



Elemental sulphur production from thiosulphate under haloalkaline conditions in a *Thioalkalivibrio versutus* amended fluidized bed bioreactor

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ABSTRACT

Concentrated sulphurous and saline streams, produced for example by pulp and paper and petrochemical industries, pose challenges for both environmental and processes management. In this study, the potential of biological recovery of S^0 from haloalkaline thiosulphate solution in a *Thioalkalivibrio versutus* amended continuous-flow fluidized bed bioreactor (FBBR) was investigated using different (12–5 h) hydraulic retention times (HRT) as well as physico-chemical means to separate the S^0 produced. $S_2O_3^{2-}$ was biotransformed to SO_4^{2-} and S^0 with the highest biotransformation efficiency of 99.9 %. At 7 h HRT, the capacity of the FBBR was reached, seen as incomplete thiosulphate conversion. S^0 production rate increased up to 6.3 ± 0.6 g S/l/d at HRT 7 h, whilst the average S^0 yield was 27 ± 2 %. The presence of biologically produced S^0 was visual and identified by scanning electron microscopy. Separation of S^0 from the effluent by centrifugation at 3417 relative centrifugal force (rcf) resulted in 93 % separation, while among the four tested coagulants, $FeCl_2$ at 0.5 g/l resulted in 40 % separation. Also, $FeCl_2$ enhanced thiosulphate biotransformation rates. In summary, continuous biological S^0 production followed by separation by centrifugation indicates potential for sulphur recovery from alkaline and saline industrial streams.

1. Introduction

Concentrated gaseous and liquid sulphurous streams are common in many industries, such as petrochemical and pulp and paper industries (PPI) (for a review, see [1]). For instance, the kraft pulping used for wood delignification, employs alkaline liquors that are made up of sulphurous and sodium containing compounds ($NaOH$, Na_2S , Na_2SO_4 , $Na_2S_2O_3$) [2]. After delignification, spent liquors enter a recovery cycle in which chemicals are recovered and recycled with an efficiency up to 97 % [3]. Such efficiency can affect the sodium hydroxide-sodium sulphide ratio (sulphidity) in the cooking liquor of the pulping process [4], which is a fundamental parameter for the quality of the pulp. In fact, sulphur accumulates more than sodium [5], resulting in increasing need for $NaOH$ addition to maintain a constant ratio. In order to reduce the sulphurous emissions and the operational costs due to the surplus of chemicals needed, removal of excess sulphur from pulping industry is desirable. Eventually, it represents a valuable potential source of re-usable sulphur.

Today, biological approaches are gaining increasing attention as

alternatives to the established physico-chemical sulphur recovery processes, such as the Claus process (for a review, see [6]). However, the main concern of biotechnical processes is associated with the harsh conditions of these sulphurous streams, such as highly alkaline pH and high concentration of chemicals, which are inhibitory for many microorganisms. Some haloalkaliphilic sulphur oxidizing bacteria (SOB), oxidize reduced sulphur compounds and cope with conditions (for a review, see [7]) similar to those of the streams in pulping industry. The natural habitats of these bacteria are soda and salt lakes, characterised by pH in the range of 9–11 and high concentrations of total salts (up to 380–475 g/l) [7,8]. Among the various genera of haloalkaliphilic SOB, of interest for this study is the genus *Thioalkalivibrio*, that has extremely salt tolerant species [7]. In particular, the *Thioalkalivibrio versutus* grows at pH up to 10.6 and salinity up to 92 g/l Na^+ [8,9]. This aerobic obligate chemolithoautotrophic microorganism uses oxygen as electron acceptor to oxidize sulphurous compounds like sulphide and thio-sulphate to sulphate, with globular elemental sulphur as metabolic intermediate [8]. Once thiosulphate has been removed, elemental sulphur is used as electron donor (for a review, see [1]). During

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biotransformation of thiosulphate, minor formation of sulphite has been reported by Ang et al. [10]. The main reactions of thiosulphate biotransformation and their Gibbs-free energy changes have been described in the supplementary materials (Table S1).

Potential of haloalkaliphilic SOB in recovering sulphur from industrial streams has been demonstrated in the THIOPAQT[™] process [11] in the petroleum industry and in a wastewater treatment plant for PPI [12]. The main advantage of these biological approaches is that the operational costs are reduced since less chemicals and energy are needed as compared to traditional physico-chemical processes (for a review, see [1]).

