Kinetics and physiological characteristics of autotrophic dentrification by denitrifying sulfur bacteria

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Abstract To study the kinetics and physiology of autotrophic denitrifying sulfur bacteria, a steady-state anaerobic master culture reactor (MCR) was operated for over six months under a semi-continuous mode and nitrate limiting conditions using nutrient/mineral/buffer (NMB) medium containing thiosulfate and nitrate. Characteristics of the autotropic denitrifier were investigated through the cumulative gas production volume and rate, measured using an anaerobic respirometer, and through the nitrate, nitrite, and sulfate concentrations within the media. The bio-kinetic parameters were obtained based upon the Monod equation using mixed cultures in the MCR. Nonlinear regression analysis was employed using nitrate depletion and biomass production curves. Although this analysis did not yield exact biokinetic parameter estimates, the following ranges for the parameter values were obtained: μ_{max} =0.12-0.2 hr^{-1}; k=0.3-0.4 hr^{-1}; K_s=3-10mg/L; Y_{NO3}=0.4-0.5mg Biomass/mg NO_3^--N. Inhibition of denitrification occurred when the concentrations of NO_3^--N, and SO_4^{2-} reached about 660mg/L and 2,000mg/L, respectively. The autotrophic denitrifying sulfur bacteria were observed to be very sensitive to nitrite but relatively tolerant of nitrate, sulfate, and thiosulfate. Under mixotrophic conditions, denitrification by these bacteria occurred autotrophically; even with as high as 2 g COD, autotrophic denitrification was not significantly affected. The optimal pH and temperature for autotrophic denitrification was about 6.5-7.5 and 33-35 °C, respectively.

Keywords Autotrophic sulfur bacteria; Denitrification; Inhibition; Kinetics; Thiobacillus

Introduction

Many heterotrophic bacteria can reduce nitrate by utilizing organic substrates such as methanol, ethanol, and acetate for the conversion of nitrate to nitrogen gas under anoxic conditions. Denitrification can also be carried out by autotrophic sulfur bacteria which use a variety of reduced sulfur compounds (S^{2-}, S^o, $S_2O_3^{2-}$, $S_4O_6^{2-}$, SO_3^{2-}) instead of organic compounds while reducing nitrate. These autotrophs may be used as a part of water and/or wastewater treatment processes to remove nitrate if an exogenous sulfur source and an anaerobic environment are provided (Koenig *et al.*, 1996; Bill Batchelor *et al.*, 1978; Günter Claus *et al.*, 1985). Many researchers studied autotrophic denitrification in the presence of elemental sulfur or thiosulfate as electron donors to denitrify nitrate ion using pure cultures of *Thiobacillus denitrificans, Thiomicrospira denitrificans, Thiobacillus versutus, Thiosphaera pantotropha,* and *Paracoccus denitrificans* (Kerry L. Sublette *et al.*, 1987; Brian A. Till *et al.*, 1998; Anje Timmer-Ten Hoor, 1981; J. G. Kuenen, 1979; Jan M. Visser *et al.*, 1997; Ying-Chien Chung *et al.*, 1997). This approach of using a pure culture has little practical value because real biological treatment processes employ mixed cultures, while there exist no previous publications concerned with the autotrophic denitrification kinetics and physiology using an acclimated mixed culture grown in thiosulfate and nitrate medium.

The specific objectives of the work presented here were 1) to find the bio-kinetic constants based upon the Monod model, and 2) to seek information on the characteristics of autotrophic sulfur denitrifying bacteria, such as the effect of chemicals, pH and temperature. To accomplish these objectives, the authors adapted the methodology of using an

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automatic anaerobic respirometer. This offers a useful means of precisely and continuously measuring gas production by the microorganisms within a specific wastewater for any duration of time. Also, this continuous readout of gas production provides an indication of lag, toxicity, or any abnormalities in the biodegradation reactions. This study was part of a broad research program examining the biological denitrification methods for the treatment of wastewaters with low C/NO_3^--N ratios using reduced sulfur compounds (hydrogen sulfide, thiosulfate, and elemental sulfur).

Materials and methods

Steady-state anaerobic culture.

