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Potential of biological sulphur recovery from thiosulphate by haloalkaliphilic *Thioalkalivibrio denitrificans*

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ABSTRACT

The aim of this study was to investigate the potential for elemental sulphur recovery from sulphurous solutions under aerobic and anoxic conditions by haloalkaliphilic *Thioalkalivibrio denitrificans* at 0.8–19.6 g S₂O₃²⁻-S L⁻¹ and 0.2–0.58 g NO₂⁻ L⁻¹, respectively. The experiments were conducted as batch assays with haloalkaline (pH 10 and ≥ 14 g Na⁺ L⁻¹) thiosulphate solution. Aerobically, the highest biotransformation rate of thiosulphate obtained was 0.03 h⁻¹ at 8.5 g L S₂O₃²⁻-S. Based on Monod model, the maximum substrate utilisation rate (q_m) was 0.024 h⁻¹ with half saturation constant (K_s) 0.42 g S₂O₃²⁻-S L⁻¹ at initial [S₂O₃²⁻-S] of 14 g L⁻¹. S⁰ accumulated at [S₂O₃²⁻-S] ≥ 1.5 g L⁻¹ (10% yield at initial 9.5 g S₂O₃²⁻-S L⁻¹) and the highest S⁰ yield estimated with the model was 61% with initial [S₂O₃²⁻-S] of 16.5 g L⁻¹. Anoxically, the maximum nitrite removal rate based on Monod modelling was 0.011 h⁻¹ with K_s = 0.84 g NO₂⁻ L⁻¹. Aerobically and anoxically the maximum specific growth rates (μ_m) were 0.046 and 0.022 h⁻¹, respectively. In summary, high-rate aerobic biotransformation kinetics of thiosulphate were demonstrated, whereas the rates were slower and no S⁰ accumulated under anoxic conditions. Thus, future developments of biotechnical applications for the recovery of S⁰ from haloalkaline streams from the process industry should focus on aerobic treatment.

Highlights

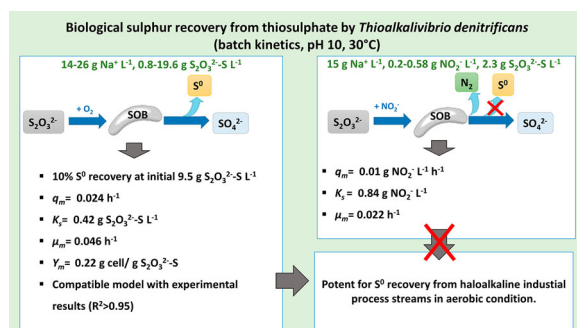
- Haloalkaline S₂O₃²⁻ biotransformations kinetics by *Thioalkalivibrio denitrificans*
- Aerobic thiosulphate-S bioconversion up to 0.024 h⁻¹ with K_s = 0.42 g S₂O₃²⁻-S L⁻¹
- 10% S⁰ yield with initial 9.5 g S₂O₃²⁻-S L⁻¹ in aerobic condition
- Anoxic NO₂⁻ removal up to 0.01 h⁻¹ with K_s = 0.84 g NO₂⁻ L⁻¹

ARTICLE HISTORY

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Biotransformation of sulphurous compounds; denitrification; haloalkaline sulphurous solution; sulphur oxidising bacteria; sulphur recovery



1. Introduction

To support circular economy, the recovery and recycling of sulphurous compounds (i.e. HS⁻, S₂O₃²⁻) from different industrial streams (i.e. pulp and paper, petrochemical, mining and fertiliser) are gaining increasing

attention [1–5]. These compounds can also cause environmental and health concerns if released without treatment [6]. From the economic point of view, the use of sulphurous compounds contributes directly to operational costs, by raising the need for other chemicals and water as well as by causing corrosion [7]. As

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an example, maintaining the Na/S balance is crucial for efficient chemical pulping, and recovery of sulphur compounds from the process would be beneficial. It would decrease the requirement of additional Na supply because S accumulates in the process more than Na [8]. In pulping, the Na/S balance is generally controlled by purging the electrostatic precipitator-ash (ESP-ash) from the recovery boiler which results in reduction of Na and S in the recycled streams [9]. Complementing large-scale chemical processes with the biological recovery of excess sulphur is a promising approach [10]. Oftentimes the industrial streams, like the ones from pulp and paper industry, have alkaline pH and high salt content [11]. Moreover, the oxygen supply in aerobic treatment of these concentrated solutions might become process limiting. Therefore, biological processes for sulphur recovery would preferably be based on anoxic or microaerophilic processes. Haloalkaline process/wastewater streams may contain other constituents in addition to sulphurous and sodium-based compounds. These streams such as spent sulphidic caustic from the petrochemical industry contains phenols and benzene that may be toxic to chemolithotrophic SOB as well as organo-sulphur compounds (for example, methanethiol) that potentially interfere with biotransformations of sulphurous compounds [12–14].

Haloalkaliphilic sulphur oxidising bacteria (SOB) are potent organisms for sulphur recovery from industrial streams. Haloalkaliphilic SOB use inorganic sulphur compounds including sulphide, polysulphide, thiosulphate, polythionates and elemental sulphur as electron donor [15]. Most SOB grow aerobically. Biooxidation of partially oxidised sulphurous compounds coupled to NO_3^- or NO_2^- reduction is possible under anoxic conditions by some haloalkaliphilic species of SOB [16,17]. Oftentimes, oxygen supply is rate limiting in industrial scale and also results in high costs, thus using SOB that biotransforms HS^- under anoxic condition is of interest.

Haloalkaliphilic SOB are abundant in salt and soda lakes. Among these microorganisms, bacteria in the genus *Thioalkalivibrio* can use a wider range of reduced sulphurous compounds (HS^- , $\text{S}_2\text{O}_3^{2-}$, S_6^{2-} , S_8 , SO_3^{2-} , $\text{S}_3\text{O}_6^{2-}$, $\text{S}_4\text{O}_6^{2-}$ and $\text{S}_5\text{O}_6^{2-}$) as a source of energy than for example those in the genus *Thiomicrospira* (HS^- , $\text{S}_2\text{O}_3^{2-}$, S_6^{2-} , S_8 , $\text{S}_4\text{O}_6^{2-}$ and $\text{S}_5\text{O}_6^{2-}$) [16]. Moreover, *Thioalkalivibrio* spp. has tolerance to high salinity (e.g. up to 4.3. M Na^+) and alkaline pH (up to 10.6) [15,18]. *T. denitrificans* uses oxygen or nitrite/nitrous oxide as an electron acceptor during oxidation of sulphurous compounds in microaerophilic and anoxic environments, respectively [17] (for a review, see [16]). Therefore, it can be a better

option with haloalkaline sulphurous solutions than for example aerophilic *T. versutus*. Besides oxidising sulphide to sulphate, *T. denitrificans* can also disproportionate partially oxidised sulphur oxyanions to elemental sulphur and sulphate [17] and was, for these reasons, selected as a model organism for this study. Elemental sulphur would be the desired product of sulphide conversion due to its separability from liquid phase and potential uses in various fields [19]. For example, the produced elemental sulphur could be used as a fertiliser or electron acceptor for denitrification [19–21]. The biological sulphur recovery process Thiopaq (Shell-Paques) in which HS^- is converted to S^0 by SOB in the presence of oxygen. This process can be used internally or for fertiliser production has been applied to recover sulphur from natural gas, refinery gas and synthetic gas since 1993 [22]. Moreover, S^0 can be applied in mining and metallurgy for the recovery and removal of metals from wastewaters via biosulphidogenesis [23]. Due to the hydrophilic nature of biologically produced sulphur, it is more readily biologically available than chemically produced sulphur [19,20].