Among the technologies used for high-rate biological treatment, fluidized bed bioreactors (FBBR) play a significant role in supporting a number of various biotransformations and have found several applications. These systems are based on fully mixed conditions and on biomass retainment as biofilm on large specific surface carrier materials, such as activated carbon. Some of the advantages of using FBBRs for concentrated sulphurous streams include high loading rates, efficient mass transfer and long sludge retention time enhancing high rates of biotransformation. In addition, the sulphur recovery can be accomplished from the recycle stream of the FBBR by installing a solid-liquid separation unit. (For a review, see [13])

Biological sulphur recovery from thiosulphate and sulphide solutions under non-haloalkaline conditions with different experimental designs and SOB has been reported for example by Gonzalez-Sanchez et al. [14] and Janssen et al. [15]. Both studies used *Thiobacillus* spp. in bioreactors with packing material and pH around 5 and 7.5, respectively. Under haloalkaline condition, Mu et al. [16] reported partial sulphur recovery from sulphide in a bioreactor with suspended biomass of *T. versutus*. So far, biotransformation of thiosulphate by *Thioalkalivibrio versutus* under haloalkaline conditions has only been studied in shake flask bioassays and batch mode chemostat experiments [9,10,17]. In this work, for the first time in literature, the *T. versutus* was used in a continuous flow FBBR. The aim of this study was to investigate biotransformations of thiosulphate by *T. versutus* in the FBBR, by varying the hydraulic retention time. Of particular interest was the conversion of thiosulphate to elemental sulphur and its separation from the liquid phase, aiming at developing a novel bioprocess for excess sulphur removal and recovery from concentrated industrial sulphurous streams.

2. Materials and methods

2.1. Model microorganism and growth medium

SOB *Thioalkalivibrio versutus* strain AL 2 (DSM 13738) used during this study was purchased from DSMZ GmbH (German Collection of Microorganisms and Cell Cultures GmbH). The stock culture was pre-cultivated in aseptic conditions in Erlenmeyers on an orbital shaker (150 rpm) at 30 ± 1 °C. The flasks contained 90 % (v/v) of 925 Alkaliphilic sulphur respiring medium and 10 % (v/v) *T. versutus* inoculum. The medium consisted of mineral base (20 g/l Na₂CO₃, 10 g/l NaHCO₃, 5 g/l NaCl, 1 g/l K₂HPO₄), 0.5 g/l KNO₃, 0.048 g/l MgCl₂, 2% (v/v) trace element solution (TES) and Na₂S₂O₃. The mineral base and TES were sterilized by autoclaving at 110 °C for 20 min and at 121 °C for 20 min, respectively. The stock solutions of KNO₃, MgCl₂, and Na₂S₂O₃ were sterile filtered (0.2 µm polyethersulfone membrane syringe filter, VWR International, U.S.A.) [17]. This medium was also used during the FBBR operations. Due to the high Na⁺ concentration and pH (~10) of the feed, the contamination of the culture in the bioreactor was highly improbable, and, therefore, the mineral base was prepared with tap water. The thiosulphate concentration was approximately 4.5 g/l in the pre-cultivation, 8 g/l in the semi-batch operation and 10 g/l in the continuous operation of the FBBR. The FBBR was inoculated with 10 % (v/v) stock culture. The volume of the inoculum was calculated from the total working volume of the FBBR and recirculation unit.

2.2. Bioreactor design and operation

The bioreactor was preliminary operated in semi-batch mode (14 days), to allow biofilm formation onto the carrier material, and then changed to continuous operation (71 days), to observe sulphur recovery. The setup of the system was slightly changed between the two modes (Fig. 1).

The main units of the system (Fig. 1) consisted of an FBBR, a recirculation unit (RU) and a gravity settling tank. The total volume of both the FBBR and RU was approximately 1 l. The bottom of the FBBR was filled with one 16 mm diameter size and several small (~8 mm diameter) glass beads below the carrier material bed, to prevent the granular activated carbon (AC) (Filtrisorb 200, Calgon Carbon Corporation, USA) leaking to the tubing below. The reactor was kept at 30 ± 2 °C by using a heating blanket that was controlled by a temperature probe. The probe was immersed from the top of the FBBR into the liquid-phase. Aeration was supplied from the bottom of the recirculation unit (approximately 21 % O₂, 78 % N₂ and 1% CO₂), in order to minimize air bubbles entering the FBBR or the solid-liquid separation unit, and was controlled by a manual flow meter. The flow-meter was adjusted several times during operation to achieve steady gas flow. The RU was connected back to the FBBR and the recirculation flow was controlled by a peristaltic pump (Master flex, Cole-Parmer, USA).