A steady-state enriched master culture reactor (MCR) was maintained to provide consistent and repeatable cultures for serum bottle batch tests to determine the fate and effects of chemicals on denitrification. A schematic diagram of the experimental setup is shown in Figure 1. The MCR was initially inoculated with the anaerobic sludge from a municipal wastewater plant and the ensuing culture was enriched with 4g/l of $Na_2S_2O_3 \cdot 5H_2O$ and 2g/l of KNO3 in the nutrient/mineral/buffer (N/M/B) medium mentioned below. The MCR, with a 4L culture volume, was operated in a constant temperature room (33–35oC) and according to the fill-and-draw mode with a 2-day feeding cycle of wasting and feeding 25% of the total volume (1L) semi-continuously. The solid retention time (SRT) was fixed at 8 days. The MCR was mixed with a magnetic stirrer. Since the anaerobic culture was enriched with thiosulfate and nitrate without organics, most of the biomass population consisted of autotrophic denitrifying sulfur bacteria including obligately and facultatively autotrophic colorless sulfur bacteria as well as heterotrophs. Necessary nutrients, minerals, and buffer (NMB) were fed, with $Na_2S_2O_3 \cdot 5H_2O$, and KNO_3, and contained the following chemicals (per litre of distilled water): 1g NH_4Cl, 2g KH_2PO_4, 0.8g $MgSO_4 \cdot 7H_2O$, 2g $NaHCO_3$, 50.0mg Disodium EDTA, 11.0mg NaOH, 7.34mg $CaCl_2 \cdot 7H_2O$, 5.0mg $FeSO_4 \cdot 7H_2O$, 2.5mg $MnCl_2 \cdot 2H_2O$, 2.2mg $ZnSO_4 \cdot 7H_2O$, 0.5mg $CoCl_2(6H_2O$, 0.5mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.2mg $CuSO_4 \cdot 5H_2O$, (Ronald M. ATLAS, 1993). During the feeding cycle, the pH consistently dropped from 7.0 to about 6.7. The denitrification process was monitored by gas production and nitrate and sulfate concentrations in the MCR. Also

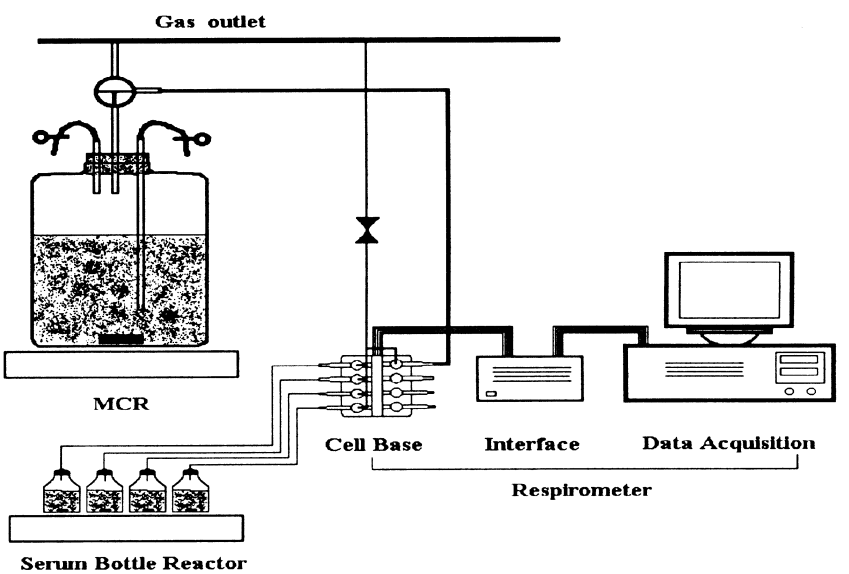

Figure 1 Schematic diagram of the MCR, batch serum bottle reactors and respirometer system

the suspended solids (SS), volatile suspended solids (VSS) and dissolved organic carbon (DOC) were measured. The draw-and-fill operation lasted for 5 months. Nitrate was completely depleted within each feeding cycle while the VSS concentrations remained relatively constant.

Experimental methods.

