Kinetics and modelling of thiosulphate biotransformations by haloalkaliphilic *Thioalkalivibrio versutus*

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Abstract

Biotransformation of thiosulphate by *Thioalkalivibrio versutus* was studied under haloalkaline conditions (pH 10, 0.66-1.2 M Na^+) using batch assays and modelling tools for possible sulphur recovery from haloalkaline industrial streams. The thiosulphate was fully biotransformed to sulphate or to sulphate and elemental sulphur at initial $S_2O_3^{2-}$-S concentrations of 25-550 mM within 10 days. The highest biotransformation rate of 2.66 mM [$S_2O_3^{2-}$-S] h^{-1} was obtained at initial $S_2O_3^{2-}$-S concentration of 550 mM with half saturation constant (K_s) of 54.5 mM [$S_2O_3^{2-}$-S]. At initial concentrations below 100 mM $S_2O_3^{2-}$-S, the main product was sulphate whilst at above 100 mM also elemental sulphur with up to 29% efficiency was produced. The model approach developed incorporated $S_2O_3^{2-}$ biotransformation to SO_4^{2-} and S^0. The kinetic modelling results were compatible (R^2> 0.90) with the experimental data. The maximum growth rate (μ_m) was 0.048 h^{-1} (0.40 mM $C_5H_7NO_2$ h^{-1}) and the maximum growth yield 0.15 mM $C_5H_7NO_2$/mM $S_2O_3^{2-}$-S (4.0 g cell/mol $S_2O_3^{2-}$-S). The high rate thiosulphate biotransformation and elemental sulphur recovery results together with the developed kinetic model can be used for bioprocess design and operation. The potential industrial applications would aim at sustainable resource recovery from industrial haloalkaline and sulphurous process and/or effluent streams.

Keywords: *Thioalkalivibrio versutus*, haloalkaliphilic sulfur oxidizing bacteria, thiosulfate biotransformation, sulfur disproportionation, resource recovery, kinetics

1. Introduction

Various obligate haloalkaliphilic microorganisms thrive in soda lakes that are unique alkaline habitats with high salinity and pH up to 11. These lakes are extremely well buffered because of their high sodium carbonate concentration (for a review, see [1]). In addition, the sodium concentration in these lakes can even reach the level of saturation. Haloalkaliphiles need nutrients such as sulphur, nitrogen and carbon to gain energy for growth (for a review, see [2]). As long as nutrients are present and the environment is hospitable in terms of pH and salinity, haloalkaliphiles will gain energy from the redox reactions. Thus, both sulphur oxidation and sulphidogenesis occur in these extreme environments (for reviews, see [1,2]).

Haloalkaliphilic sulphur-oxidizing bacteria (SOB) found from soda lakes use inorganic sulphur compounds (i.e. sulphide, polysulphide, thiosulphate, polythionates and elemental sulphur) as primary source of energy [3]. Haloalkaliphilic SOB belong to the family of *Gammaproteobacteria* among which the *Thioalkalivibrio* is the metabolically most flexible genus. *Thioalkalivibrio spp*.tolerate salt (Na^+) concentration even up to 4.3 M, while their minimum requirement for growth is 0.2 M [3]. *T. versutus* is able to grow at pH up to 10.6 (optimal pH 9.5) and accumulate sulphur globules in the periplasmic space [4–6]. The products of the sulphide and thiosulphate biotransformation by *T. versutus* are sulphate with elemental sulphur and minor levels of sulphite as the intermediates [7].

Haloalkaliphilic SOB are potent catalysts for sulphur recovery from industrial streams such as effluents and process waters from petroleum industry, pulp and paper industry, food preparation, mining, and mineral processing [8–11]. Sulphurous compounds are important process chemicals in many industries and require recycling [12]. For example, up to 97% of the chemicals (including inorganic sulphur compounds) used in the Kraft pulping are

recovered and recycled within the pulp mill [13]. Due to this recycling, different sulphurous compounds including thiosulphate, accumulate within the process. In the pulping process, maintaining the Na/S balance is essential for achieving high efficiency [14] and the surplus of recycled sulphurous compounds increases the need for sodium addition. Removal of sulphur from the process, could reduce the sodium requirement, and thus, the operational costs. De Graaf et al. [15] reported conversion of sulphide and thiosulphate from sulphidic spent caustic of oil refining into sulphate with elemental sulphur as an intermediate by haloalkaliphilic SOB in a continuous two-step process. Elemental sulphur is the most desired sulphurous product in this kind of conversion process because of its ability to be separated from liquid streams and its wide range of industrial and agricultural uses. Elemental sulphur can, for example, be used for the production of fertilizers, fungicides and in mining and metallurgy [16,17]. In addition, elemental sulphur has been used as an electron source for biological processes such as authotrophic denitrification [18].

To our knowledge, three studies [6,7,19] have reported thiosulphate biotransformation kinetics by haloalkaliphilic *T. versutus*. These studies focused on the effects of different Na^+ concentrations [6,19]; the growth kinetics of *T. versutus* [7,19], thiosulphate removal [6] and expression of sulphur oxidation genes [7]. Banciu et al. [19] reported maximum specific growth rate of 0.29 and 0.2 h^{-1} for *T. versutus* in a continuous fermentor with 40 mM $S_2O_3^{2-}$ at 35°C at Na^+ concentrations of 0.6 and 2 M, respectively. They did not report the thiosulphate biotransformations rates but instead used oxygen uptake as an indicator of activity and they obtained maximum specific oxygen uptake rate ($qO_{2\,max}$) of 0.74±0.06 µM O_2/ mg protein min^{-1} (at 0.6 M Na^+) and 0.65 ±0.05 µM O_2/ mg protein min^{-1} (at 2 M Na^+) at 10% air saturation and thiosulphate concentration of 50 µM. The $qO_{2\,max}$ with initial 34 µM elemental sulphur as substrate was 0.30 ±0.02 µM O_2/ mg protein min^{-1} and 0.21±0.02 µM O_2/ mg

protein min^{-1} at 0.6 and 2 M Na^{+}, respectively. The apparent affinity constant (K_s) for thiosulphate reported in their study was 6±3 μM. In their study, the respiration rates of washed and centrifuged cells collected from the fermentor were determined in a magnetically stirred glass chamber (5 mL) with a fitted oxygen electrode [19]. In a shake flask batch study by Makzum et al. [6], thiosulphate removal rate at 30°C was 0.76 mM h^{-1} with initial concentration of 40 mM $S_2O_3^{2-}$. Based on the protein content, they reported specific growth rate of 0.069 h^{-1} at initial concentration of 100 mM $S_2O_3^{2-}$ (200 mM $S_2O_3^{2-}$-S), but they did not report thiosulphate biotransformation kinetics. The highest elemental sulphur yield reported by Ang et al. [7] was 3.5 mM when using initial thiosulphate concentration of 40 mM in shake flasks at 200 rpm and 30°C.

These previous studies did not comprehensively report the thiosulphate biotransformation kinetic coefficients and especially not up to 550 mM $S_2O_3^{2-}$-S concentration. Also, the disproportionation of thiosulpate to elemental sulphur was reported only at very low thiosulphate concentration. Moreover, the kinetic modelling approach was not attempted for optimization of sulphate and elemental sulphur production. Therefore, the aim of this study was to delineate thiosulphate biotransformation kinetics including oxidation to sulphate and disproportionation to elemental sulphur and sulphate, and their modelling for possible uses in processes for sustainable sulphur recovery from haloalkaline industrial streams such as pulping industry [20].