In the continuous mode (Fig. 1b) an upper valve of the recirculation unit was used for the removal of the treated effluent. Furthermore, a settling tank was set between the FBBR and RU, to separate elemental sulphur from the liquid phase before the recirculation of the liquid. The flow-rate of the recirculation was set to provide 17 % expansion (536 ml) of the AC bed. After 21 days of operation, the recirculation was increased to achieve 20 % expansion (550 ml) which was maintained until the end of the continuous operation. The percent expansion was determined based on the volume increase from the non-fluidized to fluidized bed carrier material

The performance of the FBBR was studied by varying the hydraulic retention time (HRT) between 12 and 5 h in the FBBR. The HRT was referred to the fluidized bed volume.

The elemental sulphur production in the bioreactor was estimated by the sulphur balance Eq. (1):

$$[S^0]_{\text{out}} (\text{g/l}) = [S_2O_3^{2-}\text{-S}]_{\text{in}} + [SO_4^{2-}\text{-S}]_{\text{in}} - [S_2O_3^{2-}\text{-S}]_{\text{out}} - [SO_4^{2-}\text{-S}]_{\text{out}} \quad (1)$$

where the concentrations are in g/l and the small amount of sulphate detected in the feed was considered. During continuous operation, 2 ml samples were taken both from the effluent line of the RU and from the feed tank, 4 times a week, for determination of DO, pH and concentration of sulphur constituents. Also, biomass carrier samples (2 x 1.5 ml) and liquid sample with culture suspension (2 x 1.5 ml) were collected before each HRT change for biomass quantification. Some AC samples from the initial stage of continuous operation (HRT 12 h) and from day 69 (HRT 6 h) were taken for biofilm and elemental sulphur visualization by scanning electron microscopy (SEM). Moreover, sulphur precipitate from the settler on day 69 was also taken for SEM.

2.3. Elemental sulphur separation

During continuous operation, elemental sulphur produced by *T. versutus* was visually characterised as small, whitish particles suspended in the liquid. To enhance the efficiency of settling, centrifugation and coagulation were investigated.

2.3.1. Centrifugation tests

The centrifugation tests were implemented to find a combination of relatively low speed and short time that enables efficient separation of elemental sulphur from the effluent. Centrifugation with 4–16KS centrifuge (Sigma, Germany) was tested by using duplicate samples from the FBBR effluent. First, different rotational speeds (2, 53, 214,

