Optimum Cultivation and Denitrification Efficiency of *Thiobacillus denitrificans* in Batch Experiments

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**Abstract**

*Thiobacillus denitrificans* is widely used in the autotrophic denitrification process to remove nitrate. The results from batch experiments indicated that the optimum cultivation for the growth and bioactivity of *T. denitrificans* could be obtained under anaerobic condition at 30°C. Besides the addition of ordinary basal mineral salts, the optimal culture medium should be composed of 40 ml of trace element solution, 15 g/l Na₂S₂O₃·5H₂O and 5.0 g/l KNO₃. Under the optimum conditions, the average ratios of ΔS₂O₃²–-S/ΔNO₃⁻-N and ΔSO₄²⁻/ΔNO₃⁻-N were 3.8 g/g and 10.9 g/g, respectively, which were comparable with the theoretical values of the autotrophic denitrification using thiosulfate as electron donor, and the SO₄²⁻ generation, S₂O₃²⁻-S consumption and NO₃⁻-N denitrification rates were 306.0, 104.9 and 47.3 mg/l·d, respectively. After 12-day cultivation, about 80% of NO₃⁻-N could be removed from the culture with 700 mg/l of initial NO₃⁻-N contained.

**Key words:** *Thiobacillus denitrificans*, thiosulfate, nitrate, denitrification, cultivation

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**INTRODUCTION**

Nitrate contamination is becoming a widespread environmental problem. Although some physical-chemical processes have been described to be the best available technologies to remove nitrates, e.g., reverse osmosis, ion exchange and electrodialysis, they are rarely used to treat nitrate-contaminated water because of their poor selectivity, high operation costs and disposal problem resulted from the generation of concentrated nitrate waste¹. A promising and attracting alternative, biological denitrification, appears to be the most suitable solution from the point of environmental, practical and economical aspects.

Biological denitrification process, including autotrophic denitrification and heterotrophic denitrification, has been commonly used for the treatment of wastewater. Heterotrophic denitrification is very efficient in nitrate removal under anaerobic or anoxic conditions when adequate amounts of organic carbon are available²-³. Compared with heterotrophic denitrification, autotrophic denitrification is attracting increasing interest in recent years due to the following three major advantages: (1) no residual organics problems because of the utilization of inorganic substances as electron donors; (2) low operation and maintenance costs due to no external organic carbon needed and (3) lower cell yield, which minimizes sludge handling or lessens the risk of biological regrowth in distribution systems and disinfection byproduct for-
Among the denitrifying microorganisms, only a few species of autotrophic bacteria can reduce nitrate to nitrogen gas while oxidizing elemental sulfur or reduced sulfur compounds \((S^2-, S_2O_3^2-, SO_3^{2-})\) to sulfate, and *Thiobacillus denitrificans* is the most commonly used. Up to now, many researches were focused on the efficiency and influence factors of autotrophic denitrification processes by *T. denitrificans* (enriched sludge or pure culture) with reduced sulfur compounds as electron donors in the treatment of nitrate-contaminated drinking water, groundwater or wastewaters\(^3\)\(^{-17}\). Among the several reduced sulfur compounds used, thiosulfate is proved to be more suitable than elemental sulfur or reduced sulfur compounds \((KNO_3, 0.5 g \ NaHCO_3 \ and \ 100 \ ml \ of \ 10% \ (10 \ g/100ml) \ Na_2S_2O_3 \cdot 5H_2O, \) which was suggested by RIKEN. Before use, the basal mineral medium (except \(Na_2S_2O_3\) solution) was autoclaved at 125°C for 15 min and the \(Na_2S_2O_3\) solution was sterilized by filtration.