The specific objectives of this study were the following:

(i) determination of the biotransformation rates of thiosulphate by *T. versutus*,

(ii) determination of yields and production kinetics of elemental sulphur and sulphate formation at different initial concentrations of thiosulphate,

(iii) determination of qPCR-based growth kinetics and yields of *T. versutus*,

(iv) kinetic modelling and overall model validation.

2. Materials and methods

2.1. Inoculum and growth medium

Thioalkalivibrio versutus strain AL2 (DSM No. 13738) was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures GmbH). The strain was maintained in Medium 925 recommended by DSMZ [21]. The medium consisted of mineral base (189 mM Na_2CO_3, 119 mM $NaHCO_3$, 86 mM NaCl, 6 mM K_2HPO_4), 2% (v/v) trace element solution (TES), 40 mM S_2O_3, 5 mM KNO_3 and 0.5mM $MgCl_2$. The mineral base and TES were sterilized by autoclaving at 110°C for 20 min and at 121°C for 20 min, respectively, while the S_2O_3, KNO_3 and $MgCl_2$ stocks were sterile-filtered (0.2 μm polyethersulfone membrane syringe filter, VWR International, North America). The pure culture of *T. versutus* was maintained as duplicates in 250 mL (100 mL working volume) shake flasks on an orbital shaker (150 rpm) at 30±1°C and the stock cultures were transferred (10% v/v inoculum) into fresh medium every seventh day.

2.2. Kinetic experiments

All kinetic experiments were carried out in 250 mL shake flasks (100 mL working volume) at 30°C and 150 rpm. The caps of the shake flasks were kept loose to enable air transfer. The growth medium for the kinetic experiments was as described in section 2.1 but with different concentrations of $S_2O_3^{2-}$-S. The concentrations of $S_2O_3^{2-}$-S used were 25, 50, 100, 200, 350, 450, and 550 mM. All assays were inoculated with 10% (v/v) of 6 days old stock culture suspension to ensure similar initial microbial activity. During the experiment, 2 mL samples

were taken for the determination of thiosulphate and sulphate concentrations. Furthermore, at the end of the experiment, additional 3x2 mL samples were withdrawn for biomass quantification. The samples for the determination of initial biomass concentration were taken from the stock culture inoculum. The duration of each kinetic experiment was 10 days. Due to the experimental design (shake flasks, pure culture), aseptical monitoring of dissolved oxygen (DO) concentration was not possible.

2.3. Monitoring sulphur formation during thiosulphate biotransformation

To determine the quantity of elemental sulphur formation from thiosulphate by *T. versutus*, a separate batch experiment was carried out. The experiment was started with 12 identical cultures that were inoculated with 10% (v/v) of stock culture suspension and had initial $S_2O_3^{2-}$-S concentration of 300 mM (a middle range concentration used in the kinetic experiments, which resulted in full thiosulphate biotransformation in 10 days). The conditions and duration (10 days) of the experiment were the same as in the kinetic experiments described in the previous section. Every second day, 6 mL samples were taken from two of the shake flasks to analyse thiosulphate and sulphate concentrations. After sampling, the rest of the culture volume from these two shake flask was vacuum filtered using 1.2 μm glass microfiber filter (GF/C, Whatman) to enable quantification of elemental sulphur.

2.4. Analyses

The thiosulphate ($S_2O_3^{2-}$) and sulphate (SO_4^{2-}) concentrations were measured from filtered (0.45 μm Chromafil Xtra polyester membrane filters, Macherey-Nagel, Germany) samples by ion-chromatography (IC) as described by Di Capua et al. [18]. The quantity of elemental sulphur (S) was determined from 1.2 μm vacuum filtered (GF/C glass microfiber filter, Whatman) and dried (105°C overnight) samples by using elemental analyser (Flash Smart, Thermo Fischer Scientific) with thermal conductivity detector (TCD) and helium as carrier gas with flow rate of 140 mL/min (65°C oven, furnace temperature Left: 950°C and Right: 1060°C). To ensure full oxidation of the sulphur sample, approximately 10 mg vanadium pentoxide (V_2O_5) was added to each sample. At the beginning and end of the experiments, the culture pH was measured using a pH 3210 meter (WTW, Germany) equipped with a SenTix 81 pH-electrode (WTW, Germany).

The initial and endpoint 16S rRNA gene copy numbers were analysed from DNA extracted samples by using quantitative polymerase chain reaction (qPCR). Prior to DNA extraction with DNeasy PowerSoil Kit (Qiagen), cell pellet was formed by centrifuging 2 mL sample at 2800 rcf and 4°C for 15 min [7]. The qPCR was conducted withStep One Plus Real-Time PCR (AB Applied Biosystems) using the primers and PCR programme described by Rinta-Kanto et al.[22]. For the estimation of the biomass concentration, the average 16S Gammaproteobacterial qPCR gene copy number (5.8) was used [23].

During all of the kinetic experiments, a pure culture of *T. versutus* was used in shake flasks, and thus, following aseptically the change of DO concentration was not possible.

2.5. Kinetic model development

In this study, kinetic calculations were performed in the following manner:

(i) Substrate utilization rates (SURs) were calculated as mM $[S_2O_3^{2-}\text{-S}]$ h^{-1} from the batch assays conducted with varying initial thiosulphate concentrations from 25 mM to 550 mM.

(ii) Monod kinetics model was applied to data from (i), then maximum SUR (q_m) and half saturation constant (K_s) were calculated using non-linear regression using Solver Add-in tool in Microsoft Excel.

(iii) Differential equation ($d[S_2O_3^{2-}\text{-S}]/dt$) describing SUR was solved with POLYMATH 6.1 computer program using kinetic constants (q_m and K_s) obtained in the previous step for the varying substrate concentrations depending on time.

(iv) In order to model SO_4^{2-} and S^0 production rates (SPRs), a new *f* (fraction) term was defined. It was used to calculate the thiosulphate biotransformation products as follows: f_1 (fraction of $[S_2O_3^{2-}\text{-S}]$ to $[SO_4^{2-}\text{-S}]$) and f_2: (fraction of $[S_2O_3^{2-}\text{-S}]$ to $[S^0]$) using ($d[S_2O_3^{2-}\text{-S}]/dt$) modelling data and measured $[SO_4^{2-}\text{-S}]$ data with the Solver add-in program in Microsoft Excel. It was assumed that f_1+f_2 equals to 1 (or 100%). Thus, all oxidized $[S_2O_3^{2-}\text{-S}]$ was assumed to be transformed to sulphate and elemental sulphur.

(v) Finally, the SURs and SPRs were verified with an independent experimental data set of thiosulphate, sulphate and elemental sulphur (section 2.3) at the end of steps from (i) to (iv). In addition to the SUR, also elemental sulphur recovery rate was estimated using independent data set with the previously constructed model (steps from (i) to (iv)). Given this validation,

constructed model was tested for the SPR, needed for the design, modelling, and operation of a bioprocess.

These steps are more thoroughly described in the following subsections.

2.5.1. Utilization kinetics

The kinetics of S_2O_3-S consumption by *T. versutus* was described using Monod equation [24]:

$$q = \frac{q_m\,[S_2O_3 - S]}{K_s + \,[S_2O_3 - S]} \qquad (1)$$

where, q is specific thiosulphate consumption rate [mM (mM biomass·h)$^{-1}$], q_m is maximum specific thiosulphate oxidation rate [mM (mM biomass·h)$^{-1}$] and K_s (mM) is half saturation concentration [25,26].