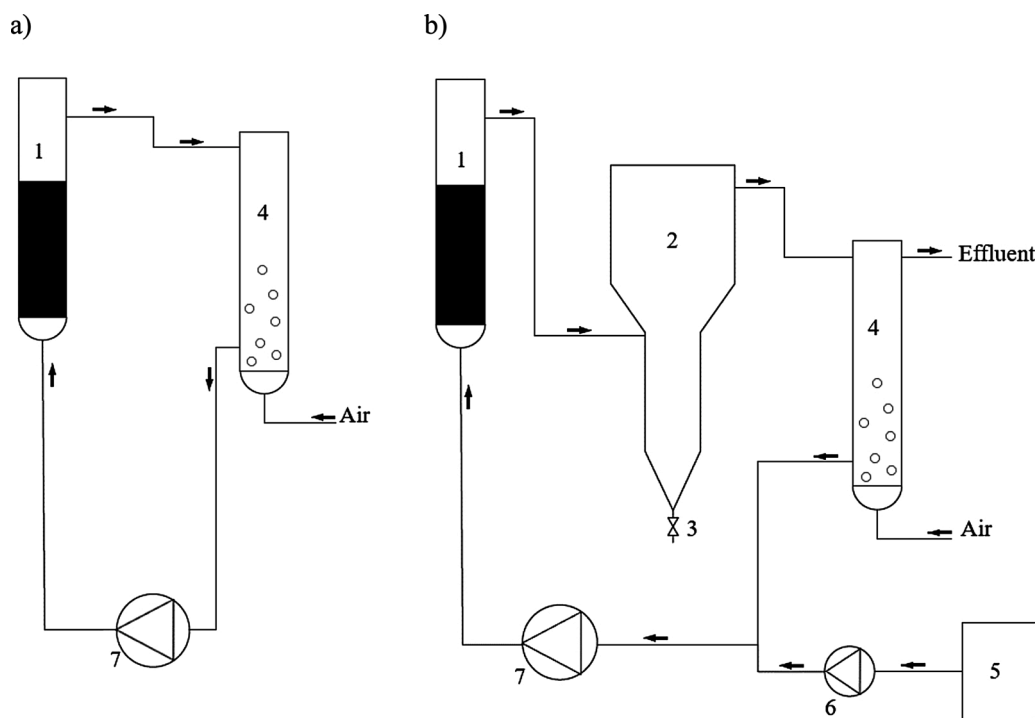


Fig. 1. Schematic diagram of the fluidized bed bioreactor system: (1a) during the semi-batch operation, (1b) during the continuous operation. Units: (1) fluidized bed bioreactor (FBBR), (2) settling tank, (3) S^0 outlet, (4) recirculation unit (RU), (5) feed tank, (6) feed pump, (7) recirculation pump. Not drawn to scale.

480, 854, 1335 and 3417 relative centrifugal force (rcf) with fixed duration (5 min) and then different durations (1, 2.5, 5, 7.5, 10, 12.5 and 15 min) with fixed rotational speed (214 rcf) were used. The speed of the duration test was selected based on the results of the speed test. The effluent from the FBBR (HRT 6 h) was collected overnight, manually mixed, filled into falcon tubes (50 ml) and mixed by vortexing before centrifugation.

2.3.2. Coagulation tests and their effect on the biotransformation

Settling enhanced by coagulation was done similarly as in the study of Chen et al. [18]. First (Test 1), different coagulants with concentration of 0.5 g/l were studied: $Al_2(SO_4)_3$, $Fe_2(SO_4)_3$, $FeCl_2$ and $FeCl_3$. Based on the results of Test 1, the coagulant that was most efficient with the separation of elemental sulphur was tested at 0.1, 0.25 and 0.5 g/l (Test 2). Similarly, as prior to the centrifugation tests, the effluent from the FBBR was collected overnight. For Test 1 and 2, separate batches of effluent were used, collected at HRT 5 h and 6 h, respectively. Prior to the tests, the effluent was continuously mixed with a magnetic stirrer. The protocol followed was similar to the Jar test, except for the working volume used. Testing with larger volume (1000 ml/sample) as it was suggested in the protocol would have required longer effluent collection time, thus resulting in further oxidation of elemental sulphur to sulphate. Therefore, duplicate beakers with working volume of 100 ml (90% v/v effluent) were used with each coagulant and concentration. To reach 100 ml working volume, MilliQ-water was supplemented over the volumes of the coagulants. After the addition of the coagulant, the mixture was stirred rapidly at 400 rpm for 10 s and then at 100 rpm for 20 min. After the stirring, the mixture was transferred to volumetric cylinders (100 ml) and let to settle for 30 min. Finally, the amount of the floc was recorded, and the turbidity and the pH of the liquid phase measured.

For both the centrifugation tests and coagulation Test 2, turbidity was measured before and after the experiments and the TS separation efficiency (SE) was calculated by using Eq. (2):

$$SE(\%) = \frac{TS_{out} - TS_{calc}}{TS_{out}} \cdot 100 \quad (2)$$

Where TS_{out} and TS_{calc} are the concentrations (g/l) of total solids of the FBBR effluent sample prior and after the separation, respectively. The TS_{calc} was estimated by using standard turbidity-TS curve. The same percentage efficiency was assumed for the elemental sulphur separation.

After the tests, the possible toxicity of different concentrations of $FeCl_2$ (0.1, 0.25 and 0.5 g/l) on *T. versutus* was investigated. Duplicate cultures in shake flasks with each $FeCl_2$ concentration and two inoculated control flasks without coagulant addition were prepared. Each flask had 100 ml culture (10 % (v/v) stock culture as inoculum and 90 % (v/v) medium with approx. 10 g/l $S_2O_3^{2-}$). The coagulant was added over the culture volume and supplemented with sterile MilliQ-water to reach 101 ml working volume. The flasks were placed to an orbital shaker (KS 4000i Control, IKA, USA) at 150 rpm and 30°C for 7 days. A 2 ml sample was taken from each flask daily.