At the end of the MCR feeding cycle, the acclimated MCR culture and NMB with different known chemical concentrations were added to each of a number of 550mL serum bottle reactors according to Table 1. After transferring the culture, vessels were flushed with nitrogen gas and connected to an automatic respirometer to measure gas production over time. The batch assay was carried out in a constant temperature room (33–35°C). Phase 1 tests were conducted to determine the bio-kinetic parameters in the absence of substrate inhibition. To obtain complete nitrate conversion and cell production profiles, two reactors with nominal initial nitrate concentrations were monitored in terms of their biomass production and nitrate, nitrite, and sulfate ion concentrations until the nitrate was depleted. The remaining seven phases of the serum batch tests were conducted to obtain the characteristics of the sulfur-oxidizing autotrophic denitrifier. Phase 2 tests were conducted to determine the optimum NO_3^--N/$S_2O_3^{2-}$ ratio under real conditions (the theoretical NO_3^--N/$S_2O_3^{2-}$ ratio is 5.0 from the stoichiometric calculation). During phases 3 and 4, inhibition due to nitrate and sulfate, respectively, were studied using the optimum NO_3^--N/$S_2O_3^{2-}$ ratio from phase 2. The effect of organics (glucose) and nitrite on the autotrophic denitrification were tested in phases 5 and 6. Finally, phases 7 and 8 were performed to determine the effect of temperature and pH. All tests were duplicated with a total liquid volume of 520mL. Detailed experimental conditions for each step are shown in Table 1. Abiotic (i.e., biomass-free), and blank (i.e., substrate-free) controls were also prepared as previously described. Initial and final nitrate, nitrite, and sulfate concentrations were measured at each phase.

Analytical methods.

The mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were measured as a biomass concentration according to the Standard Methods (APHA, 1992). Analysis of nitrite, nitrate, and sulfate ion concentrations was carried out by

Table 1 Set-up for batch test.

Phase	Materials added	Remarks
I	Culture(50 mL) + 2 levels of NO_3^--N (230, and 140 mg/L)	$S_2O_3^{2-}/NO_3^-$-N = 8.15
II	Culture(20 mL) + 6 levels of $S_2O_3^{2-}/NO_3^-$-N ratios (1.63, 3.26, 4.89,6.51, 8.15, and 9.77)	Theoretical $S_2O_3^{2-}/NO_3^-$-N = 5 (266 mg NO_3^--N/L)
III	Culture (20 mL)+5 levels of NO_3^--N (95.2, 285.6, 475.9, 666.4, and 856.7 mg /L)	$S_2O_3^{2-}/NO_3^-$-N = 6.51
IV	Culture (20 mL)+ 6 levels of SO_4^{2-} (0, 1, 2, 4, 6, and 8 g/520mL)	$S_2O_3^{2-}/NO_3^-$-N = 6.51 (266 mg NO_3^--N/L)
V	Culture (20 mL) + 6 levels of COD (0, 50, 100, 150, 200, and 2000 mg/L)	$S_2O_3^{2-}/NO_3$N = 6.51 (266 mg NO_3N/L)
VI	Culture (20 mL)+ 5 levels of NO_2N (0-200 mg/520mL)	$Na_2S_2O_3$(5H_2O 2 g/520mL
VII	Culture (20 mL)+ 6 levels of pH(6, 6.5, 7, 7.5, 8, and 9)	$S_2O_3^{2-}/NO_3$N = 6.51 (190 mg NO_3N/L)
VIII	Culture (20 mL)+ 4 levels of Temp.(25, 30, 35, and 40 °C)	$S_2O_3^{2-}/NO_3$N = 6.51 (190 mg NO_3N/L)

Ion Chromatography (model DX-120, USA). Dissolved organic carbon (DOC) for measuring the excreted organic carbon was measured after filtration with a 0.45μm mixed cellulose ester filter using a TOC analyzer (model of Dohrmann DC-180). Cumulative gas production from the MCR and serum bottles was measured by using the respirometer system (AER-200, Challenge Environmental Systems, Inc., USA).

Results and discussion

Enriched Master Culture Reactor (MCR).