The aim of this study was to determine thiosulphate biotransformation potential by microaerophilic/denitrifying *T. denitrificans* under haloalkaline conditions and for the recovery of elemental sulphur from saline and alkaline sulphurous streams. In case of toxic concentration of HS^- , chemical oxidation of to $\text{S}_2\text{O}_3^{2-}$ could be used as pre-treatment prior to thiosulphate bioconversion step [21]. Therefore, thiosulphate was selected as a model sulphurous compound of this study. The biotransformation kinetics of *T. denitrificans* have not been comprehensively studied and especially not at high (up to 19.6 g L^{-1}) thiosulphate concentrations. The earlier studies on *T. denitrificans* have focused on the growth kinetics [17], pH limitation and N_2O reducing activity [17]. The sensitivity of *T. denitrificans* to NO_2^- has been reported by Sorokin et al. [17], but anoxic kinetics or the potential of elemental sulphur production have not been investigated. Therefore, the specific objectives of this study were the following: (i) determination of the biotransformation rates of thiosulphate and nitrite by *T. denitrificans* under aerobic and anoxic conditions, respectively; (ii) determination of the kinetics of elemental sulphur and sulphate formation at different initial concentrations of thiosulphate in aerobic condition; (iii) determination of elemental sulphur production yield by *T. denitrificans* at a chosen concentration by in aerobic conditions; (iv) determination of qPCR-based growth kinetics and yields of *T. denitrificans* in presence of oxygen or nitrite, and (v) model fitting of aerobic biotransformations.

2. Materials and methods

2.1. Inoculum and growth medium

Thioalkalivibrio denitrificans strain ALJD was obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ). The stock culture was routinely grown with the 925 alkaliphilic sulphur respiring medium recommended by DSMZ on an orbital shaker at $30 \pm 1^\circ\text{C}$ and 150 rpm under aerobic condition [24]. The growth medium included: $20 \text{ g L}^{-1} \text{ Na}_2\text{CO}_3$, $10 \text{ g L}^{-1} \text{ NaHCO}_3$, $5 \text{ g L}^{-1} \text{ NaCl}$, $1 \text{ g L}^{-1} \text{ K}_2\text{HPO}_4$, $0.5 \text{ g L}^{-1} \text{ KNO}_3$, $0.05 \text{ g L}^{-1} \text{ MgCl}_2$ and 2% (v/v) trace element solution (see preparation from [24]). As energy source, 4.5 g L^{-1} sterile filtered ($0.2 \mu\text{m}$ polyethersulfone membrane syringe filter, VWR International, North America) S_2O_3 stock solution was added to the medium of the stock culture. The inoculum used during both aerobic and anoxic experiments was actively growing. The biotransformation activity of the stock culture under anoxic condition (nitrite as electron acceptor) was seen to decrease by repeatedly transferring the culture to a fresh anoxic medium (data not shown), thus the aerobically grown stock culture was used as inoculum during all experiments.

The stock solution of nitrite (as NaNO_2) that was added to the cultures at the beginning of the anoxic experiments, was purged with N_2 gas and sterile filtered ($0.2 \mu\text{m}$ polyethersulfone membrane syringe filter, VWR International, North America) in an anoxic chamber. In addition, the cultures (including the inoculum) were purged with N_2 gas prior to the addition of nitrite stock in the anoxic chamber.

2.2. Aerobic biotransformation experiments with thiosulphate

2.2.1. Kinetic experiments

The experiments in the presence of air were implemented as liquid cultures with a working volume of 100 mL in 250 mL Erlenmeyer flasks [24]. To enable air transfer to the flasks, the caps were loosened slightly. The alkaline growth medium was the same as described in Section 2.1. The culture preparation was done as previously described by Hajdu-Rahkama et al. [24]. In brief, different concentrations (0.8, 1.5, 3, 6, 8.5, 14, 16.5 and 19.6 g L^{-1}) of thiosulphate-S were added to duplicate cultures with 10% (v/v) inoculum taken from the stock culture. All flasks were incubated for 14 days in an orbital shaker at 150 rpm and 30°C . In order to ensure comparability of the kinetic results at the different thiosulphate-S concentrations, the inoculum used was in the same growth phase.

2.2.2. Determination of sulphur formation

Quantitative determination of elemental sulphur formation under aerobic conditions was implemented as a separate batch experiment to enable validation of kinetic modelling of the elemental sulphur formation. The contents of the medium and growth phase of the 10% (v/v) inoculum were the same as used during the aerobic kinetic experiments (Section 2.2.1). Altogether 12 identical cultures were prepared with 9.5 g L^{-1} concentration of $\text{S}_2\text{O}_3^{2-}\text{-S}$ which was a mid-range concentration used during the kinetic experiments. After the removal of sample (6 mL) for sulphur compounds determination by ion-chromatography, the full solid content was collected by vacuum filtration of the whole culture volume. This sacrificial sample collection was carried out from duplicate cultures every second day. During this incubation, the temperature and shaking were the same as of the inoculum and the duration of the experiment was 10 days.

2.3. Anoxic experiments in the presence of nitrite

Anoxic experiment was implemented to compare the thiosulphate transformation kinetics with nitrite as an electron acceptor to the rates obtained with oxygen as the terminal electron acceptor. Nitrite was used because *T. denitrificans* is missing nitrate reductase and can thus utilise nitrate only as nitrogen source for biomass formation. *T. denitrificans* can use nitrite as electron acceptor at concentration at least up to $4.14 \text{ g NO}_2^- \text{ L}^{-1}$, but only after prolonged adaptation to increasing nitrite concentrations [17].

The anoxic experiment was conducted in 160 mL serum bottles (60 mL working volume) at 30°C and 150 rpm in an arbitrary shaker. The growth medium was the same as used for the aerobic experiments. To enable the investigation of the toxicity of nitrite only, the initial concentration of $\text{S}_2\text{O}_3^{2-}$ in the serum bottles was $3.9 \pm 0.2 \text{ g L}^{-1}$, as this concentration was found to be non-inhibitory (similar as of stock culture) during the aerobic experiments and resulted in excess thiosulphate concentration based on the stoichiometry of nitrite biotransformation. The used initial concentrations of nitrite were 0.2, 0.3 and 0.58 g L^{-1} . The 10% (v/v) inoculum added to each culture was in the same growth phase. The length of the incubations depended on the timing of full nitrite consumption. This duration was 3, 6 and 8 days for the cultures with initial nitrite concentrations of 0.2, 0.3 and 0.58 g L^{-1} , respectively.

2.4. Analytical methods

The concentration of thiosulphate ($\text{S}_2\text{O}_3^{2-}$), sulphate (SO_4^{2-}) and nitrite (NO_2^-) were measured with ion

chromatograph from 0.45 µm sterile filtered (Chromafil Xtra polyester membrane filter, Macherey-Nagel, Germany) samples as reported by Di Capua et al. [19]. Dionex IonPac AS22 anion exchange column (Thermo Scientific) was used with the ion-chromatography. The quantity and quality of the elemental sulphur formation were measured from samples that were vacuum filtered (1.2 µm GF/C glass microfiber filter, Whatman) and dried overnight at 105°C. The sulphur content of these samples was determined by using elemental analyser (Flash Smart, Thermo Fischer Scientific) coupled to a Thermal Conductivity Detector (TCD and supplied with helium as carrier gas [24]. The initial and end pH of the batch cultivations was measured with a pH 3210 metre (WTW, Germany) and SenTix 81 pH-electrode (WTW, Germany).