The culture medium (400 ml in total) was composed of the basal mineral medium and a certain volume of trace element solution. The latter solution contained (mg/l): EDTA (500.0), CaCl\(_2\) (55.4), CuSO\(_4\)•5H\(_2\)O (15.7), CoCl\(_2\)•6H\(_2\)O (16.1), MnCl\(_2\)•4H\(_2\)O (50.6), ZnSO\(_4\)•7H\(_2\)O (220.0), (NH\(_4\)\(_2\))\(_6\)Mo\(_7\)O\(_{24}\)•H\(_2\)O (11.0) and FeSO\(_4\)•7H\(_2\)O (49.9)\(^{20}\).

**Experiment setup** All the batch experiments were started at initial culture pH=7.0 (adjusted by 0.1M NaOH) and conducted at 30°C in duplicates in flasks (500 ml) sealed with butyl rubber stoppers on a shaking table, supplemented with 400 ml of culture medium and then inoculated in a sterilization chamber with 10% (v/v) of *T. denitrificans* enrichment culture, which was the fourth-day culture of *T. denitrificans* being enriched with the culture medium in the same kind of flask. An aliquot amount of initial biomass was applied in one group of experiments, although the initial biomass, indicated by the optical density of the culture at 650 nm (OD\(_{650}\)), varied from 0.156 to 0.202 for all groups of our experiments.

As for anaerobic cultivation, the anaerobic conditions were established by flushing the flasks with nitrogen gas at least three times, each in 5 min, through the diffuser set beneath the culture, and then the flasks were kept in vacuum for 2 min. As for aerobic test, 0.2 μm filter membrane instead of butyl rubber stopper was used to seal the flask and air could go freely through the filter.

\[S_2O_3^{2-} (Na_2S_2O_3, \ about \ 3900 \ mg \ (S_2O_3^{2-}-S)/l) \ and \ NO_3^- (KNO_3, \ about \ 700 \ mg \ (NO_3^-N)/l) \] were used as electron donor and electron acceptor, respectively, in the experiments to investigate the effects of cultivation conditions on the growth and denitrification efficiency of *T. denitrificans* and the \(SO_3^{2-}\) generation rate, \(S_2O_3^{2-}-S\) consumption rate and \(NO_3^-\)N denitrification rate were compared under...
different conditions. All the conditions of the experiments were listed in Table 1.

In this study, SO$_4^{2-}$ generation rate was defined as the increased SO$_4^{2-}$ concentration per day, and S$_2$O$_3^{2-}$–S consumption and NO$_3$–N denitrification rates were the decreased S$_2$O$_3^{2-}$–S concentration and NO$_3$–N concentration per day in the culture, respectively.

Analytical methods All the following indices were analyzed once every other day in duplicate, and the chemicals used were of chemical grade.

Biomass was indicated by optical density at the wavelength of 650 nm (OD$_{650}$). OD$_{650}$ (DR/4000, Hach) and pH (Twin B-212, Horiba) could be measured directly with the obtained culture.

For the determinations of nitrate nitrogen (NO$_3$–N), sulfate (SO$_4^{2-}$) and thiosulfate sulfur (S$_2$O$_3^{2-}$–S), the sampled culture should be centrifuged at 5000 rpm for 15 min and the supernatant was used for the corresponding measurements.

The concentration of NO$_3$–N was measured with the method of UV spectrophotometry (DR/4000, Hach). Besides, SO$_4^{2-}$ was determined with Sulfaver 4 turbidimetric kit following the procedures developed by Hach company, and the classical iodimetry was used to quantify the amount of S$_2$O$_3^{2-}$–S remained in the culture.

All the data present were the average values of duplicate experiments.

RESULTS AND DISCUSSION

Influence of aeration on the growth of *T. denitrificans* The OD values increased to 0.100 and 0.200 after 10-day cultivation under aerobic and anaerobic conditions, respectively (Fig. 1). It seems that anaerobic condition is more suitable for the growth of this selected *T. denitrificans*.

The result also indicated that *T. denitrificans* 3870 used in this study was a kind of facultative microorganism. Compared with O$_3$, NO$_3$–N seemed to be more easily utilized by *T. denitrificans*, and the growth rate of *T. denitrificans* under anaerobic condition was two-times of that under aerobic condition. Thus anaerobic cultivation was applied in the following experiments.