The MCR was operated semi-continuously by wasting 1L of the culture and feeding the same volume every other day for 5 months allowing a steady-state condition. A simplified stoichiometry for the MCR's energy-producing reactions, using thiosulfate as an electron donor and nitrate as the electron acceptor, is as follows:

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

$$2HCO_3^- + 2H^+ \rightarrow 2CO_2 + 2H_2O \quad (2)$$

The molar ratio of $S_2O_3^{2-}$ to NO_3N in the medium was adjusted to 6.51 under nitrate-limiting conditions. From the equations, when 8 moles of nitrate react, 4 moles of nitrogen gas are produced by biological denitrification and 2 moles of CO_2 by the production of hydrogen ions. Results from the steady-state operation of the MCR showed 340(30 mg/L of MLSS, 130(20 mg/L of MLVSS, 4000(40 mg/L of SO_4^{2-}, 1100-1200 mL/4L of gas production (Figure 2), 30-40 mg C/L of DOC, a pH of 6.8-6.6, and a NO_3N concentration of less than 5mg/L when the gas production ceased. Nitrite did not accumulate during operation. Although the MCR operated with the medium of thiosulfate and nitrate without organics, a DOC of 30-40 mg C/L accumulated in the steady-state MCR due to cell death and/or lysis.

Estimation of kinetic constants.

The Monod model was tentatively proposed to describe the rate of substrate oxidation (equivalent nitrate reduction in this case) and cell growth during exponential growth. When nitrate was provided as substrate under nitrate limiting conditions, the kinetics of anaerobic growth of the sulfur oxidizing denitrifier and the biodegradation rates in the batch reactor were described by the Monod expression.

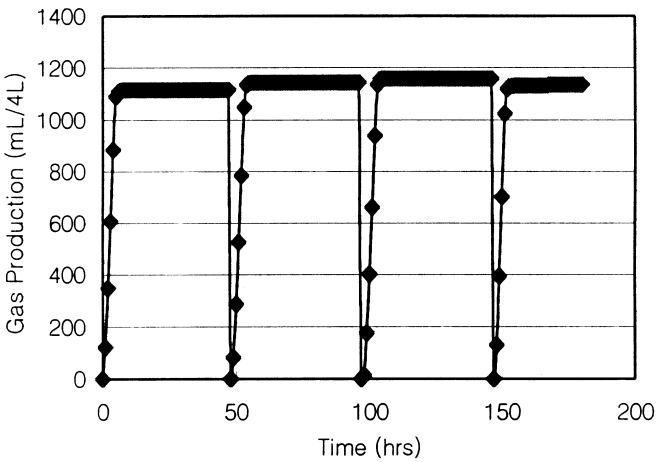

Figure 2 Gas production during feeding cycles of the MCR

$$dS/dt = -k S X/(K_s + S) \quad (3); \quad dX/dt = Y(-dS/dt) - k_d X = Y k S X/(K_s + S) - k_d X \quad (4)$$

where, dS/dt = substrate conversion rate, mg substrate/L-hr
dX/dt = net rate of bacterial growth, mg VSS/L-hr
S = concentration of substrate, mg/L
X = specific biomass concentration, mg/L
K_s = substrate concentration at one half the maximum rate, mg/L
k = maximum substrate utilization rate, mg substrate/mg VSS-hr
Y = growth yield coefficient, mg VSS formed/mg substrate converted
k_d = decay coefficient, hr^{-1}

The sequential reactions of denitrification can be simplified as $NO_3^- \rightarrow NO_2^- \rightarrow N_2$. This model, however, assumed one reduction step from nitrate to nitrogen gas because very low concentrations of nitrite were detected throughout the reaction time in the serum bottle. The decay term (k_d) in eq. (4) was neglected when the growth was in the exponential phase. When using batch culture techniques, equations (3) and (4) must be solved simultaneously for S and M to model the change of substrate and biomass concentrations in the reactor. This model is a system of two initial-value, non-linear ordinary differential equations that were solved numerically using the Runge-Kutta method. A search for the values of the model parameters was carried out using an algorithm for least-squares estimation. This algorithm finds a local minimization of the sum of the square weighted errors (SSWE). As shown in Figure 3, a very good fit between the data and the model predictions was obtained for two batch studies. The best fit mathematical-model solution to the data from the batch test was obtained with the following parameters : μ_{max} =0.12-0.2 hr^{-1}; k=0.3-0.4 hr^{-1}; K_s=3-10mg/L; Y_{NO3}=0.4-0.5mg Biomass/mg NO_3^--N. The best-fit solution of the mathematical model and the experimental data are shown in Figure 3.