The change in biomass concentration was estimated from the starting and endpoint culture sample 16S rRNA copy numbers. For modelling purposes, the biomass concentration is typically measured as dry weight (volatile suspended solids, VSS), protein content and/or total nitrogen [12,18,25,26]. However, measuring the VSS content is not possible in the presence of elemental sulphur which has a boiling point of 440°C. Total-N content quantification can be challenging from low biomass concentration. Therefore, quantitative real-time polymerase chain reaction (qPCR) was used for the determination of the biomass concentration as it has been commonly used in microbial ecology studies [26,27]. The DNA samples were taken after the same durations (14 days) from all aerobic cultures, while in the case of anoxic cultures after all, nitrite was consumed (4, 6, 9 days with 0.2, 0.3 and 0.58 g L⁻¹ NO₂⁻ concentrations, respectively). The copy numbers were measured with quantitative polymerase chain reaction (qPCR). Prior to the qPCR, the DNA was extracted from cell pellets (2 mL samples centrifuged at 2800 g and 4°

C for 15 min) by using DNeasy PowerSoil Kit (Qiagen). The qPCR was conducted with Step One Plus Real-Time PCR (AB Applied Biosystems) as reported by Rinta-Kanto et al. [27]. The qPCR gene copy number of 5.8, which is the average of *Gammaproteobacteria* [28], was used for the conversion of copy numbers to cell numbers.

Monitoring the dissolved oxygen (DO) concentration in the shake flasks with pure culture was not possible aseptically and therefore was not done in this study.

2.5. Kinetic calculations

The kinetic calculations applied on the results of aerobic experiments were similar as described by Hajdu-Rahkama et al. [24]. The calculations used were as summarised in Table 1.

2.5.1. Thiosulphate and nitrite utilisation kinetics

The biological substrate utilisation rate (SUR) is directly proportional to the active microorganism concentration [29] which was also the case in our bio-assays (Figure S1). Factors such as concentration of the substrate, possible inhibitory compounds, and the environmental conditions (temperature, pH, pressure, etc.) that influence the concentration of microorganisms also increases the volumetric reaction rate as, for example, demonstrated in our earlier research [30,31]. Moreover, aerobic substrate utilisation can also be limited by the mass transfer of oxygen and nutrient availability [32,33].

The kinetics of both S₂O₃²⁻-S and NO₂⁻ utilisation by *T. denitrificans* were described by Monod equation (Equation (1)) [29–31]:

$$q = -\frac{q_m [S_2O_3^{2-}-S]}{K_s + [S_2O_3^{2-}-S]} \quad \text{and} \quad q = -\frac{q_m [NO_2^-]}{K_s + [NO_2^-]} \quad (1)$$

Table 1. Kinetic calculations used during aerobic ([24]) and anoxic experiments.

Kinetic calculation	Aerobic experiments	Anoxic experiment	Software used	Function used
(i) Substrate utilisation rates (SUR) – Monod kinetics (q, q _m , K _s) ^a	yes	no	Microsoft Excel	Non-linear regression of Solver add-in/ 'fminsearch'
(ii) Haldane kinetics (K _i) ^b	yes	yes	Microsoft Excel/	Non-linear regression of Solver add-in/ 'fminsearch'
(iii) Differential equation (d[S ₂ O ₃ ²⁻ -S]/dt) using constants from (i)	yes	no	POLYMATH 6.1	DEQ Differential equations
(iv) Estimation of SO ₄ ²⁻ and S ⁰ production rates (SPR) by using fractions (f ₁ and f ₂) ^c	yes	no	Microsoft Excel	Solver add-in/ 'fminsearch'
(v) Nitrite utilisation kinetics (q, q _m , K _s) ^a	no	yes	Microsoft Excel	
(viii) Growth kinetics (μ, μ _m , Y _m) ^d	yes	yes	Microsoft Excel	
(xi) Verification for SUR and SPR	yes	no	POLYMATH 6.1 and Microsoft Excel	DEQ Differential equations and Solver add-in/ 'fminsearch'

^aq_m is the maximum SUR; K_s is the half saturation constant.

^bK_i is the inhibitory substrate concentration (g L⁻¹).

^cf₁ is the fraction of [S₂O₃²⁻-S] to [SO₄²⁻-S]; f₂ is the fraction of [S₂O₃²⁻-S] to [S⁰].

^dμ is the specific growth rate, μ_m is the maximum specific growth rate and Y_m is the yield. The yield was only calculated with the aerobic experiments.

where q is specific SUR ($\text{g-S} / (\text{g-VSS h})^{-1}$ or simply h^{-1}), q_m is the specific maximum substrate ($\text{S}_2\text{O}_3^{2-}\text{-S}$) utilisation rate or specific nitrite (NO_2^-) reduction rate (h^{-1}), respectively, and K_s is half saturation concentration (g L^{-1}) of the analyte. As it was mentioned before, the SUR is proportional of the biomass concentration, thus the kinetic equation takes the following form:

$$\frac{d[\text{S}_2\text{O}_3\text{-S}]}{dt \cdot X} = q = -\frac{q_m \cdot [\text{S}_2\text{O}_3\text{-S}]}{K_s + [\text{S}_2\text{O}_3\text{-S}]} \quad (2)$$

and

$$\frac{d[\text{S}_2\text{O}_3\text{-S}]}{dt} = q \cdot X = -\frac{q_m \cdot [\text{S}_2\text{O}_3\text{-S}]}{K_s + [\text{S}_2\text{O}_3\text{-S}]} \cdot X \quad (3)$$

where X is the biomass concentration as g-VSS L^{-1} . More information about how the kinetic constants were obtained can be seen from S1.

Besides using Monod modelling for the kinetic calculations of $\text{S}_2\text{O}_3^{2-}\text{-S}$ utilisation, Haldane model has been also applied to see if there is inhibition by the substrate (Equation (4)) with the aerobic batch cultures:

$$q = -\frac{q_m [\text{S}_2\text{O}_3\text{-S}]}{K_s + [\text{S}_2\text{O}_3\text{-S}] + \frac{[\text{S}_2\text{O}_3\text{-S}]^2}{K_i}} \quad (4)$$

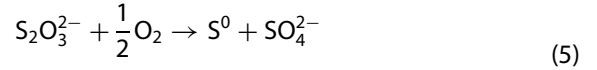
where K_i is the inhibitory concentration of $[\text{S}_2\text{O}_3^{2-}\text{-S}]$ in g L^{-1} .

The lag phases of biotransformations observed at different initial thiosulphate concentrations were omitted in the model fitting to the experimental data.

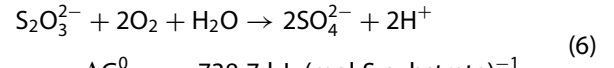
2.5.2. Product formation kinetics under aerobic conditions

The aerobic biotransformation reactions of thiosulphate together with their Gibbs-free energy changes have been given in Equations (5)–(9). Depending on the available oxygen concentration, thiosulphate is mainly converted to elemental sulphur (Equation (5)) and sulphate (Equation (6)) by *T. denitrificans*. In case oxygen is not limited, elemental sulphur is further converted to sulphate (Equation (7)). Although unlikely, some of the elemental sulphur may first become oxidised to sulphite (Equation (8)) which is then further oxidised to sulphate by *T. denitrificans* (Equation (9)). [25,34,35]. Moreover, Ang et al. [25] has also reported the formation of thiosulphate as metabolic intermediate of elemental sulphur oxidation to sulphate by

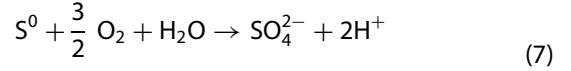
Thioalkalivibrio versutus:



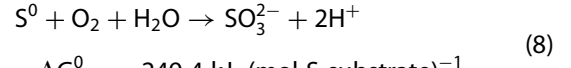
$$\Delta G^0 = -231.6 \text{ kJ. (mol S-substrate)}^{-1}$$



$$\Delta G^0 = -738.7 \text{ kJ. (mol S-substrate)}^{-1}$$



$$\Delta G^0 = -507.4 \text{ kJ. (mol S-substrate)}^{-1}$$



$$\Delta G^0 = -249.4 \text{ kJ. (mol S-substrate)}^{-1}$$

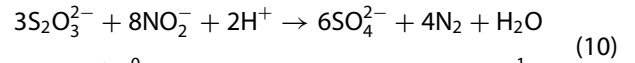


$$\Delta G^0 = -258.0 \text{ kJ (mol S-substrate)}^{-1}$$

According to Equations (3) and (4), conversion of thio-sulphate produces two fractions that are SO_4^{2-} (f_1) and S^0 (f_2). A detailed description of this calculation of the two fractions was reported by Hajdu-Rahkama et al. [24].