Due to our interest in thiosulphate consumption rate and the fact that biomass growth is fuelled by this consumption, we prefer to regard the rate of thiosulphate consumption as the basic rate, while cell growth is derived from this. Thus, the Monod equation takes the form:

$$r_{ut} = \frac{q_m\,[S_2O_3 - S]}{K_s + \,[S_2O_3 - S]} \cdot X \qquad (2)$$

where r_{ut} is the rate of thiosulphate consumption and X is the cell concentration (mM cell). Thiosulphate consumption and biomass growth are connected by the following equation:

$$\mu_m = q_m \cdot Y \qquad (3)$$

where Y is true yield for cell synthesis (mM biomass/mM consumed thiosulphate) and μ_m is maximum specific growth rate (h^{-1}). Here, Y value was calculated by converting the unit of μ_m

from (h^{-1}) to (mM cell h^{-1}) using Eq. 13-14. The μ_m is considered from growth kinetic as (h^{-1}), 1 cell = $6.25 \cdot 10^{-10}$ g, 112 g/mol cell ($C_5H_7NO_2$) and q_m is from substrate utilization kinetic as (mM S_2O_3-S h^{-1}). The maximum growth rate was converted to (mM cell h^{-1}) to calculate Y value as mM cell/mM $S_2O_3^{2-}$-S to represent the fraction of electron-donor electrons converted to biomass electrons during synthesis of new biomass.

In the batch bottle assays, the substrate is biotransformed while no substrate is added or removed from the system. Therefore, over the duration of the assay, the mass of product accumulation is proportional to the mass of substrate consumption [27]. First, substrate consumption was modelled as shown in the following equation:

$$V\frac{d[S_2O_3 - S]}{dt} = V \cdot r_{ut} \quad (4)$$

in which, V is the culture volume volume; r_{ut} is the rate of thiosulphate consumption.

The rate of thiosulphate consumption (*dS/dt*) is assumed to follow the kinetics as given by following Equations:

$$V \cdot \frac{d[S_2O_3 - S]}{dt} = V \cdot \left(- \frac{q_m \cdot [S_2O_3 - S]}{K_s + [S_2O_3 - S]} \cdot X \right) \quad (5)$$

and

$$\frac{d[S_2O_3 - S]}{dt \cdot X} = q = \left(- \frac{q_m \cdot [S_2O_3 - S]}{K_s + [S_2O_3 - S]} \right) \quad (6)$$

In the current study, kinetic constants (q_m and K_s) shown in Equation 1 were fitted to substrate utilization data by nonlinear regression using Solver add-in program in Microsoft Excel. This search method minimizes the sum of the squares of the differences between the predicted and measured values, the model results and coefficients (with 95% confidence interval). Thereafter, substrate utilization kinetics (*dS/dt*) were predicted by obtained kinetic constants and differential equation 4. This equation was numerically solved using POLYMATH 6.1 and the

Runge-Kutta-Fehlberg (RFK) numerical integration routine. The program integrates the system of differential equations using the RKF algorithm. Thus, differential equation of S_2O_3-S utilization given in Equation 4 was mathematically solved as a function of time.

2.5.2 Sulphate and elemental sulphur production kinetics

In this section, we propose a new model to express SO_4^{2-} and S^0 production rates (SPRs) based on our constructed kinetic model in this work to calculate product formation for use in industrial scale bioreactor applications.

In order to calculate SO_4^{2-} and S^0 production rates (SPRs), a new f (fraction) term was defined to calculate [$S_2O_3^{2-}$-S] to [SO_4^{2-}-S] (f_1) and [$S_2O_3^{2-}$ -S] to [S^0] (f_2) during the incubations using modelling data. These fractions are the ratios calculated for each initial thiosulphate concentration. These *f* values are expected to be constant as the function of initial concentration. Solver add-in program in Microsoft Excel was the main actor to calculate f_1 and f_2 values based on the measured and the predicted data.

Ang et al. [7] reported that thiosulphate was mainly converted to elemental sulphur and sulphate by *T. versutus*. During the conversion of thiosulphate, only minor level of sulphite was formed. Based on the findings of Ang et al. [7], it can be assumed that the conversion of [$S_2O_3^{2-}$-S] by *T. versutus* follows the Equations 7, 8 and 9:

$$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2\ SO_4^{2-} + 2H^+ \quad (7)$$

$$S_2O_3^{2-} + ½\ O_2 \rightarrow S^0 + SO_4^{2-} \quad (8)$$

$$S^0 + 1½\ O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+ \quad (9)$$

When *T. ver*sutus uses $S_2O_3^{2-}$-S as an electron donor, a portion of [S_2O_3-S] (f_1) is transformed to SO_4^{2-} and the rest of [S_2O_3-S] (f_2) into S^0. Therefore, it was assumed that the sum of f_1 and f_2 equals to 1 (or 100%). Thus, all consumed S_2O_3-S was assumed to be transformed to sulphate and elemental sulphur.

In the batch bottle assays, sulphate production rate was defined as:

$$\frac{d[SO_4 - S]}{dt} = \left([S_2O_3 - S]_0 - \frac{d[S_2O_3 - S]}{dt}\right) \cdot f_1 \quad (10)$$

and the elemental sulphur production rate as:

$$\frac{d[S^0]}{dt} = \left([S_2O_3 - S]_0 - \frac{d[S_2O_3 - S]}{dt}\right) \cdot f_2 \quad (11)$$

where $[S_2O_3^{2-}\text{-}S]_0$ is the initial substrate concentration, $d[S_2O_3^{2-}\text{-}S]/dt$ is the biotransferred thiosulphate-sulphur concentration as a function of time t, f_1 and f_2 are the conversionfractions of [$S_2O_3^{2-}$-S] to [SO_4^{2-}-S] and to [S^0], respectively. The f_2 was calculated as $1 - f_1$.

First, f_1 was calculated with Microsoft Excel add-in Solver program using the measured and the predicted [SO_4^{2-}-S] and [$S_2O_3^{2-}$-S] data. The predicted [SO_4^{2-}-S] was calculated using Equation 10. In the calculation of f_1, this program was used to find an optimal value by minimizing the sum of the squares (SSE) of the differences between the predicted and the

measured values the model results with 95% confidence interval. This program adjusts the values in the decision variable cell (SSE) to produce the result wanted for the objective cell (*f*). These two reactions produce both SO_4^{2-} and S^0. Therefore, two different production rates were defined as SO_4^{2-} and S^0 production rates. At the end of all experimentations, f_1 and f_2 were verified with the independent experimental data from the experiments described in section 2.3.

More detailed explanation for the calculation of the fractions expressing SO_4^{2-}-S and S^0 production for initial $S_2O_3^{2-}$-S concentration of 200 mM is available in the supplementary materials (S1, Fig. S1).