Günter Claus *et al.* (1985) made use of a pure culture of *Thiobacillus denitrificans* in a continuous run to derive its biokinetic parameters, which were found as μ_{max} =0.11 hr^{-1}; K_s=0.2 mg/L; Y_{NO3}=0.57 mg Biomass/mg NO_3^--N. These kinetic parameters were similar to those of this work although here they were obtained in a batch run using a mixed culture. These kinetic parameters may be used as a guide for developing and designing an autotrophic denitrification process using thiosulfate as an electron donor.

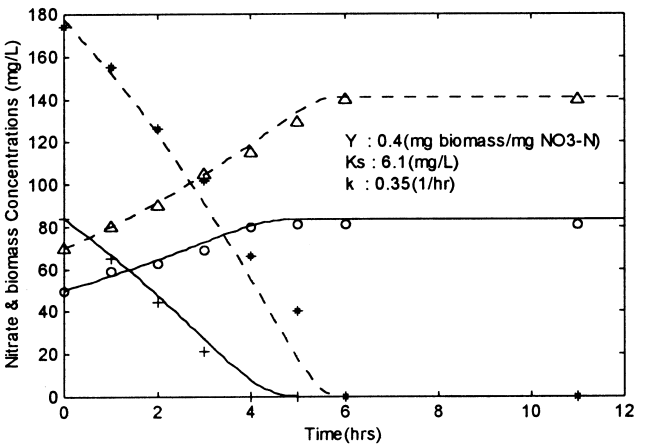

Figure 3 Nitrate depletion and biomass growth profiles in two batch reactors
(The symbols show measured data; lines show model prediction)

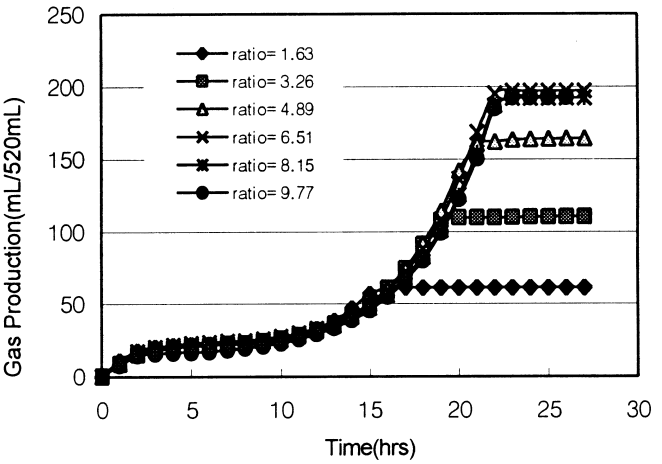

Figure 4 Gas production profile for different $S_2O_3^{2-}/NO_3^-$-N ratios

Effect of S_2O3^{2-}/NO3$^-$-N ratio.

In all figures with gas production data throughout the paper, a rapid gas production was seen during the initial periods (0-3hrs). That gas was identified as carbon dioxide, which was formed when the hydrogen ions react with bicarbonate existing in the NMB solution. After this, a lag period of about 10 hrs, log growth, and then the stationary phase can be seen. Since nitrite did not accumulate and the amount of gas, mostly N_2 and CO_2, produced in the serum bottle agreed closely with the amount from the NO_3^--N conversion, it was easy to observe nitrate removal from the gas production results. In order to find the suitable ratio of $S_2O_3^{2-}/NO_3^-$-N for denitrification, phase 2 was conducted. As shown in Figure 4, in the case where the ratios are 6.51, 8.15, 9.77, the denitrification reached completion, but in all other cases the nitrate was incompletely denitrified due to a shortage of thiosulfate as an electron donor (Figure 4). Thus, the following experiments were conducted under nitrate-limiting conditions ($S_2O_3^{2-}/NO_3^-$-N ratio of 6.51).