2.4. Analytical methods

The thiosulphate ($S_2O_3^{2-}$) and sulphate (SO_4^{2-}) concentrations were analysed with ion chromatography according to di Capua et al. [19]. The only modification was that Dionex IonPac AS22 anion exchange column (Thermo Scientific) was installed to the ion chromatograph. The samples were diluted with MilliQ-water (~ pH 10) to prevent auto-oxidation. The pH and DO of the samples (reactor system and toxicity test) were measured with pH-meter (pH 3110, WTW, Germany) and HQ40d portable multimeter equipped with an intellical LDO101 probe (HACH, USA), respectively.

The turbidity was measured by using portable turbidimeter (TN-100, Eutech instruments, Singapore). Standard turbidity-total solid (TS) curves of the effluent were interpolated for the centrifugation and the coagulation (Test 2) batches. For both batches, TS were determined on a 12 ml sample, after 24 h at 105 °C, according to the EPA protocol.

2.5. Quantification of biomass

The biomass as biofilm and suspension were estimated from the AC and the effluent, respectively, by using Bradford protein analysis. To

maintain the same volume of AC in the FBBR, the removed sample volume was each time replaced with new one, that was overnight soaked in the mineral base. The effluent samples were centrifuged (5417R, Eppendorf, Germany) at 2800 rcf and 4 °C for 15 min. After removal of the supernatant, the AC and cell pellets were stored at -80 °C until analysis. Prior to the Bradford analysis, the cell pellets and 1 g AC samples were pre-treated by adding 1 ml 1 M NaOH, vortexing and keeping them at 90 °C water bath for 10 min. After the heating, the samples were cooled down in an ice bath, centrifuged at 2000 gravity force (g) for 2 min and the supernatant diluted first with sterile MilliQ-water and then with phosphate-saline buffer (PBA). The protein quantification was performed by using Plate Chameleon microplate reader (Hidex).

2.6. Particle analysis and visualisation of biomass attached on AC

The carrier material (AC) (HRT 12 and 6 h) and sulphur samples from the settler were visualized by using scanning electron microscopy (SEM). Prior to the SEM the AC sample from HRT 12 h and the sulphur samples were oven dried at 150 °C. The carrier material from HRT 6 h was let to settle on double-sided carbon tape that was stick to a Petri dish. After fixing the AC, primary fixation took place with 2.5 % glutaraldehyde in 0.025 M phosphate buffered saline (PBS) containing 0.15 % Alcian Blue for 2 h according to Kaksonen et al. [20]. The liquid was removed after 2 h and the sample washed with 0.025 M PBS for 5 min and sequentially dehydrated in ethanol series (50, 70, 90 and 100 %) for 15 min in each concentration. Finally, the samples were critical-point dried in ethanol-acetone series (75–15, 50–50, 25–75 and 0–100) and stored in a desiccator.

The oven dried AC and sulphur samples were stick to double-sided carbon tape on an SEM sample tubs. Also, the carbon tape with the AC sample was stick on another SEM sample tub and then both samples were carbon coated with carbon evaporator. The coated samples were visualized with a high-resolution Jeol JSM-IT500 scanning electron microscope (Japan) equipped with energy-dispersive X-ray spectrometer (EDS).

3. Results

3.1. FBBR continuous operation

During the 71 days of continuous operation, thiosulphate was continuously supplied to the FBBR system at different HRTs resulting in different loading rates (from 11.9 ± 1.2 to 33.0 ± 1.1 g $S_2O_3^{2-}$ -S/l/d). The HRT was gradually decreased from 12 h to 5 h, and then increased back to 6 h. Performances of the FBBR were as shown in Fig. 2. Thiosulphate was completely removed at HRTs from 12 to 9 h. During the period from 11 to 9 h HRTs the average sulphate and elemental sulphur (estimated by the mass balance) concentrations were 4.8 ± 0.1 g/l and 1.8 ± 0.1 g/l, respectively (Fig. 2a). The increase in the biotransformation rate and sulphur production rate (Table 1) corresponded with the increase in the loading rate at HRT from 11 to 9 h (Fig. 2b). In this period the removal efficiency remained at 99.9 % and the conversion efficiency to elemental sulphur was averagely $27 \pm 2\%$ (Fig. 2c). The effluent pH slightly decreased from the pH 10 of the feed and remained stable at 9.7, while the average DO was 4.5 ± 0.2 mg/l (Fig. 2d and e).