2.5.3. Product formation kinetics under anoxic conditions

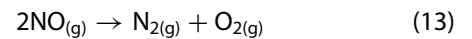
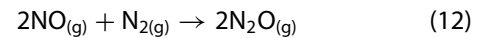
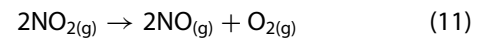
Under anoxic conditions with nitrite as electron acceptor, biotransformation of thiosulphate by *T. denitrificans* is shown in Equation (10) [10]:



$$\Delta G^0 = -5515.4 \text{ kJ (mol S-substrate)}^{-1}$$

Based on this pathway, the formation of 1 mol SO_4^{2-} requires 1.33 mol of NO_2^- .

Sorokin et al. [17] reported nitrous oxide (N_2O) formation during reduction of NO_2^- to N_2 gas by *T. denitrificans*. The equations of denitrification of gaseous NO_2^- to N_2 gas (Equations (11)–(13)) are as follows:



2.5.4. Growth of *T. denitrificans* at different thiosulphate and nitrite concentrations

Similarly, as in Hajdu-Rahkama et al. [24] the cell growth was estimated based on the results of qPCR copy numbers. The copy number was converted to g L^{-1} by using 6.25×10^{-10} g dry weight for cell formula of

$C_5H_7NO_2$ [36]. Then, the specific growth rates (μ , h^{-1}) were calculated by using Monod (Equation (14)). It was not possible to calculate the K_s from the results of $[S_2O_3^{2-}-S]$ of the anoxic experiments, thus also with the anoxic specific growth rate calculation, the K_s from the aerobic experiments were used. The yield with aerobic condition was calculated as reported by Hajdu-Rahkama et al. [24]:

$$\mu = \frac{\mu_m [S_2O_3^{2-}-S]}{K_s + [S_2O_3^{2-}-S]} \quad (14)$$

where μ is the specific growth rate calculated from experimental data and μ_m is the maximum specific growth rate (h^{-1}).

The growth of the biomass and the consumption of thiosulphate are connected as follows:

$$\mu_m = q_m \cdot Y \quad (15)$$

where Y is the biomass growth yield ($g\ L^{-1}$ biomass/ $g\ L^{-1}\ S_2O_3^{2-}-S$ or $g\ biomass/ g\ S_2O_3^{2-}-S$).

2.5.5. Model validation with experimental data

At the end of this study, the SUR, sulphate production rate (SPR_1) and elemental sulphur production rate (SPR_2) kinetic models for the aerobic experiments were statistically verified with experimental data from the sulphur formation aerobic batch experiments (see Section 2.5.2). For this verification, regression analysis was applied.

3. Results and discussion

Biotransformations of thiosulphate by *T. denitrificans* in aerobic and anoxic conditions were studied and the batch experimental data was used to derive the SUR model. With aerobic biotransformation results, this SUR model was further used to create the SPR models (sulphate and elemental sulphur production). The aerobic biotransformation models were validated with the data of an independent batch experiment. Moreover, both aerobic and anoxic growth kinetics of *T. denitrificans* were determined.

3.1. Thiosulphate biotransformation under aerobic condition

3.1.1. Kinetics of thiosulphate biotransformation

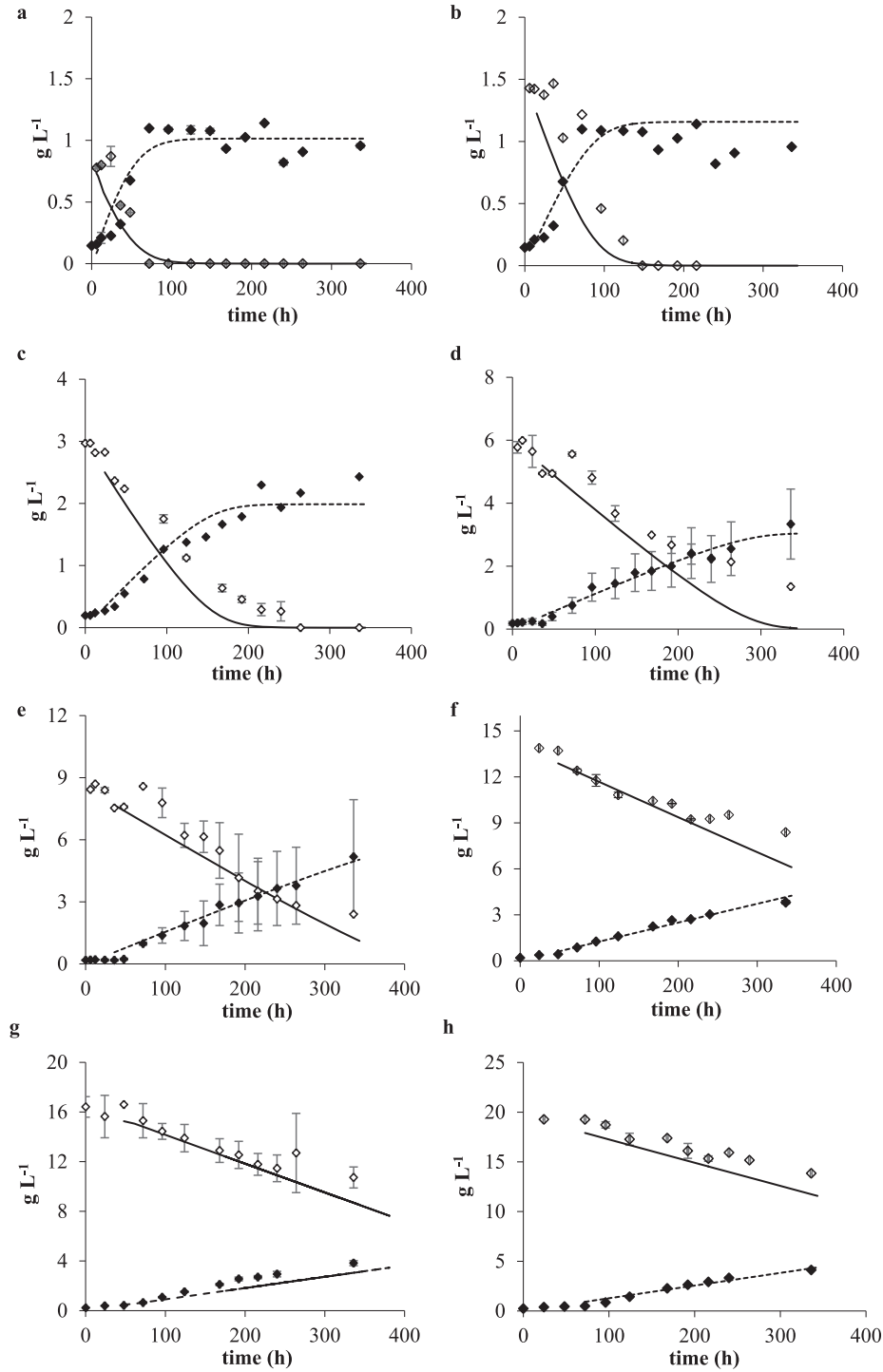
As shown in Figure 1, thiosulphate was biotransformed at all studied initial substrate concentrations (0.8 – $19.6\ g\ S_2O_3^{2-}-S\ L^{-1}$). The specific SUR increased with initial 0.8 – $8.5\ g\ S_2O_3^{2-}-S\ L^{-1}$ and at higher concentrations, it decreased (Figure 2(a)). The highest q measured was $0.051\ h^{-1}$ with initial $8.5\ g\ S_2O_3^{2-}-S\ L^{-1}$. At initial $[S_2O_3^{2-}-S]$