Effect of trace element dosage on *T. denitrificans* As it can be observed in Fig. 2, the OD values increased to 0.24 after 10-day cultivation at the dosages of 40ml (OD=0.240) and 120 ml (OD=0.244) of trace element solution, with the OD value being 0.207 at the same time under no addition of trace element. In Fig. 2, the decreased OD value at 400 ml addition of trace element solution indicated that the role of basal mineral medium could not be replaced by trace element.

<table>
<thead>
<tr>
<th>No</th>
<th>Composition of culture medium</th>
<th>Characteristics of the culture and performance of <em>T. denitrificans</em></th>
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<tr>
<td>Trace</td>
<td>Na$_2$SO$_4•$5H$_2$O</td>
<td>KNO$_3$</td>
</tr>
<tr>
<td>element</td>
<td>(g/l)</td>
<td>(g/l)</td>
</tr>
<tr>
<td>solution</td>
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<td>(%)</td>
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<td>40(10%)</td>
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<td></td>
<td>0</td>
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</tbody>
</table>

All the above experiments were conducted in flasks with effective volume of 400 ml at 30°C, with basal mineral medium being added under anaerobic condition. The initial pHs were set at 7.0.

$^1$Data referred to the volume ratio of trace element solution to the total 400 ml of culture medium

$^2$OD values were the results of the cultures at 12$^{th}$ day after being inoculated with *T. denitrificans*
elements and too much trace elements might inhibit the growth of *T. denitrificans*. From the viewpoint of cost and efficiency, 40 ml of trace element solution was suggested to be the appropriate dosage for this kind of denitrificans, in which the contents of trace metals were more than those utilized by Sierra-Alvarez *et al.* in their culture media for the sulfur/limestone autotrophic denitrification process.

Equation (1) suggested that SO$_4^{2-}$ generation rate had some stoichiometric relationship with S$_2$O$_3^{2-}$-S consumption and NO$_3^-\text{-N}$ denitrification rates in the culture with *T. denitrificans* inoculated.

From Fig. 3, it can be seen that *T. denitrificans* exhibited more active under the condition of 40 ml dosage of trace element, resulting in higher SO$_4^{2-}$ generation, S$_2$O$_3^{2-}$-S consumption and NO$_3^-\text{-N}$ denitrification rates about 429.3, 147.6 and 37.3 mg/l·d, respectively, which were about 1.2 times of the corresponding rates under the condition of no trace element addition (Table 1). From Eq.(1), the stoichiometric values of the ΔS$_2$O$_3^{2-}$-S/ΔNO$_3^-\text{-N}$ and ΔSO$_4^{2-}$/ΔNO$_3^-\text{-N}$ ratios were calculated as 3.86 g/g and 11.58 g/g, respectively, which were quite similar to our experimental values (ΔS$_2$O$_3^{2-}$-S/ΔNO$_3^-\text{-N}$ = 3.8–3.9 g/g, ΔSO$_4^{2-}$/ΔNO$_3^-\text{-N}$=11.4–11.5 g/g) (Table 1). The total NO$_3^-\text{-N}$ removals at the 12th day were 54.1%, 65.9%, 68.9% and 38.8% for the four trace element additions, respectively (Table 1).

After 12-day cultivation, the theoretical generation of SO$_4^{2-}$ would be about 4224 mg/l and 5313 mg/l under the conditions of 0 ml and 40 ml of trace element solution added, with relative deviation (RD) from the measured SO$_4^{2-}$ concentrations being 0.19% and 3.03%, respectively. This difference might be largely contributed by the turbidimetric method applied for SO$_4^{2-}$ determination. The results of sulfur balance over cultivation time indicated that SO$_4^{2-}$ was the only one product in the cultivation and no other sulfur compounds generated. The final SO$_4^{2-}$ concentration, much higher than 1500 mg/l (500 mg (SO$_4^{2-}$-S) /l) and 2000 mg/l obtained in enriched mixed culture of autotrophic organisms, was in agreement with the initial inhibition concentration (5000 mg/l) found by Claus and Kutzner* for the pure culture of *T. denitrificans*. No inhibition was observed during the cultivation in all the experiments. In addition, the terminal average values of pHs, varied between 6.57
and 6.84 for all the conditions with initial pH=7, which lied between 6–9, the optimum pH range for *T. denitrificans*²⁹, implying that the microorganisms were in their normal state of growth.