2.5.3. Growth Kinetics

During the exponential growth phase, a bacterial culture follows first-order reaction kinetics. The rate of increase of cells is proportional to the number of bacteria present at that specific time. The constant of proportionality, μ, is an index of the growth rate as h^{-1} and it is called the growth rate constant as:

$$rate\ of\ increase\ of\ cells = \mu \cdot number\ of\ cells \quad (12)$$

The value of μ can be determined from the following equation:

$$\mu = \frac{lnC_2 - lnC_1}{t_2 - t_1} \quad (13)$$

in which C_1 and C_2 are the numbers of cells estimated from qPCR copy numbers at t_1 and t_2, respectively. The expected 16S rRNA gene copy number per cell was similar as the average

16S Gammaproteobacterial qPCR gene copy number (5.8) [23]. The dry weight (d.w.) of the cell mass was estimated based on the weight of one cell equalling to 9.5 x 10^{-13} g [28]. Finally, the d.w. was converted to mM by using the simplified molecular cell formula of $C_5H_7NO_2$ [28].

In order to determine the growth kinetics in this study, qPCR results of the batch assays were firstly used to estimate the specific growth rate (μ, h^{-1}) in terms of cell numbers/mL in samples taken at the beginning and end of the batch incubations. Then Monod model [24] was applied to express the effect of substrate concentration on specific growth rate:

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

where μ is experimental specific growth rate (h^{-1}), μ_m is maximum specific growth rate (h^{-1}), and K_s is half saturation concentration (mM). As it is shown in Eq. 3, substrate utilization and biomass growth are interlinked. The growth yield (Y) represents the fraction of electron donor electrons [S_2O_3-S] converted to biomass electrons [$C_5H_7NO_2$] during synthesis of new biomass.

2.5.4. Model validation of experimental data

All the kinetic models for SUR, sulphate production rate (SPR_1) and elemental sulphur production rate (SPR_2) were statistically verified with the experimental resultson $S_2O_3^{2-}$-S, SO_4^{2-}-S and S^0 obtained in the separate experiment with the initial $S_2O_3^{2-}$-S concentration of 300 mM. This was done to examine the relationship between two or more variables of interest with regression analysis.

3. Results and discussion

Biotransformation of thiosulphate by *T. versutus* was studied for the determination of biotransformation rate, sulphate and sulphur production rates as well as the growth rate.

3.1. Effect of thiosulphate concentration on biotransformation kinetics by *T. versutus*

Thiosulphate was biotransformed by *T. versutus* at all the studied initial thiosulphate concentrations ranging from 25 mM to 550 mM and the results were as shown in Fig 1. Based on visual observations, elemental sulphur was not formed at thiosulphate concentrations of 25 and 50 mM. At 100 mM and higher thiosulphate concentrations, elemental sulphur production increased with the increasing initial thiosulphate concentration. Makzum et al. [6] also reported increase of elemental sulphur accumulation with increasing thiosulphate concentrations in batch assays. In the study by Makzum et al. [6], at 100 mM thiosulphate concentration, elemental sulphur was further oxidized to sulphate after depletion of thiosulphate. This may have also occurred in our study especially at 25 and 50 mM $S_2O_3^{2-}$-S. These results indicate that optimization of elemental sulphur production requires that the thiosulphate is not completely oxidized in the system.

[Fig. 1 here]

Model results were based on Eq. 6 (SUR) and Eqs. 10 and 11 (SPRs) describing biotransformation and production kinetics solved by differential equation using Polymath 6.1 computer program. Both biotransformation and production kinetics gave excellent fit and correlation with the R^2 values ranging from 0.90 to 0.98 and the confidence bound to 0.95.

Complete consumption of thiosulphate required from 30 hours (1.25 days) to 240 hours (10 days) at initial $S_2O_3^{2-}$-S concentrations from 25 mM to 550 mM, respectively (Fig. 1). A lag phase before any thiosulphate consumption occurred was observed at all studied initial thiosulphate concentrations (Fig. 2b). The lag phase varied from approximately 1 hour at 25 mM, to 38 hours at 300 mM to 550mM $S_2O_3^{2-}$-S concentration.

Monod fitting of the results (Fig. 2a) showed that the thiosulphate biotransformation rate reached maximum of 2.66 mM [$S_2O_3^{2-}$-S] h^{-1} at 550 mM. Monod based substrate utilization kinetics produced half saturation constant of 54.5 mM [$S_2O_3^{2-}$-S] for *T. versutus* with a good correlation (R^2=0.95) (Fig. 2a). The K_s value (6±3 μM) reported by Banciu et al. [19] was considerably smaller than that reported in this study. This may be due to oxygen-controlled kinetics in their respiration experiment (10% air saturation) and differences in the experimental designs (e.g., fermenter vs. shake flask). In this study the thiosulphate consumption rate increased with increasing initial thiosulphate concentrations, thus indicating that the oxygen mass transfer did not control the rate of consumption with a possible exception of the highest initial thiosulphate concentration of 550 mM. Similarly to our results Makzum et al. [6] reported at the studied thiosulphate concentrations: *T. versutus* grew up to 750 mM thiosulphate concentration and utilized thiosulphate at a rates of 0.76 mM h^{-1} at 80 mM [$S_2O_3^{2-}$-S].

[Fig. 2 here]

Oxygen is of key importance in aerobic processes also under haloalkaliphilic conditions and thus a potentially rate controlling factor. In this study, the highest SUR determined based on the Monod model was 2.66 mM [$S_2O_3^{2-}$-S] h^{-1}. Considering the reaction stoichiometry (Eq. 7),

this corresponds with oxygen consumption rate of 2.66 mM [O_2] h^{-1}, which was the highest oxygen demand and the highest oxygen uptake rate that occurred in the shake flasks at the highest substrate concentration of 550 mM [$S_2O_3^{2-}$-S] used. The same amount of oxygen was supplied by shaking of flasks containing lower $S_2O_3^{2-}$-S concentrations. An important evidence for no oxygen limitation is the Monod curve (Fig. 2a). The experimental data corresponded with the Monod model with a high correlation and the data did not comply with the Haldane equation [27]. Therefore, substrate, oxygen or product inhibition can be ruled out in the shake flasks. Since the shake flasks were open to the surrounding air, the amount of dissolved oxygen in a liquid is directly proportional to the oxygen consumption rate d(O_2)/dt according to Henry's law. If the oxygen uptake rate would have been limiting, the resulting SUR (dS/dt) (which is directly proportional to oxygen uptake rate) would rather have matched the Haldane model than the Monod model, especially at high thiosulphate concentrations.

3.2. Stoichiometry of elemental sulphur production from thiosulphate

At 300 mM initial $S_2O_3^{2-}$-S concentration, the elemental sulphur production in the batch assays was as shown in Fig. 3. The $S_2O_3^{2-}$-S bioconversion efficiency to S^0 yied was 29% on day 6. On the same day, 67% of the $S_2O_3^{2-}$-S was biotransformed to SO_4^{2-}-S.

[Fig. 3 here]

In the shake flask study by Ang. et al. [7], the highest reported S^0 concentration was 3.5 mM from the initial 40 mM $S_2O_3^{2-}$-S. Thus, the elemental sulphur yield that they obtained was 4.4% and was lower than the yield obtained in this study. This shows that the elemental

sulphur production can be favoured by using high thiosulphate feed concentrations and short enough retention time not to enable full oxidation to sulphate.

3.3. Modeling of the experimental data

Figure 4 (a) shows elemental sulphur production as a function of time with the initial $S_2O_3^{2-}$-S concentrations from 100 mM to 550 mM, while elemental sulphur did not accumulate at the initial $S_2O_3^{2-}$-S concentrations ranging from 25 mM to 50 mM. Figure 4 (b) shows elemental sulphur yield (mM $[S^0]$ h^{-1}) as a funtion of the initial thiosulphate concentration. The results show that elemental sulphur production rate and the initial thiosulphate concentration had a linear correlation.