Effect of nitrate concentrations on denitrification.

Figure 5 shows the gas production with different initial nitrate concentrations over time. In these experiments, inhibition of denitrification occurred at concentrations greater than 660 mg/L NO_3N, which was indicated by the final nitrate concentrations, different cumulative gas production rates, and gas production volume. In the case of 95.2, 285.6, and 475.9mg NO_3^--N/L, complete denitrification was seen. In the case of 666.4, 856.7mg NO_3-N/L, the remaining nitrate concentrations were 75.9 and 290mg NO_3-N /L, respectively, while the sulfate concentrations were lower than 2g/L in all cases. This result is certainly due to a drop in the pH to lower than 6.1 resulting from H^+ ion production in the autotrophic denitification process. Some researchers have suggested that nitrate inhibition of heterotrophic denitrification is actually due to the toxicity of accumulated nitrite, specifically the unionized nitrous acid species (Van Versefeld, 1977). The concentration of nitrite, however, was maintained at very low levels in all cases throughout the experiment. Thus, it may be assumed that this inhibitory mechanism for this autotrophic sulfur denitrifier is not due to product inhibition, but rather a substrate inhibition due to excessive concentrations of nitrate.

Effect of sulfate ion concentration on denitrification.

Sulfate is the end product of the sulfur oxidizing denitrification process. As shown in Figure 6, the inhibition began at concentrations above 2g/L, which was indicated by a lower

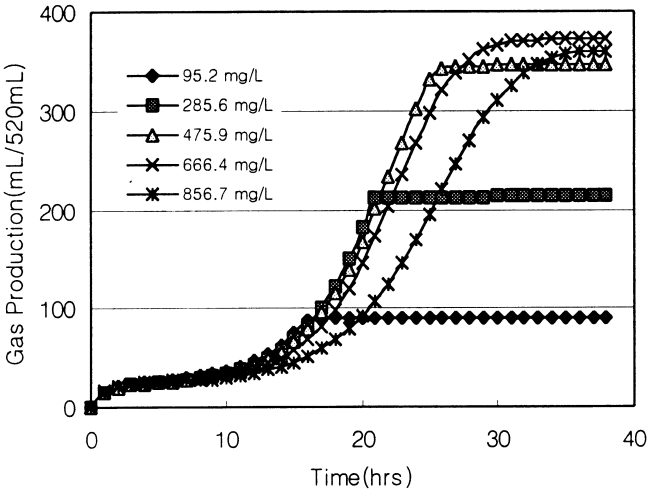

Figure 5 Gas production profile for different NO_3^--N concentrations

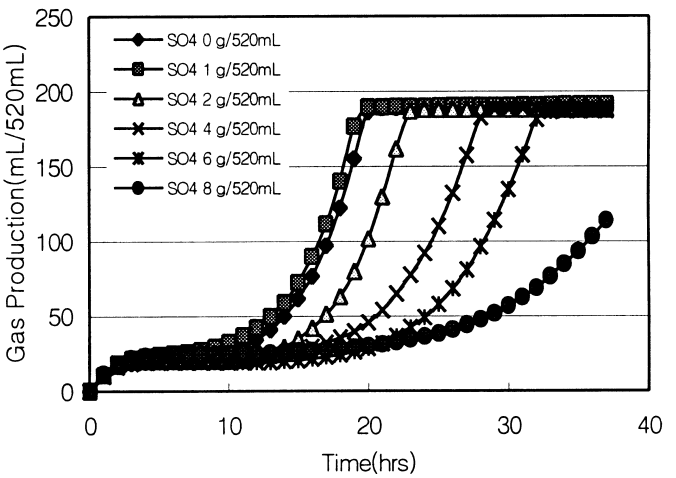

Figure 6 Gas production profile for different sulfate concentrations

gas production rate with a longer lag phase. Autotrophic denitrifying sulfur bacteria were less sensitive to sulfate than to nitrate or nitrite. Ten moles of sulfate correspond to the reduction of 8 moles of nitrate from the stoichiometry. This process results in the production of considerable amounts of sulfate when high nitrate containing wastewater is treated, thus confining the applicability of the process to certain areas. However, sulfate is not injurious to health and it is much easier to precipitate in contrast to nitrate (Günter Claus *et al.*, 1985).