**Effect of initial Na₂S₂O₃ concentration on *T. denitrificans*** In this study, S₂O₃²⁻–S was used as electron donor. So the selection of initial Na₂S₂O₃ concentration was important in the enhancement of the performance of *T. denitrificans*. According to the stoichiometry of Eq. (1), 10 g/l Na₂S₂O₃·5H₂O was appropriate for the denitrification of nitrate-contaminated water with 700 mg/l of NO₃⁻–N. Considering the utilization efficiency of S₂O₃²⁻–S by *T. denitrificans*, besides 10 g/l of Na₂S₂O₃·5H₂O, another two cases, with 15 g/l and 20 g/l of Na₂S₂O₃·5H₂O being added respectively, were investigated in parallel in the experiments.

Figure 4 showed the variations of OD values in the cultures under different initial Na₂S₂O₃ concentrations. It was obviously observed that 10 g/l Na₂S₂O₃·5H₂O was the optimal initial concentration with OD value =0.300 after 12-day cultivation, about 1.5 times of the OD value of 15 g/l Na₂S₂O₃·5H₂O condition after the same duration of cultivation. The biomass increment partly confirmed the validity of Na₂S₂O₃·5H₂O concentration in the basal mineral medium suggested by RIKEN.

The stoichiometric values of the ΔS₂O₃²⁻–S/ΔNO₃⁻–N and ΔSO₄²⁻/ΔNO₃⁻–N ratios under the conditions of 10 g/l and 15 g/l of Na₂S₂O₃·5H₂O varied between 3.7–3.8 g/g and 10.9–11.3 g/g (Table 1), which were also similar to the results of Oh *et al.*²⁹ and in accordance with the results of above section. Seen from Fig. 5, *T. denitrificans* showed very active under the condition of 10 g/l of initial Na₂S₂O₃·5H₂O, which could be obtained from the higher SO₄²⁻ generation rate, 536.7 mg/l·d, and S₂O₃²⁻–S consumption rates, 175.2 mg/l·d (Table 1), about 1.7 times of the corresponding rates under the condition of 15 g/l of initial Na₂S₂O₃·5H₂O. However, 15 g/l of initial Na₂S₂O₃·5H₂O was more suitable for *T. denitrificans* with NO₃⁻–N denitrification and NO₃⁻–N removal rate being considered. Too much S₂O₃²⁻–S addition (20 g/l Na₂S₂O₃·5H₂O) had an inhibitory effect on the growth and activity of *T. denitrificans*. And the total NO₃⁻–N removals after the 12th day were 47.7%, 80.7% and 25.0% under the conditions of 10, 15 and 20 g/l of Na₂S₂O₃·5H₂O, respectively (Table 1).

According to Eq. (1), the theoretical generation of SO₄²⁻ would be 6306 mg/l and 3777 mg/l under the conditions of 10 g/l and 15 g/l of initial Na₂S₂O₃·5H₂O, respectively. The results of the minor difference between the theoretical value and measured SO₄²⁻ concentration (with RD about 2.12% and 2.78%, respectively) also indicated that SO₄²⁻ was the only one product in the cultivation and no other sulfur compounds generated.

After 12-day cultivation, no inhibition was observed for the SO₄²⁻ concentration generated (6440 mg/l for 10g/l and 3670 mg/l for 15 g/l of initial Na₂S₂O₃·5H₂O) during the
cultivation period. And the terminal average pHs varied between 6.50 and 6.73 for all the conditions with initial pH=7, which also lied between 6–9, the optimum pH range for T. denitrificans27).