[Fig. 4 here]

Fig. 5 shows the calculated fractions (f_1 and f_2) of $S_2O_3^{2-}$-S transformation to SO_4^{2-}-S and to S^0 during the thiosulphate biotransformation assays. At thiosulphate concentrations below 100 mM only sulphate was produced, whereas at higher concentrations elemental sulphur production increased. The model results were compatible with experimental results at an R^2 of 0.88. At higher concentrations, close to half of the oxidized thiosulphate was recovered (i.e. 45% recovery with initial 550 mM $S_2O_3^{2-}$- S) as elemental sulphur and the other half as sulphate. Elemental sulphur accumulation did not affect $S_2O_3^{2-}$-S biotransformation. The results indicate that the increasing of initial thiosulphate concentration resulted in increased elemental sulphur yields.

[Fig. 5 here]

3.4. Model validation

All equations used in the modelling were verified using the data from the independent batch assay with an initial $S_2O_3^{2-}$-S concentration of 300 mM. In this experiment, $S_2O_3^{2-}$-S, SO_4^{2-}-S and elemental sulphur (S^0) were analysed and validated using kinetic models developed in this study. All modelling equations and the modelling pathway consisting of the six main steps are presented in Fig. 6.

[Fig. 6 here]

Model was validated for initial $S_2O_3^{2-}$-S concentration of 300 mM with model parameters, constants and equations obtained from this study (Table 1). The model was run with the help of kinetic equations obtained in this study. The validation results were as given in Fig. 7. The experimental results obtained from the batch assay operated for the validation showed that the model is compatible with high correlation (R^2>0.90) and the proposed kinetic models (SUR and SPRs) can be confidently used in reactor design and operation.

[Table 1 here]

[Fig. 7 here]

3.5 Growth of *T. versutus* at different thiosulphate concentrations

The maximum growth rate (μ_m) for each $S_2O_3^{2-}$-S concentration (Fig. 8) was determined in terms of the cell numbers estimated using the qPCR results (Fig. S1) and the estimated gene copy number per cell. Sampling for qPCR took place at the beginning and end of the incubation (day 10). The maximum specific growth rate was 0.048 h^{-1} (0.40 mM $C_5H_7NO_2$ h^{-1}), corresponding a maximum yield of 0.15 mM $C_5H_7NO_2$/mM $S_2O_3^{2-}$-S (4.0 g $C_5H_7NO_2$/mol $S_2O_3^{2-}$-S or 6.98 g cell/mol $S_2O_3^{2-}$), which was calculated by Eq. 13 and 14 considering kinetic constants q_m and K_s of substrate uptake rates. There was no inhibition at any $S_2O_3^{2-}$-S concentrations tested. The Table 2 summarizes all kinetic constants and compares them with values reported for *T. versutus* and other SOB. In a fermenter study, Banciu et al. [19] reported growth yields of 13.6 and 9.6 g cell/ mol $S_2O_3^{2-}$ (0.6 and 1.2 M Na^+) at 35°C. Furthermore, Makzum et al. [6] showed that thiosulphate biotransformation and growth rates increased by increasing the temperature from 25 to 35 °C. Operation at higher temperature (> 30°C of this study) would be beneficial for applications with industrial streams as less cooling prior to biological treatment would be needed.

[Fig. 8 here]

[Table 2 here]

In this study, the kinetics of sulphate and elemental sulphur production were reported at intial thiosulphate concentrations ranging from 25 to 550 mM. To our knowledge this is the first comprehensive study combining all these aspects of thiosulphate biotransformation. Furthermore, a model that not only reveals the kinetics of substrate utilization but also kinetics of sulphate and elemental sulphur production was developed. The findings of this study especially about the rate and yield of elemental sulphur accumulation indicated that

development of a bioprocess for recovery of elemental sulphur from industrial streams is possible. Although sulphide containing streams would be more inhibitory to *T. versutus* than thiosulphate containing streams [29–32], application of a two-step process would enable elemental sulphur recovery also from these streams. The two-step process would include first chemical oxidation of sulphide to thiosulphate followed by the biological transformation of thiosulphate [15]. De Graaff et al. [15] demonstrated successful conversion of sulphide from spent caustic to sulphate by a *Thioalkalivibrio* dominated culture in a continuously fed two-step process.

Our experimental design (shake flask incubations, pure culture) did not allow monitoring of DO concentration. However, controlling and monitoring the DO concentration in continuous flow bioreactors is a standard methodology. The DO concentration in bioreactors can be maintained at a desired level that favours elemental sulphur formation and bioreactor studies are needed for this optimization. Based on the results of Makzum et al. [6] and Banciu et al. [19], operation temperature should be set to 35°C to enhance thiosulphate biotransformation and biomass growth. Industrial process streams often have elevated temperatures and thus, would require less cooling making the higher operation temperature feasible. Future validation of the created model with sulphurous stream, such as process stream from pulp industry, is also needed. Moreover, separation of the biologically produced sulphur from liquid phase needs further development. The kinetic constants obtained in this study can be used for design and experimental operation of continues bioreactors.

4. Conclusions

Under haloalkaline conditions (~pH 10, 0.66-1.2 M Na^+), thiosulphate at initial concentration of 25-550 mM was completely biotransformed by *Thioalkalivibrio versutus* within 10 days. The highest biotransformation rate was 2.66 mM [$S_2O_3^{2-}$-S] h^{-1} with K_s of 54.5 mM [$S_2O_3^{2-}$-S] at 550 mM. The highest growth rate and yield were 0.048 h^{-1} (0.40 mM $C_5H_7NO_2$ h^{-1}) and 4.0 g $C_5H_7NO_2$/mol $S_2O_3^{2-}$-S, respectively. Elemental sulphur accumulation was observed at initial concentration of ≥100 mM $S_2O_3^{2-}$-S . A model incorporating $S_2O_3^{2-}$-S biotransformation and product formation was developed. High-rate biotransformations and the modelling results indicate that bioprocesses can be developed for the sustainable recovery of S^0 from haloalkaline industrial process streams.

E-supplimentary data of this work can be found in the online version of the paper.

Aknowledgement

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Fig. captions

Fig. 1. Biotransformation kinetics of $S_2O_3^{2-}$-S and production kinetics of SO_4^{2-}-S for *T. versutus* at initial $S_2O_3^{2-}$-S concentrations of (a): 25 mM; (b): 50 mM; (c) 100 mM; (d) 200 mM; (e): 350 mM; (f): 450 mM; (g): 550 mM. (◊): $S_2O_3^{2-}$-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.

Fig. 2 (a) Monod based thiosulphate utilization kinetics (q_m=2.60 mM [$S_2O_3^{2-}$-S] h^{-1} ; K_s= 54.5 mM [$S_2O_3^{2-}$-S] for *T. versutus* and (b) lag phases of thiosulphate utilization at different initial concentrations.

Fig. 3. Thiosulphate disproportionation to elemental sulphur and sulphate. The symbols are (◊): $S_2O_3^{2-}$-S, (▲) S^0, (♦): SO_4^{2-}-S and (■) S^0+ SO_4^{2-}-S.