Effect of COD.

Figure 7 shows the effects of COD (glucose) on autotrophic denitrification. These tests were carried out under anaerobic, mixotrophic conditions using excess thiosulfate concentrations and different initial CODs as electron donors with fixed nitrate concentrations. COD values below 200 mg/L did not inhibit autotrophic denitrification and at concentrations as high as 2 g, denitrification was not significantly affected, though it showed a slightly lower gas production rate with a longer lag phase compared with the other cases. This information is very important because most wastewater contains a certain level of organics.

Figure 7 showed that the gas production increased with an increase in the COD even though the initial nitrate concentration was the same in all serum bottles, which may be explained by the fermentation of the glucose resulting in a greater pH drop and alkalinity decrease. Under heterotrophic conditions without thiosulfate, denitrification occurred after an acclimation period of 20 hrs (data not shown).

Inhibition of Nitrite.

The different gas production rates at low initial nitrite concentrations (shown in Figure 8) indicates that nitrite, as an intermediate of denitrification, exhibited a strong inhibition at rather low concentrations but complete denitrification occurred in the range of 0-150mg/L. Glass *et al.* (1998) observed that nitrite concentrations of 250 mg/L NO_2^--N could inhibit heterotrophic denitrification at a near-neutral pH.

Effects of pH and temperature on denitrification.

The pH optimum for most environmental strains of denitrifying bacteria has been reported to be between 7 and 8 (Knowles, 1982). Also, all autotropic colorless denitrifying sulfur bacteria are mesophillic (25-35 ºC) and have an optimum pH range of 6-9 (John *et al.*, 1994). As can be seen from Figure 9, the optimal pH condition for autotrophic denitrification was about 6.5-7.5. At a pH of 6 and 9, the denitrification reaction was completely

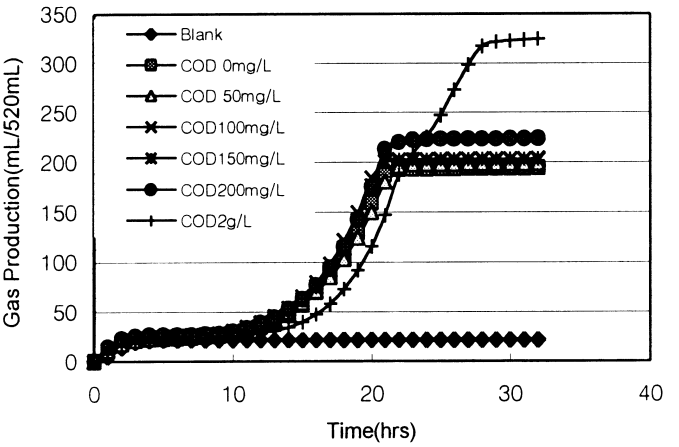

Figure 7 Gas production profile for different COD concentrations under mixotrophic conditions