of $1.5\ g\ L^{-1}$ and higher, elemental sulphur was produced and based on visual observations the quantity increased with increasing thiosulphate concentration. Once thiosulphate was removed at low (1.5 – $6\ g\ S_2O_3^{2-}-S\ L^{-1}$) initial concentrations, the elemental sulphur was further oxidised to sulphate, resulting in the removal of sulphur particles (Figure S4). At initial thiosulphate-S concentrations from 0.8 to $3\ g\ L^{-1}$, thiosulphate was completely biotransformed within 14 days while this thiosulphate conversion efficiency was only 76%, 70%, 39%, 35% and 30% at initial substrate concentrations of 6, 9, 14, 16.5 and $19.8\ g\ S_2O_3^{2-}-S\ L^{-1}$, respectively. Hajdu-Rahkama et al. [24] studied the thiosulphate biotransformations of *T. versutus* using similar experimental design conditions (pH 10 and 0.6 – $1.2\ Na^+$) and thiosulphate concentrations (0.8 – $17.6\ g\ S_2O_3^{2-}-S\ L^{-1}$) at 150 rpm and $30^\circ C$. In their study, the thiosulphate utilisation rate increased from 0.03 to $0.08\ h^{-1}$ by increasing the thiosulphate concentration while in this study with *T. denitrificans*, the highest SUR was only $0.03\ h^{-1}$. Sorokin et al. [17] reported severe growth inhibition of *T. denitrificans* by forced aeration in batch culture. Thus, *T. denitrificans* as a microaerophile is likely more sensitive to oxygen than the aerobic *T. versutus*.

Monod fitting of the experimental data resulted in q_m of $0.025\ h^{-1}$ and K_s of $0.42\ g\ S_2O_3^{2-}-S\ L^{-1}$ (Figure 2(a) and Equation (3)). The model fitted well the $S_2O_3^{2-}-S$ and $SO_4^{2-}-S$ results with initial substrate concentrations of 0.8 and 8.5 – $19.6\ g\ S_2O_3^{2-}-S\ L^{-1}$ (Figure 2(a, e–h)) and had a worse fit with 1.5 – $6\ g\ S_2O_3^{2-}-S\ L^{-1}$ (Figure 1(b–d)). The Haldane model (Equation (4)) showed no substrate inhibition at any of the studied concentrations. The substrate inhibition constant or K_i value estimated by the Haldane model was high, $64\ g\ S_2O_3^{2-}-S\ L^{-1}$. Due to the better overall fit of the Monod model and high K_i value, the kinetic constants from the Monod model were used in the further kinetic calculations. The lag-phase estimated from the thiosulphate consumption curves (Figure S3 and Figure 2(b)) increased from approximately 8–70 h with the increase of initial $S_2O_3^{2-}-S$ concentration from 0.8 to $19.6\ g\ L^{-1}$ ($R^2 = 0.93$).

3.1.2. Sulphate and elemental sulphur formation

For the quantification of elemental sulphur formation with time, an independent batch experiment with $9.5\ g\ S_2O_3^{2-}-S\ L^{-1}$ was performed (Figure S5). The results in Figure 3 showed continuous increase of both sulphate ($R^2 = 0.96$) and elemental sulphur ($R^2 = 0.90$) with time. During the experiment, 35% and 10% of the $S_2O_3^{2-}-S$ was biotransformed to $SO_4^{2-}-S$ and S^0 , respectively. Over 50% of the $S_2O_3^{2-}-S$ was not removed within 10 days. The sulphur formation started after 4 days and increased



i

$$SUR = \left(- \frac{d[S_2O_3-S]}{dt} \right) = \frac{0.024[S_2O_3-S]}{0.42 + [S_2O_3-S]} \cdot X$$

$$SPR = \left([S_2O_3-S]_0 - \frac{d[S_2O_3-S]}{dt} \right) \cdot f_1$$

Figure 1. Aerobic $S_2O_3^{2-}$ -S biotransformation and SO_4^{2-} -S production kinetics for *T. denitrificans* at initial $S_2O_3^{2-}$ -S concentrations of (a): 0.8 $g L^{-1}$; (b): 1.5 $g L^{-1}$; (c): 3 $g L^{-1}$; (d): 6 $g L^{-1}$; (e): 8.5 $g L^{-1}$; (f): 14 $g L^{-1}$; (g): 16.5 $g L^{-1}$; (h): 19.6 $g L^{-1}$. (◇): $S_2O_3^{2-}$ -S data from batch assays; (◆): SO_4^{2-} -S data from batch assays; solid line (—): $S_2O_3^{2-}$ -S biotransformation kinetics model; dashed line (---): SO_4^{2-} -S production kinetics model. At the end (i) are the equations used to calculate the substrate (thiosulphate) utilisation rate (SUR) and sulphate production rate (SPR).

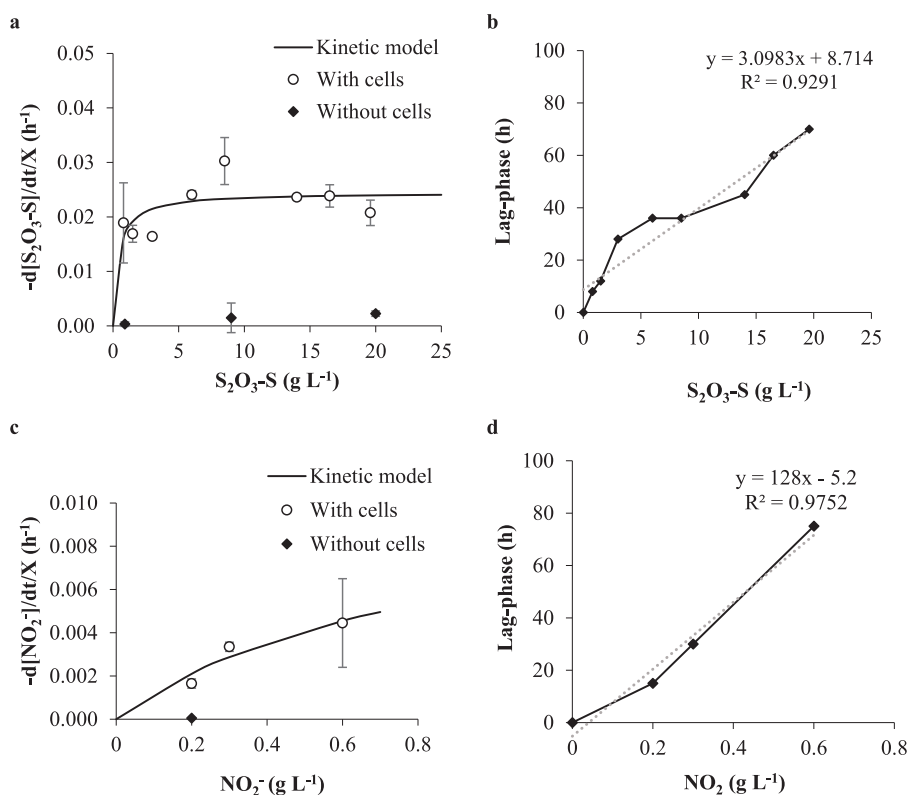


Figure 2. (a) Monod kinetics for aerobic thiosulphate utilisation ($q_m = 0.024 \text{ h}^{-1}$; $K_s = 0.42 \text{ g } S_2O_3^{2-}\text{-S L}^{-1}$ for *T. denitrificans* and (b) lag phases of $S_2O_3^{2-}$ -S removal at different initial concentrations. (c) Monod kinetics for anoxic nitrite removal ($q_m = 0.01 \text{ h}^{-1}$; $K_s = 0.84 \text{ g } NO_2^- \text{ L}^{-1}$) by *T. denitrificans* and (d) lag phases of NO_2^- removal at different initial NO_2^- concentrations and initial $2.5 \text{ g } S_2O_3^{2-}\text{-S L}^{-1}$.