Fig. 4. (a) Elemental sulphur production from thiosulphate as a function of time ; (a) the initial $S_2O_3^{2-}$-S concentration from bottom to top is 100 mM, 200 mM, 350 mM, 450 mM and 550 mM and (b) elemental sulphur yield (mM [S^0] h^{-1}) as funtion of intial thiosulphate concentration.

Fig. 5. Calculated fractions of $S_2O_3^{2-}$-S to SO_4^{2-}-S (f_1) and to S^0 (f_2) ratio during biotransformation in batch reactors depending on substrate concentration. These fractions were calculated from experimental and substrate biotransformation kinetics model data (for a detail see Eq. 7 and 8 giving SPRs, sulphate/sulphur production rates) (◊): f_1 (S_2O_3-S/SO_4-S) data from batch reactor runs; solid line (—) shows fitted curve for f_1 depending on substrate

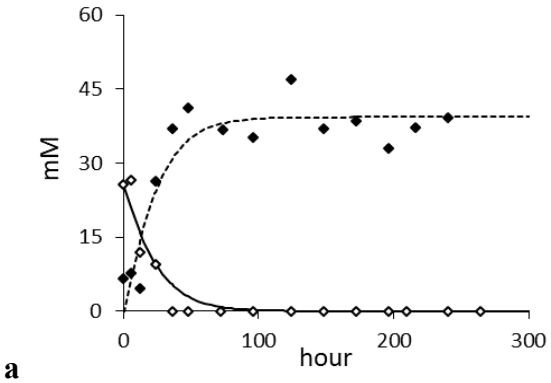
concentration (f_1=*-0.214xln [$S_2O_3^{-}$S]+1.90* with R^2 value of 0.88) and f_2 is calculated from *1-f_1* with the dashed line and black diamond.

Fig. 6. The pathway for bioreactor modelling including substrate utilization rate and sulphur production rates and equations obtained in this study.

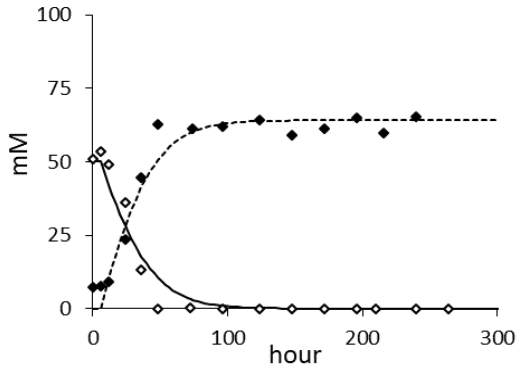
Fig. 7. Model validation for thiosulphate biotransformation to sulphur and sulphate. (Constants in Table 1 are used for calculations (SUR and SPR_1 and SPR_2), orange circle: SUR, white diamond: SPR1, black diamond: SPR2)

Fig. 8. Monod based growth kinetics μ_m= 0.048 h^{-1}; K_s= 54.47 mM [$S_2O_3^{2-}$-S], Yield (*Y*)=0.15 mM cell/mM [$S_2O_3^{2-}$-S] (4 g cell/mol [$S_2O_3^{2-}$-S] or 6.98 g cell/mol [$S_2O_3^{2-}$] for *T. versutus*). Yield value was calculated by Eq. 13 considering μ_m 0.048 h^{-1}, 1 cell = $6.25 \cdot 10^{-10}$ g, 112 g/mol cell, q_m = 2.6 mM S_2O_3-S/h. From here maximum growth rate was calculated as 0.4 mM cell h^{-1}, which corresponds to *Y* value of 0.4/2.6 = 0.15 mM cell/mM $S_2O_3^{2-}$-S considering $C_5H_7NO_2$ molecular formula of cell.