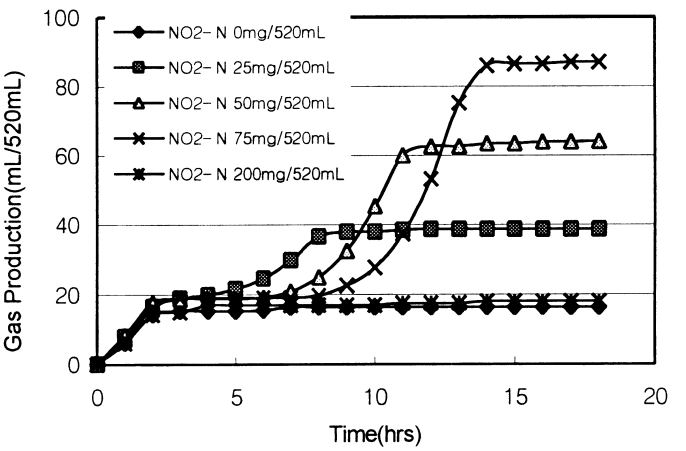

Figure 8 Gas production profile for different nitrite concentrations

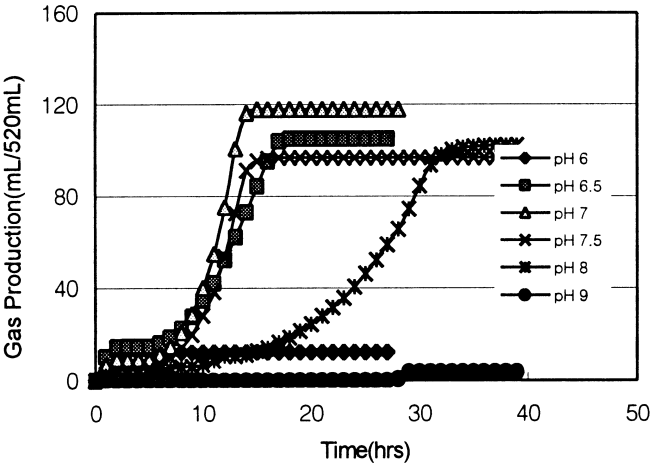

Figure 9 Gas production at various pHs

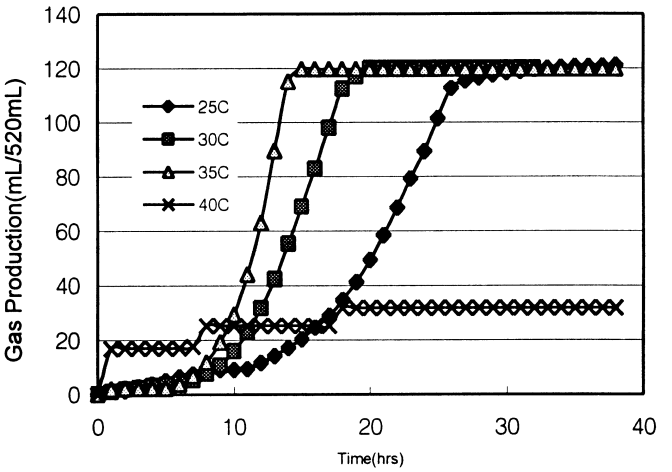

Figure 10 Gas production for different temperatures

stopped. Since two moles of H^+ are produced for every eight moles of nitrate that are reduced to nitrogen gas according to the stoichiometry of the autotrophic denitrification, the pH will drop during denitrification in an inadequately buffered system. If these bacteria are applied to an engineering treatment process, consideration has to be given to buffer the wastewaters to maintain a near-neutral pH (pH 6.5-8.0). In addition, Figure 10 shows the temperature effects. The optimal temperature condition for autotrophic denitrification was 33-35 °C while it was completely inhibited at 40 °C.

Conclusion

A steady-state mixed culture was employed to determine the bio-kinetic parameters and characteristics of autotrophic sulfur denitrifying bacteria. The following parameters were found using a batch experiment under nitrate limiting conditions: μ_{max} =0.12-0.2 hr^{-1}; k=0.3-0.4 hr^{-1}; K_s=3-10mg/L; Y_{NO3}=0.4-0.5mg Biomass/mg NO_3^--N. Inhibition of denitrification by nitrate occurred at concentrations greater than 660mg N/L, with the inhibitory mechanism being substrate inhibition due to its excessive concentrations. In the case of sulfate, the inhibition began at concentrations above 2g/L with a longer lag phase. Nitrite inhibition of denitrification occurred at very low concentrations. Under mixotrophic condi-

tions, denitrification occurred autotrophically even at as high as 2 g COD/L. The optimal pH and temperature conditions for autotrophic denitrification were about 6.5-7.5 and 33-35 ^{o}C, respectively. Denitrification was completely inhibited at a pH of 6 and 9 and a temperature of 40 ^{o}C. These kinetic parameters and physiological data provide a foundation for process analysis and design for the optimal removal of nitrate in autotrophic biological treatment systems.

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