S_2O_3$ and Na_2SO_3 had some inhibition on the oxidation of Fe^{2+}, especially the inhibition of Na_2SO_3 was more obvious. And the time needed for complete oxidation of ferrous iron was longer, for 6 days. While in the solution added with $Na_2S_2O_3$, ferrous iron had been completely oxidized in 2 d. With the growth of bacteria, the concentration of Fe^{2+} in the solution gradually decreased until it was completely oxidized, the energy utilization metabolic pathway of sulfur-oxidase started, and the pH value in the solution began to decrease significantly. The change of pH value in the solution of *T. ferrooxidans* with $FeSO_4$ and $Na_2S_2O_3$ as substrates was the same as that with $FeSO_4$ and S, and with a trend of increase first and then decrease, while the former's rangeability was bigger than that of the latter. For *T. ferrooxidans* with $FeSO_4$ and $Na_2S_2O_3$ as substrates, the pH value in the solution showed a downward trend. Though the addition of Na_2SO_3 led to an increase in the initial pH value of the solution, the pH in the solution dropped rapidly after 1 day. The reason may be that Fe^{2+} in the solution is first oxidized by *T. ferrooxidans* and results in Fe^{3+} which can oxidize SO_3^{2-}, thus decreasing the pH of the solution. Moreover, with the growth of *T. ferrooxidans*, the enzyme system that oxidizes SO_3^{2-} begins to take effect, thus accelerating the decrease of the pH.

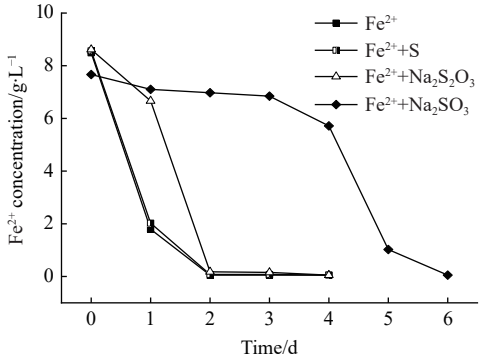

Figure 3 The Fe^{2+} concentration change of the solution with different substrates

Due to the presence of energy substance Fe^{2+}, the concentration of SO_4^{2-} in the solution with Fe^{2+} all increased to some extent. Moreover Fe^{3+} oxidized by *T. ferrooxidans* from Fe^{2+} had an oxidation effect, thus, the utilization of intermediate product of sulfur compound metabolite by *T. ferrooxidans* was better than that in the absence of Fe^{2+}. Due to oxidation of SO_3^{2-} by air, the SO_4^{2-} concentration in the solution with $FeSO_4$ and Na_2SO_3 as substrates in the first 4 d was higher than that in the solution with $FeSO_4$ and S as substrates. However, in the later stage of culture, SO_4^{2-} in the latter solution increased rapidly with the increase of the amount of *T. ferrooxidans* adsorbed on elemental S. In solutions with $FeSO_4$ and S, $FeSO_4$ and $Na_2S_2O_3$, and $FeSO_4$ and Na_2SO_3 as substrates separately, maximum concentration of SO_4^{2-} were 18.48 g/L, 18.80 g/L, 18.32 g/L separately, which, in relative to the increase amplitude of the initial value, were higher than that with absence of Fe^{2+}. At the later stage of the experiment, SO_4^{2-} concentration in the three solutions showed a downward trend due to hydrolysis and precipitation of some Fe^{3+} in the solution.

4 Conclusions

In the absence of Fe^{2+}, $Na_2S_2O_3$ was easiest to use for *T. ferrooxidans*, next was elemental S, and Na_2SO_3 was difficult to use. When ferrous iron and sulfur-containing substrates were present together, the iron oxidation system of *T. ferrooxidans* was first started. Then these two oxidation systems synergistically acted. Elemental S did not inhibit the oxidation of Fe^{2+}, while $Na_2S_2O_3$ and Na_2SO_3 had certain inhibition on the oxidation of Fe^{2+}, especially the inhibition of Na_2SO_3 was more obvious. Due to its special physiological and metabolic characteristics, *T. ferrooxidans* has become one of the most important strains in the fields of biological desulfurization of fuel and acid waste gas, biological hydrometallurgy, acid mine drainage treatment and so on[38,39]. Its research not only provides a new way for the biological removal of H_2S, but also expands the research and application of biotechnology in the field of waste gas treatment, which is of great significance for the protection of ecological environment.

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