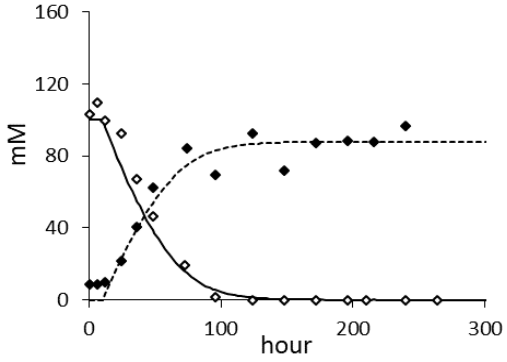
Fig. 1



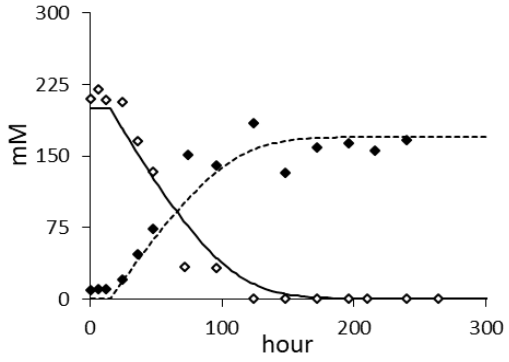
a



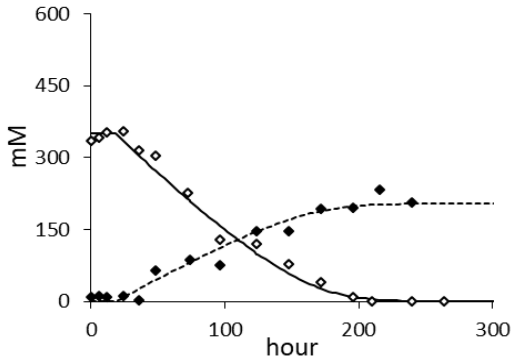
b



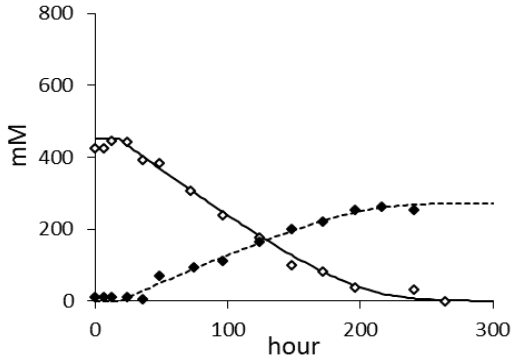
c



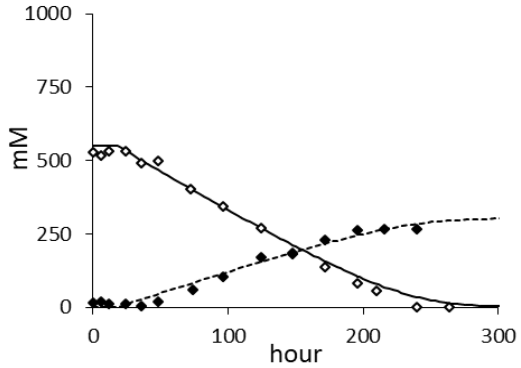
d



e



f

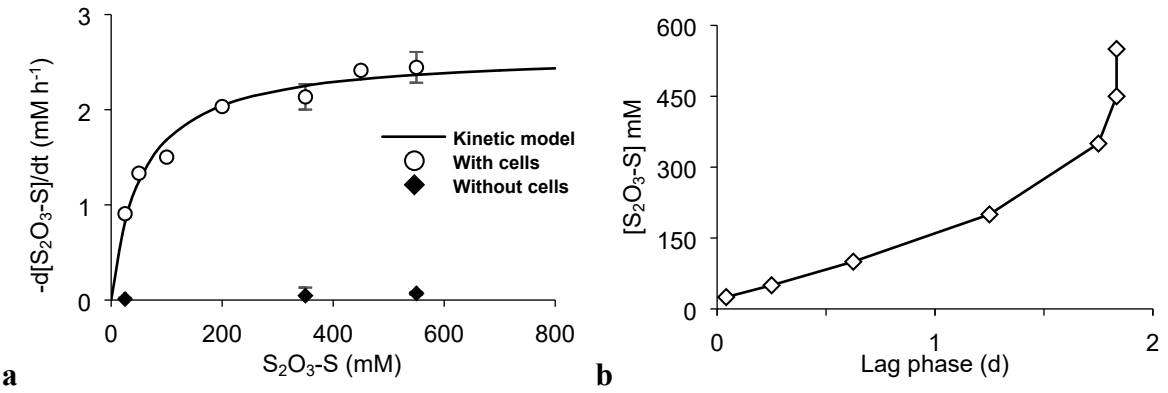


g

$$SUR: -\frac{d[S_2O_3-S]}{dt} = \frac{2.60 \cdot [S_2O_3-S]}{54.47 + [S_2O_3-S]}$$

$$SPR_1: ([S_2O_3 - S]_0 - \frac{d[S_2O_3-S]}{dt}) \cdot f_1$$

Fig. 2


-d[S2O3-S]/dt (mM h-1)
S2O3-S (mM)
Kinetic model
With cells
Without cells
[S2O3-S] mM
Lag phase (d)

a **b**

Fig. 3

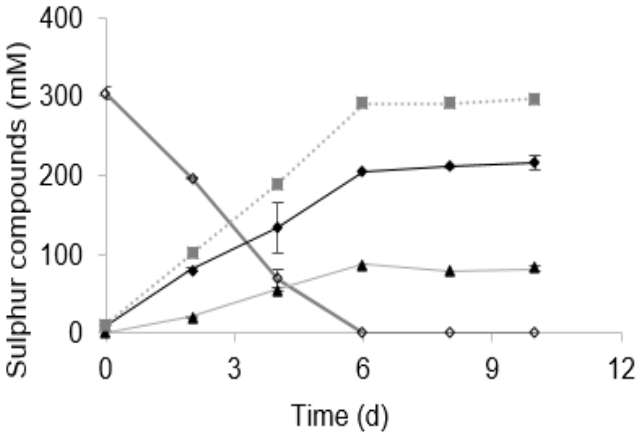

Sulphur compounds (mM)
Time (d)

Fig. 4

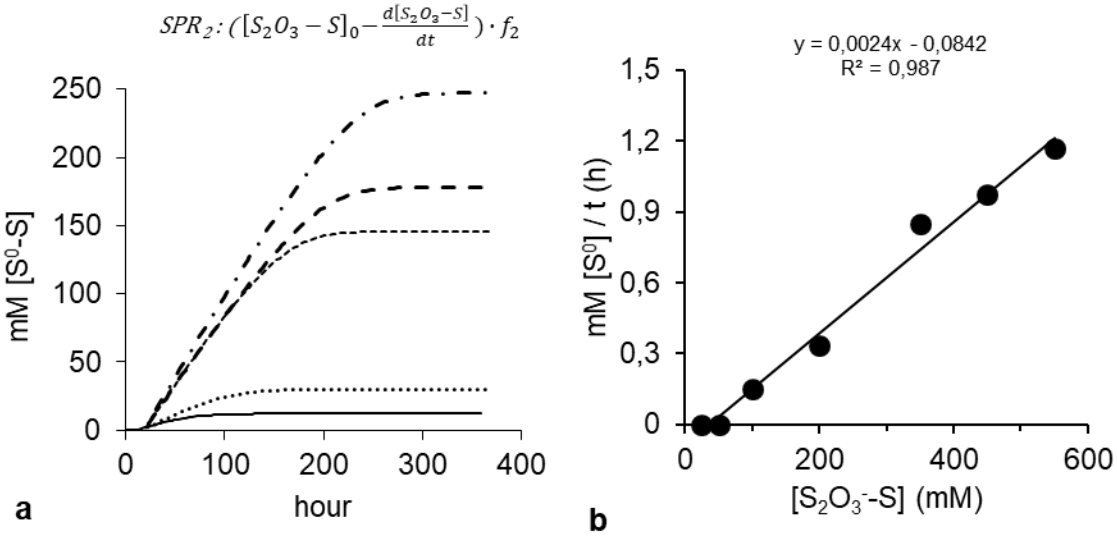

$SPR_2: ([S_2O_3 - S]_0 - \frac{d[S_2O_3-S]}{dt}) \cdot f_2$
mM [S⁰-S]
hour
a
y = 0,0024x - 0,0842
R² = 0,987
mM [S⁰] / t (h)
[S2O3-S] (mM)
b