Effect of initial nitrate concentration on T. denitrificans In this study, NO$_3^-$ was used as electron acceptor. So the initial KNO$_3$ concentration may play an important role in the denitrification of T. denitrificans. According to the stoichiometry of Eq. (1), 5 g/l of KNO$_3$ was appropriate for the denitrificans with the consideration of 3900 mg/l of S$_2$O$_3^{2-}$ contained in the culture. Another concentration of KNO$_3$, 10 g/l, was conducted in parallel to inspect the effect of initial nitrate concentration on the denitrification efficiency of T. denitrificans.

Figure 6 showed the OD values vs time in the cultures under different initial KNO$_3$ concentrations. The result showed that 5 g/l KNO$_3$ was more suitable for the growth of T. denitrificans, and a slight decreased OD value (by 13%) could be observed if 10 g/l KNO$_3$ being applied in the culture after 12-day cultivation. The result was also in agreement with the suggested KNO$_3$ concentration by RIKEN in the basal mineral medium for T. denitrificans.

It can be seen from Fig. 7 and Table 1 that the rates of S$_2$O$_3^{2-}$ generation, S$_2$O$_3^{2-}$–S consumption and NO$_3^-$–N denitrification decreased with increase of initial KNO$_3$ concentration, which were 302.9 mg/l•d, 100.4 and 27.3 mg/l•d, respectively, under the condition of 5 g/l of initial KNO$_3$.

The stoichiometric values of the ΔS$_2$O$_3^{2-}$–S/ΔNO$_3^-$–N and ΔSO$_4^{2-}$/ΔNO$_3^-$–N ratios in these tests ranged between 3.7–4.0 g/g and 11.1–11.8 g/g, which were also similar to Oh et al.26) in accordance with the results of above sections. The terminal average pHs varied between 6.70 and 6.84 for all the conditions with initial pH=7, which also lied between the optimum pH range of 6–927). The overall NO$_3^-$–N removals were 48.2% and 37.5% under 5 g/l and 10 g/l of initial KNO$_3$, respectively.

All the results obtained under anaerobic conditions were summarized in Table 1. Under the arranged cultivation conditions, the biomass and its denitrification efficiency of T. denitrificans were different. The results indicated that the impact of the influence factors studied followed the order of aeration>Na$_2$S$_2$O$_3$>trace element≥KNO$_3$. It has been found that the initial S$_2$O$_3^{2-}$–S/NO$_3^-$–N ratio have some effect on T. denitrificans23), which couldn’t be quantified in this study and has been included in our further investigation. In order to make the mechanism of this denitrification process more clear, other nitrogenous compounds such as NO$_2^-$–N, besides NO$_3^-$–N, should also be considered and measured in this process.

CONCLUSIONS

From our present study, the T. denitrificans should be cultivated under anaerobic condition...
with respect to biomass yield and denitrification efficiency.

Among the main factors investigated, aeration, Na$_2$S$_2$O$_3$ dosage and trace element addition had obvious effects on the growth of *T. denitrificans*, SO$_4^{2-}$ generation rate, S$_2$O$_3^{2-}$ –S consumption rate and NO$_3^-$–N denitrification rate, following the order of aeration > Na$_2$S$_2$O$_3$ > trace element ≥ KNO$_3$. The initial S$_2$O$_3^{2-}$–S / NO$_3^-$–N ratio should also be taken into consideration, which was helpful to determine the concentrations of initial Na$_2$S$_2$O$_3$ and KNO$_3$ in the culture medium. The stoichiometric analysis of the results indicated that the enriched *T. denitrificans* performed fully and could tolerate a higher concentration of SO$_4^{2-}$ in the culture medium under the experimental conditions. Future work is necessary to disclose the causes of the efficiency difference under different conditions and to investigate the immobilization of *T. denitrificans* for its future and actual use in the treatment of nitrate-contained water or wastewaters.

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