to 0.9 g L^{-1} (10% yield from initial $S_2O_3^{2-}$ -S) by the end of the experiment.

3.2. Anoxic nitrite reduction and sulphate formation

In the anoxic experiment, complete NO_2^- removal took approximately 3, 5 and 8 days with initial NO_2^- concentrations of 0.2, 0.3 and 0.58 g L^{-1} , respectively. The highest measured removal rate of NO_2^- was 0.004 h^{-1}

with initial $0.58 \text{ g } NO_2^- \text{ L}^{-1}$ (Figure 2). The formation of sulphate increased with the increase of initial nitrite concentration. The concentration of nitrate (NO_3^-) in the medium remained constant with all nitrite concentrations throughout the experiments.

The lag-phase increased with the increase of initial nitrite concentration (Figure 2(d)). The Monod fitting resulted in q_m of 0.01 h^{-1} and K_s of $0.84 \text{ g } NO_2^- \text{ L}^{-1}$ (Figure 2(c)). The measured sulphate formation, especially at initial 0.2 and $0.3 \text{ g } NO_2^- \text{ L}^{-1}$, was different than predicted from the stoichiometry (Equation (10)). This indicates other fates for nitrite such as reduction to gaseous N_2O followed by partial loss to gas phase due to stirring. No elemental sulphur was formed based on visual observations and therefore, SPR was not modelled. The substrate inhibition constant or K_i value estimated by using Haldane model was $30 \text{ g } NO_2^- \text{ L}^{-1}$.

During the anoxic experiments, the highest conversion of $S_2O_3^{2-}$ -S to SO_4^{2-} of 60% could be explained by nitrite reduction at initial $0.3 \text{ g } NO_2^- \text{ L}^{-1}$ (Figure 4, Equation (10)). Sulphate production reduced by 21% when increasing the initial NO_2^- concentrations to 0.58 g L^{-1} . The sulphate production with initial $0.2 \text{ g } NO_2^- \text{ L}^{-1}$ was 56%. Figure 6 shows that the calculated $S_2O_3^{2-}$ -S to SO_4^{2-} conversion was linear.

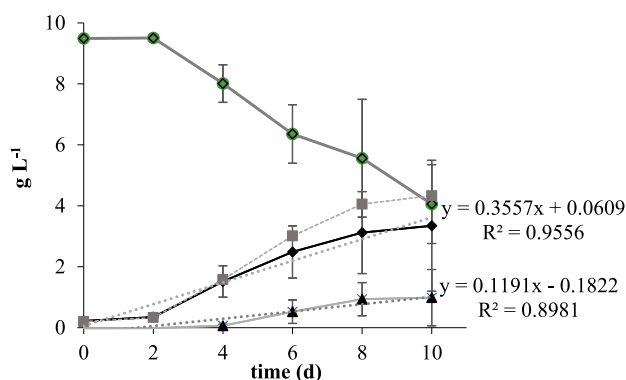


Figure 3. Thiosulphate biotransformation to elemental sulphur and sulphate by *T. denitrificans* under aerobic conditions. The symbols are (\diamond): $S_2O_3^{2-}$ -S, (\blacktriangle): S^0 , (\blacklozenge): SO_4^{2-} -S and (\blacksquare): $S^0 + SO_4^{2-}$ -S.

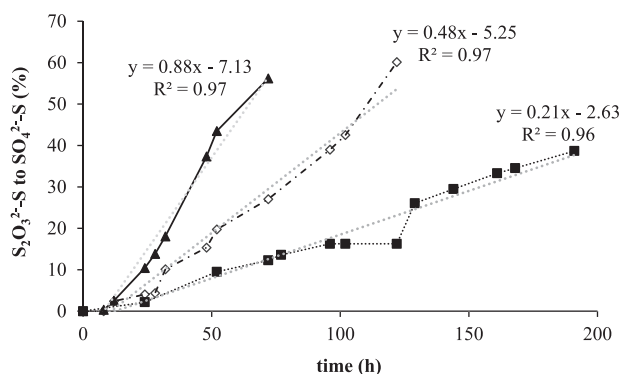


Figure 4. Sulphate formation yield as calculated based on the stoichiometry of thiosulphate biotransformation with nitrite as electron acceptor with *T. denitrificans* (Equation (10)). (▲) with 0.2 g L^{-1} , (◇): with 0.3 g L^{-1} and (■) 0.58 g L^{-1} initial NO_2^- concentrations.

3.3. Estimation of thiosulphate biotransformation under aerobic conditions

The fractions of thiosulphate biotransformed into $\text{SO}_4^{2-}\text{-S}$ and S^0 were calculated by using the results of kinetic experiments (Section 2.2.1) and the SUR models (Section 3.1.1). Finally, the models were validated with the results of the independent batch (Section 3.1.2).

3.3.1. Estimation of SPR

At initial $\text{S}_2\text{O}_3^{2-}\text{-S}$ of 0.8 g L^{-1} and below no elemental sulphur was formed, as shown by the calculated fractions of f_1 and f_2 (Figure 5(a)). The highest f_2 formation (61%) was obtained with initial $16.5 \text{ g S}_2\text{O}_3^{2-}\text{-S L}^{-1}$ while above this concentration it decreased. The yields of S^0 formation as a function of time were as shown in

Figure 5(b). In the study of Hajdu-Rahkama et al. [24] with *T. versutus*, this highest yield was 45% with initial $17.6 \text{ g S}_2\text{O}_3^{2-}\text{-S L}^{-1}$ when the lag phases were not omitted from the kinetic calculations under similar conditions. Calculating the sulphur formation yield similarly as with *T. versutus*, the highest S^0 yield by *T. denitrificans* would be close to the one by *T. versutus*.

3.3.2. SUR and SPR model validation

The validation of the SUR and SPR model parameters was done by using the experimental results with initial $9.5 \text{ g S}_2\text{O}_3^{2-}\text{-S L}^{-1}$ concentration (Section 3.1.2). The results of model validations are shown in Figure 6. The regression analysis (confidence bound to 95%) resulted in high correlation ($R^2 > 0.95$) between the kinetic models and the experimental data.

3.4. Growth of *T. denitrificans*

The kinetics of the growth of *T. denitrificans* was estimated by using qPCR copy numbers and K_s from the SUR model.

3.4.1. Growth kinetics under aerobic condition

As shown in Figure 7(a), the kinetic model fitted well the experimental growth rate results. The highest specific growth rate of *T. denitrificans* was 0.024 h^{-1} when using $K_s=0.42 \text{ g S}_2\text{O}_3^{2-}\text{-S L}^{-1}$ of the SUR model. The maximum yield (Y_m) was $0.22 \text{ g cells/g S}_2\text{O}_3^{2-}\text{-S}$.