Fig. 5

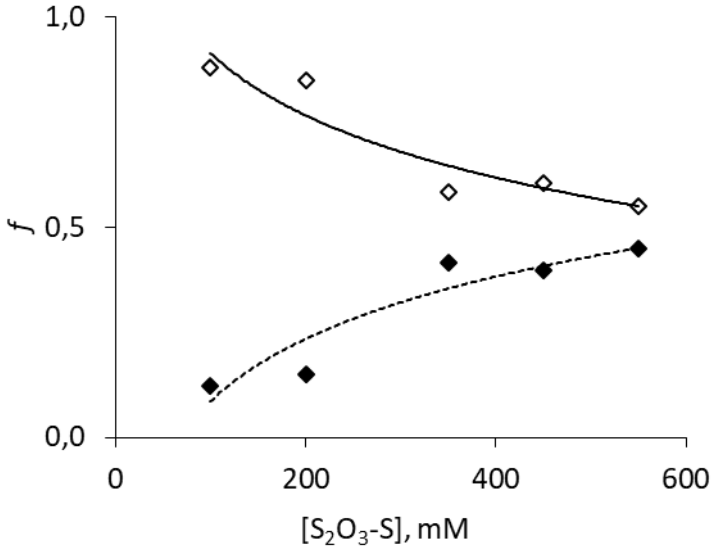

f
[S2O3-S], mM

Fig. 6

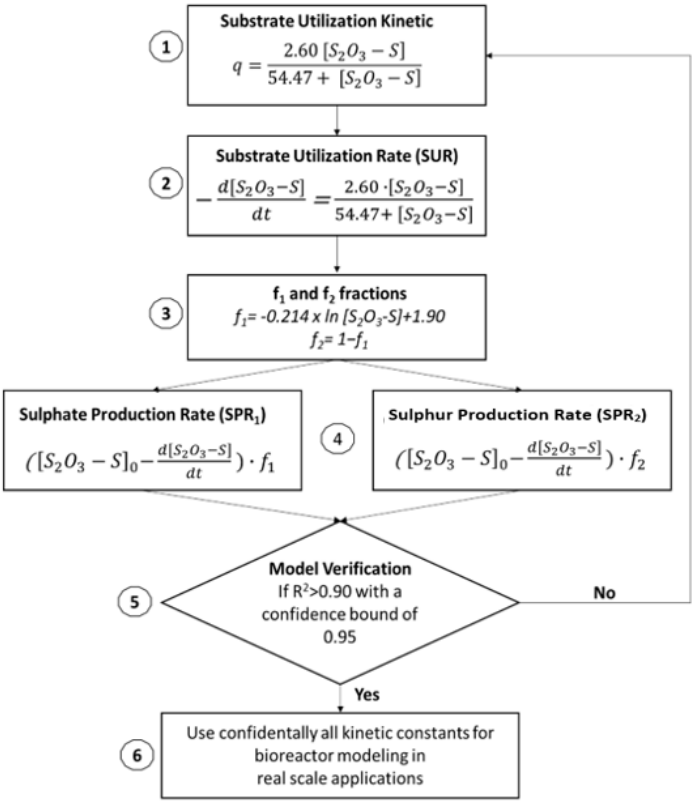

1
Substrate Utilization Kinetic
$q = \frac{2.60\,[S_2O_3 - S]}{54.47 + [S_2O_3 - S]}$
2
Substrate Utilization Rate (SUR)
$-\frac{d[S_2O_3-S]}{dt} = \frac{2.60\cdot[S_2O_3-S]}{54.47+[S_2O_3-S]}$
3
f_1 and f_2 fractions
f_1= -0.214 x ln [S_2O_3-S]+1.90
f_2= 1–f_1
Sulphate Production Rate (SPR_1)
$([S_2O_3 - S]_0 - \frac{d[S_2O_3-S]}{dt}) \cdot f_1$
4
Sulphur Production Rate (SPR_2)
$([S_2O_3 - S]_0 - \frac{d[S_2O_3-S]}{dt}) \cdot f_2$
5
Model Verification
If R^2>0.90 with a confidence bound of 0.95
No
Yes
6
Use confidentally all kinetic constants for bioreactor modeling in real scale applications

Fig. 7

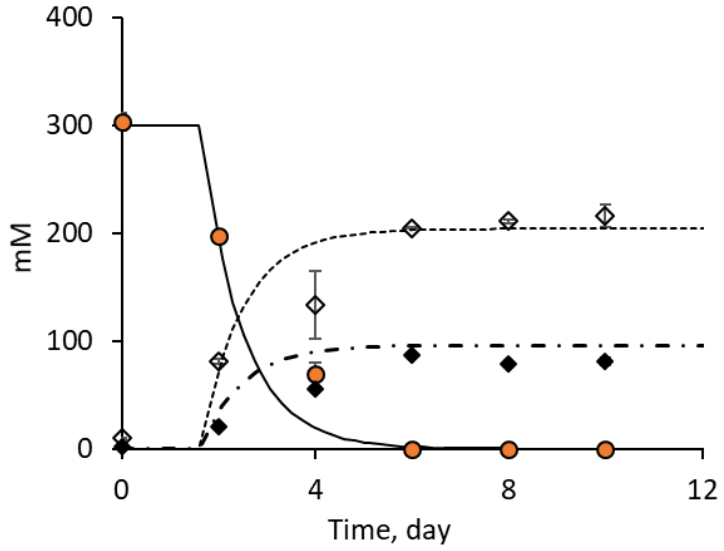
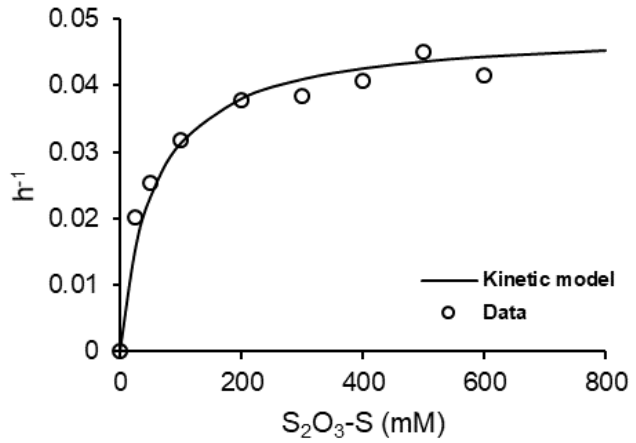

400
300
200
100
0
mM
0
4
8
12
Time, day

Fig. 8.


0.05
0.04
0.03
0.02
0.01
0
h-1
0
200
400
600
800
S2O3-S (mM)
Kinetic model
Data

Tables

Table 1. Model validation with an initial S_2O_3-S concentration of 300 mM

Model Parameters	Values	Equation
L, day	1.58	Calculated by iteration using Fig. 3
q_m, mM h^{-1}	2.60	Eq. 1 and Eq. 4
K_s, mM	54.47	Eq. 1 and Eq. 4
f_1 from Fig.5 $f_1 = -0.214 \times \ln [S_2O_3\text{-}S] + 1.90$	0.68	Eq. 7 for SPR_1
$f_2 = 1 - f_1$	0.32	Eq. 8 for SPR_2

Table 2. Summary of kinetic constants from different thiosulphate biotransformation studies in aerobic assays

Experimental conditions							Kinetic parameters				Reference
Microorganism	**Experimental system**	**Temp. (°C)**	**pH**	**rpm**	**Salinity (M Na^+)**	**Initial $S2O3^{2-}$ (mM)**	**q_m (mM h^{-1})**	**K_s (mM)**	**μ_m (h^{-1})**	**Y_m**	
T.versutus	batch assays	30	10	150	1.2	550	2.66	54.47	0.048 (0.40 mM cell h^{-1})	0.15 mM $C_5H_7NO_2$/mM $S_2O_3^{2-}$-S (4.0 g cell/mol $S_2O_3^{2-}$ -S or 6.98 g cell/mol $S_2O_3^{2-}$)	This study
T. versutus	batch assays	30	10	150	N.R.	40	0.76	N.D.	0.082	N.D.	[6]
T. versutus	batch assays	37	10	150	N.R.	40	1.0	N.D.	0.095	N.D.	[6]
Thioalkalivibrio denitrificans	batch assay	30	10	N.R	0.6	40	N.D.	N.D.	0.028	4.2 g protein/ mol $S_2O_3^{2-}$	[33]
T.versutus ALJ 15	continuous cultivation in lab-scale fermentor	35	N.R	N.R	0.6, 2 and 4	40	N.D.	N.D.	0.29, 0.21 and 0.11	13.5, 9.6 and 6.1 g cell/mol $S_2O_3^{2-}$	[19]

Thiobacillus versutus	chemostat	30	7.5	750	N.R	25	N.D.	N.D.	N.D.	8.3 g cell/ mol $S_2O_3^{2-}$	[34]
Thiobacillus neapolitanus strain C	chemostat	30	6.8	750	N.R	10-14	N.D.	N.D.	N.D.	8.6 g cell/ mol $S_2O_3^{2-}$	[34]
Thermotrix thiopara	chemostat	65 and 72	6.9	750	N.R	10-14	0.55	N.D.	N.D.	21.7 and 18.2 g cell/ mol $S_2O_3^{2}$	[34]

N.D. : not determined
N.R. : not reported

Highlights:

- $S_2O_3^{2-}$ biotransformations kinetics by haloalkaliphilic *Thioalkalivibrio versutus* was studied
- High rate bioconversion of 2.66 mM [$S_2O_3^{2-}$-S]/h with K_s of 54.47 mM [$S_2O_3^{2-}$-S] was obtained at 550 mM
- S^0 accumulated at 100-550 mM initial $S_2O_3^{2-}$ concentrations up to 29% sulphur recovery
- A model approach incorporating $S_2O_3^{2-}$ biotransformation to products (SO_4^{2-}, S^0) was developed
- This bioprocess has potential for recovery of S^0 from haloalkaline industrial process streams