3.4.2. Growth under anoxic conditions

The overall biomass growth was 10.2 and $10.7 \text{ mg cell h}^{-1}$ at $0.2\text{--}0.3 \text{ g NO}_2^- \text{ L}^{-1}$, respectively. The increase of

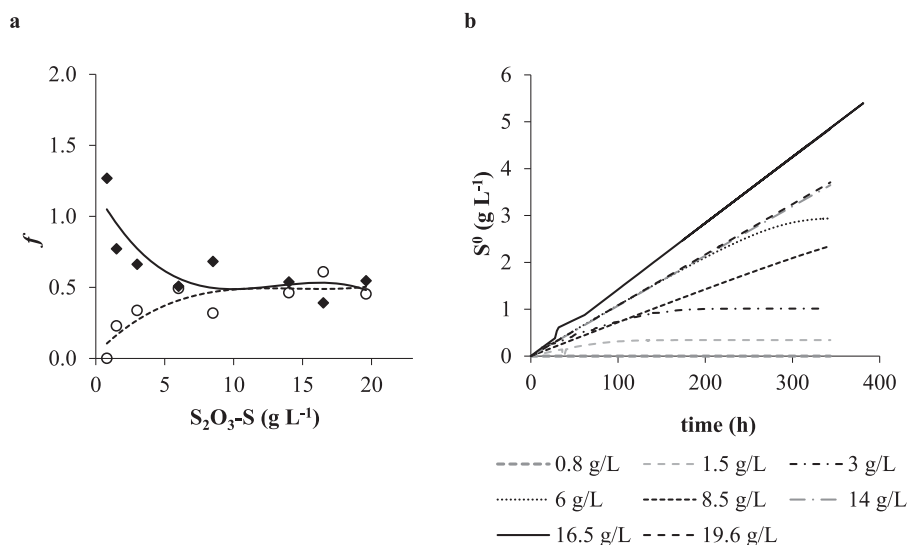


Figure 5. (a) Fractions of biotransformation of $[\text{S}_2\text{O}_3^{2-}\text{-S}]$ to $[\text{SO}_4^{2-}\text{-S}]$ (f_1 , black rhombus) and $[\text{S}_2\text{O}_3^{2-}\text{-S}]$ to $[\text{S}^0]$ (f_2 , black empty spheres) with *T. denitrificans*. The solid line (—) shows the f_1 and the dashed (---) the f_2 based on the kinetic model. (b) modelling of elemental sulphur formation by different initial thiosulphate concentrations (these concentrations are shown below the figure as legends).

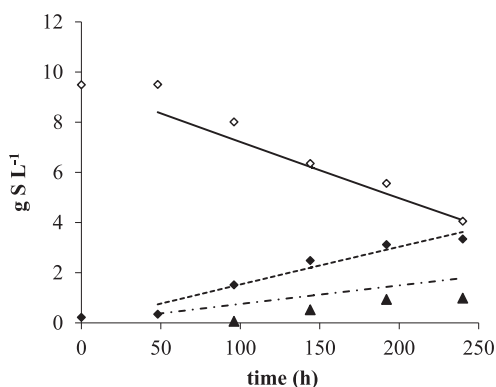


Figure 6. Validation of the SPR modelling with experimental data with initial $9.5 \text{ g S}_2\text{O}_3^{2-}\text{-S L}^{-1}$ concentration in batch incubation with *T. denitrificans*. The (\diamond): $\text{S}_2\text{O}_3^{2-}\text{-S}$; (\blacklozenge): $\text{SO}_4^{2-}\text{-S}$ and (\blacktriangle) S^0 data from batch assays. The solid line (—): $\text{S}_2\text{O}_3^{2-}\text{-S}$ bio-transformation kinetics model, dashed line (---): $\text{SO}_4^{2-}\text{-S}$ production and dotted dash line (— · —) is S^0 formation kinetics model applied with the experimental data. Here the lag-phase (38 h) was estimated from Figure 2(b).

biomass concentration of the cultures with 0.2 and 0.3 $\text{g NO}_2^- \text{ L}^{-1}$ was approximately $0.73 \text{ g cell L}^{-1}$ (5 times) and $1.31 \text{ g cell L}^{-1}$ (7 times) after 72 and 122 h, respectively. The specific growth rate was 0.022 h^{-1} with K_s of $0.42 \text{ g S}_2\text{O}_3^{2-}\text{-S L}^{-1}$. This result is similar to the μ_m of 0.022 h^{-1} reported by Sorokin et al. [17] with initial $4.14 \text{ g NO}_2^- \text{ L}^{-1}$ and $2.56 \text{ g S}_2\text{O}_3^{2-}\text{-S L}^{-1}$.

3.5. Limiting factors of aerobic biotransformations

Figure 7(b) shows that the rate of thiosulphate bio-transformation did not increase after 0.03 h^{-1} (initial $8.6 \text{ g S}_2\text{O}_3\text{-S L}^{-1}$) with the increasing biomass concentration. This indicates that a third factor, in addition to thiosulphate and biomass concentrations,

controlled the overall biotransformation rate. In shake flask bio-assays, aeration is intensive and we suggest that dissolved oxygen concentration was actually the controlling factor for the microaerophilic *T. denitrificans*. Experimentally using batch bio-assays demonstration of this phenomenon is very challenging and requires continuous-flow bioreactor experimentation. Neither sulphate nor elemental sulphur are toxic and therefore, product inhibition is out of question in this case.

Different kinetic parameters from this and other studies with similar haloalkaline SOB as *T. denitrificans* are summarised in Table 2. In aerobic condition, *T. versutus* has much higher K_s ($1.74 \text{ g S}_2\text{O}_3^{2-}\text{-S L}^{-1}$) than *T. denitrificans* ($0.42 \text{ g S}_2\text{O}_3^{2-}\text{-S L}^{-1}$), higher q_m ($+2.6 \text{ h}^{-1}$) and higher yield ($+0.09 \text{ g cell/g S}_2\text{O}_3^{2-}\text{-S}$) of sulphur formation.

Table 3 compares the results obtained with initial $2.5 \text{ g S}_2\text{O}_3^{2-}\text{-S L}^{-1}$ in aerobic and anoxic conditions (containing $0.2\text{--}0.56 \text{ g NO}_2^- \text{ L}^{-1}$). With $2.5 \text{ g S}_2\text{O}_3^{2-}\text{-S L}^{-1}$, the aerobic μ was higher (0.04 h^{-1}) than that of the anoxic with nitrite (0.02 h^{-1}). At slightly higher substrate concentration ($2.56 \text{ g S}_2\text{O}_3^{2-}\text{-S L}^{-1}$), Sorokin et al. [17] reported μ_m of 0.045 h^{-1} with N_2O (3.96 g L^{-1}) based on their batch bio-assays. Further, they also reported anoxic (with NO_2^-) growth rate of 0.038 h^{-1} but not substrate utilisation kinetics by *T. denitrificans* in a continuous chemostat culture [17]. Therefore, the chemostat growth rates were lower than those obtained in our batch assays (0.046 h^{-1}). Further studies are needed to optimise electron acceptor supply in anoxic bioreactors for thiosulphate biotransformation.

This study showed high yield elemental sulphur accumulation by *T. denitrificans* in the presence of sufficient thiosulphate and oxygen (from air) concentrations. In industrial scale applications, bioprocesses are always open systems. Based on our earlier work

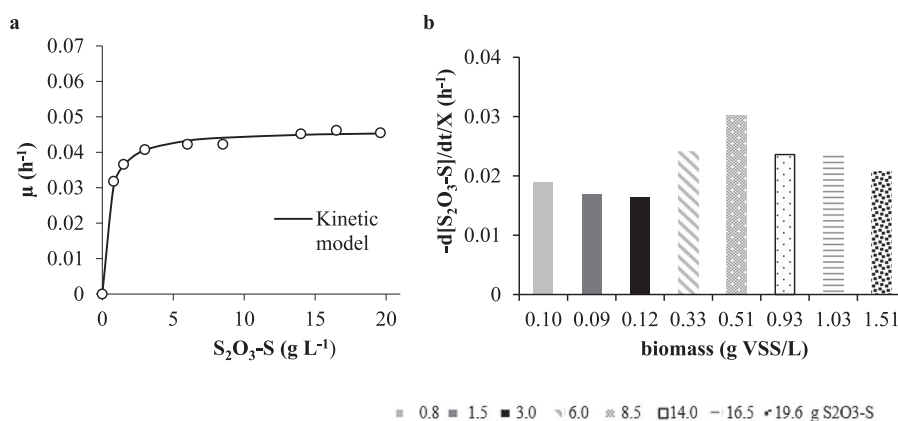


Figure 7. (a) Aerobic growth kinetics of *T. denitrificans*, $\mu_m = 0.046 \text{ h}^{-1}$; $K_s = 0.42 \text{ g S}_2\text{O}_3^{2-}\text{-S L}^{-1}$, Yield (Y_m) = $0.22 \text{ g cells (g S}_2\text{O}_3^{2-}\text{-S)}^{-1}$; and (b) specific thiosulphate-S utilisation rates and biomass concentrations with mean biomass concentrations.

Table 2. Comparison of aerobic thiosulphate biotransformation kinetic constants of studies in haloalkaline condition.

Microorganism	Experimental conditions						Kinetic parameters				Reference
	Experimental system	Temp. (°C)	pH	rpm	Salinity (g L ⁻¹ Na ⁺)	Initial S ₂ O ₃ ²⁻ -S (g L ⁻¹)	q _m (h ⁻¹)	K _s (g L ⁻¹)	μ _m (h ⁻¹)	Y (g cell/g S ₂ O ₃ ²⁻)	
<i>T. denitrificans</i>	batch assays	30	10	150	24	14	0.024	0.42	0.046	0.22*	This study [17]
<i>T. denitrificans</i>	batch assays	30	10	NR	0.6	2.56	N.D.	N.D.	0.028	0.038 g protein/ g S ₂ O ₃ ²⁻ (4.2 mg protein/ mmol S ₂ O ₃ ²⁻)	
<i>T. versutus</i>	batch assays	30	10	150	26	17.2	2.66	1.74	0.048	0.31*	[24]
<i>T. versutus</i>	batch assays	30	10	150	N.R.	2.56	0.049**	N.D.	0.082	N.D.	[18]
<i>T. versutus</i>	batch assays	37	10	150	N.R.	2.56	0.064**	N.D.	0.095	N.D.	[18]
<i>T. denitrificans</i>	chemostat (lab-scale fermentor)	30	10	N.R.	N.R.	2.56	N.D.	N.D.	0.038	0.039 g protein/ g S ₂ O ₃ ²⁻ (4.4 mg protein/ mmol S ₂ O ₃ ²⁻)	[17]
<i>T. versutus</i>	chemostat (lab-scale fermentor)	35	10	N.R.	13.8, 46 and 92	2.56	N.D.	N.D.	0.27 0.21 and 0.11	0.12, 0.086 and 0.055	[37]

*As S₂O₃²⁻-S; N.D.: not determined; N.R.: not reported.

**As g L⁻¹ h⁻¹.

with *T. versutus* [24] and the results of this study, both *T. denitrificans* and *T. versutus* would be likely catalysts of thiosulphate biotransformations. Although the rate of aerobic biotransformation is higher by *T. versutus*, in oxygen-limited conditions, the application of microaerophilic *T. denitrificans* can be more suitable.

The experimental design (shake flasks) and pure culture (a requirement for aseptic conditions) did not allow monitoring and control of DO concentration although it is an important variable that influences the final product formation in thiosulphate biotransformation. Therefore, the DO concentration effects and optimisation for elemental sulphur formation by *T. denitrificans* should be delineated in bioreactor studies that allow the possibility for DO control and continuous monitoring. Adjusting the DO concentration to an adequate level is crucial when the aim is to produce elemental sulphur, thus preventing its further oxidation to sulphate [21,38]. At low DO concentrations, which are preferred for S⁰ formation, reading the actual values is often challenging, therefore controlling the oxygen supply based on the oxidation redox potential (ORP) is a better approach [21,39]. In addition, bioreactor provides steady-state conditions and thus, gives

additional information about the practical applicability of this bioprocess. The desired DO levels can be maintained in continuous-flow bioreactors such as fluidised bed bioreactor where completely mixed conditions are maintained via high-rate recirculation [40]. This has been demonstrated under haloalkaline conditions in a *Thioalkalivibrio versutus* amended fluidised bed bioreactor [41]. In a practical application, the bioreactor would serve as a kidney removing the excess/ accumulating sulphur from the process stream. Some of the haloalkaline streams may contain organic constituents that can be toxic towards SOB [13] and therefore, the potential inhibitory effects of these constituents should be determined in future studies.

5. Conclusions

Under haloalkaline conditions (~pH 10 and 14–28 g Na⁺ L⁻¹) aerobic and anoxic thiosulphate biotransformation batch bio-assays with *Thioalkalivibrio denitrificans* used in this work resulted in the following conclusions:

1. With oxygen as electron acceptor and initial [S₂O₃²⁻-S] of 0.8–19.6 g L⁻¹, the highest biotransformation rate of thiosulphate was 0.024 h⁻¹ with K_s = 0.42 g S₂O₃²⁻-S L⁻¹.
2. Elemental sulphur accumulated at ≥1.5 g S₂O₃²⁻-S L⁻¹. The highest obtained elemental sulphur yield was 10% with an initial 9.5 g S₂O₃²⁻-S L⁻¹ and the estimated 61% with initial of 16.5 g S₂O₃²⁻-S L⁻¹.
3. Under anoxic conditions (with nitrite as an electron acceptor), only minor thiosulphate biotransformation occurred with no visual elemental sulphur formation. The highest rate of nitrite removal was 0.011 h⁻¹ with K_s = 0.84 g NO₂⁻ L⁻¹.
4. The maximum aerobic and anoxic specific growth rates were 0.046 and 0.022 h⁻¹, respectively, which

Table 3. Comparison of the kinetic constants of *T. denitrificans* under aerobic and anoxic conditions obtained in this study.

Kinetic constants and parameters	Aerobic condition (kinetics with S ₂ O ₃ ²⁻ -S) ^a	Anoxic condition (kinetics with NO ₂ ⁻)
lag-time (h)	27	15–75 ^b
highest q (h ⁻¹)	0.02	0.005
K _i (g L ⁻¹)	64	30
K _s (g L ⁻¹)	0.42	0.84
max. SO ₄ ²⁻ -S formation (%)	80	60 ^c
μ (h ⁻¹) with S ₂ O ₃ ²⁻ -S	0.04	0.02

^aThese results were calculated considering the initial 2.5 g S₂O₃²⁻-S L⁻¹ concentration of the anoxic cultures.

^bWith 0.2–0.56 g [NO₂⁻] L⁻¹.

^cBased on theoretical calculation.

may indicate partial inhibition by nitrite. The highest aerobic growth yield was 0.22 g cells/ g $S_2O_3^{2-}$ -S.

5. In summary, aerobic/microaerobic biotransformations producing elemental sulphur under haloalkaline conditions have potential for development of sulphur recovery from saline and alkaline industrial sulphurous streams.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials.

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