Once the HRT was further decreased from 9 h to 7 h, the thiosulphate in the effluent started to increase, sulphate started to decrease while the average calculated elemental sulphur and the average S^0 yield remained at 1.8 ± 0.1 g/l and $27 \pm 2\%$, respectively (Fig. 2a and c). This was also seen as partial biotransformation and decrease in % removal of thiosulphate, while sulphur production rate increased (Fig. 2b and c). The pH and DO remained stable (Fig. 2d and e). The partial biotransformation indicated that the removal capacity of the FBBR was reached.

When the HRT was decreased to 5 h, effluent thiosulphate and sulphate remained constant, while average calculated elemental sulphur

decreased to approximately 0.4 ± 0.1 g/l after day 53. (Fig. 2a) Similarly, the trend of thiosulphate biotransformation rate started to decrease, although the loading rate was increased, but sulphur production rate maintained an increasing trend until day 53 (Fig. 2b). During this period, the removal efficiency remained at $68 \pm 1\%$ (Fig. 2c). The pH remained stable, while DO uncontrollably decreased and was difficult to reliably determine (Fig. 2d and e). These results showed that at HRT of 5 h (average loading rate 33.0 ± 1.1 g $S_2O_3^{2-}$ -S/l/d) the oxygen supply became process limiting. This was partially caused by the low oxygen transfer due to the clogging in the aeration system.

The trial of recovering the process by increasing the HRT to 6 h (Fig. 2a) and above (results not shown) was not accomplished as seen by increasing thiosulphate effluent concentration and decreasing biotransformation rate.

Elemental sulphur production was confirmed not only with the consistent presence of whitish particles in the effluent but also visualized with SEM (Fig. 3). The EDS mapping of the elements (Fig. 3c,d,e) showed that sulphur was the most abundant element of the solid samples from the settler and the biologically produced S^0 particles were in the range 2–3 μ m. The sample was carbon coated, thus the second most abundant element was carbon mainly originated from there.

3.1.1. Biomass

Most of the biomass grew as biofilm on the carrier material. The attached biomass in the continuous mode gradually increased (based on protein concentration) during the period from day 0 (0.048 ± 0.004 mg/g AC) to day 22 (results not shown). In the period from 11 to 6 h HRT (49 days), both the attached and suspended protein concentration fluctuated around average values of 0.303 ± 0.007 mg/g AC and 0.055 ± 0.004 mg/ml effluent, respectively, with no significant increasing or decreasing trends (weekly sampling). The suspended protein concentration represented about 15–20 % of the total protein. These results indicate that biomass accumulation onto the carrier remained low and part of the biomass was lost with the effluent.

The biofilm formed was characterised by scanning electron microscopy. As Fig. 3a shows *T. versutus* was present on the surface and in the pores of the carrier material. Whitish globules represented elemental sulphur (Fig. 3b).

3.2. Elemental sulphur separation

In the sulphur separations tests, direct measurement of elemental sulphur was not available and therefore, the S^0 removal percentage was estimated as direct proportional to the TS separation efficiency.

In the centrifugation tests (Fig. 4), the turbidity and TS concentration of the collected effluent were 640 NTU and 58.1 g/l, respectively. By increasing the centrifugation speed (Fig. 4a), the turbidity in the effluent decreased, thus enhancing the elemental sulphur separation. More than 50 % of S^0 was removed at 214 rcf, while it reached 93 % at 3417 rcf. For the duration test, 214 rcf was selected based on the sulphur removal efficiency in the previous test. The percent sulphur removal increased by approximately 25 % with increasing centrifugation time from 1 to 7.5 min and by less than 10 % after that. The highest sulphur removal was 71 % with the centrifugation time of 15 min (Fig. 4b).

In the first coagulation test, ferrous chloride supplementation resulted in the highest separation efficiency seen as the highest turbidity removal and settled volume ($76 \pm 1\%$ and 8 ml, respectively), while ferric chloride was the second most efficient ($76 \pm 1\%$ and 7 ml, respectively). Therefore, $FeCl_2$ was selected for further studies. The results of the coagulation test with various $FeCl_2$ concentrations are shown in Fig. 5. The turbidity and TS concentrations of the collected effluent were 320 NTU and 47.5 g/l, respectively. The removal of turbidity and sulphur increased with increasing concentrations of ferrous chloride. The highest sulphur removal (40 %) was reached with a $FeCl_2$ concentration of 0.5 g/l. Also, the highest volume of settled precipitate (6.75 ml) was obtained with 0.5 g/l $FeCl_2$, while it was only 2 ml with

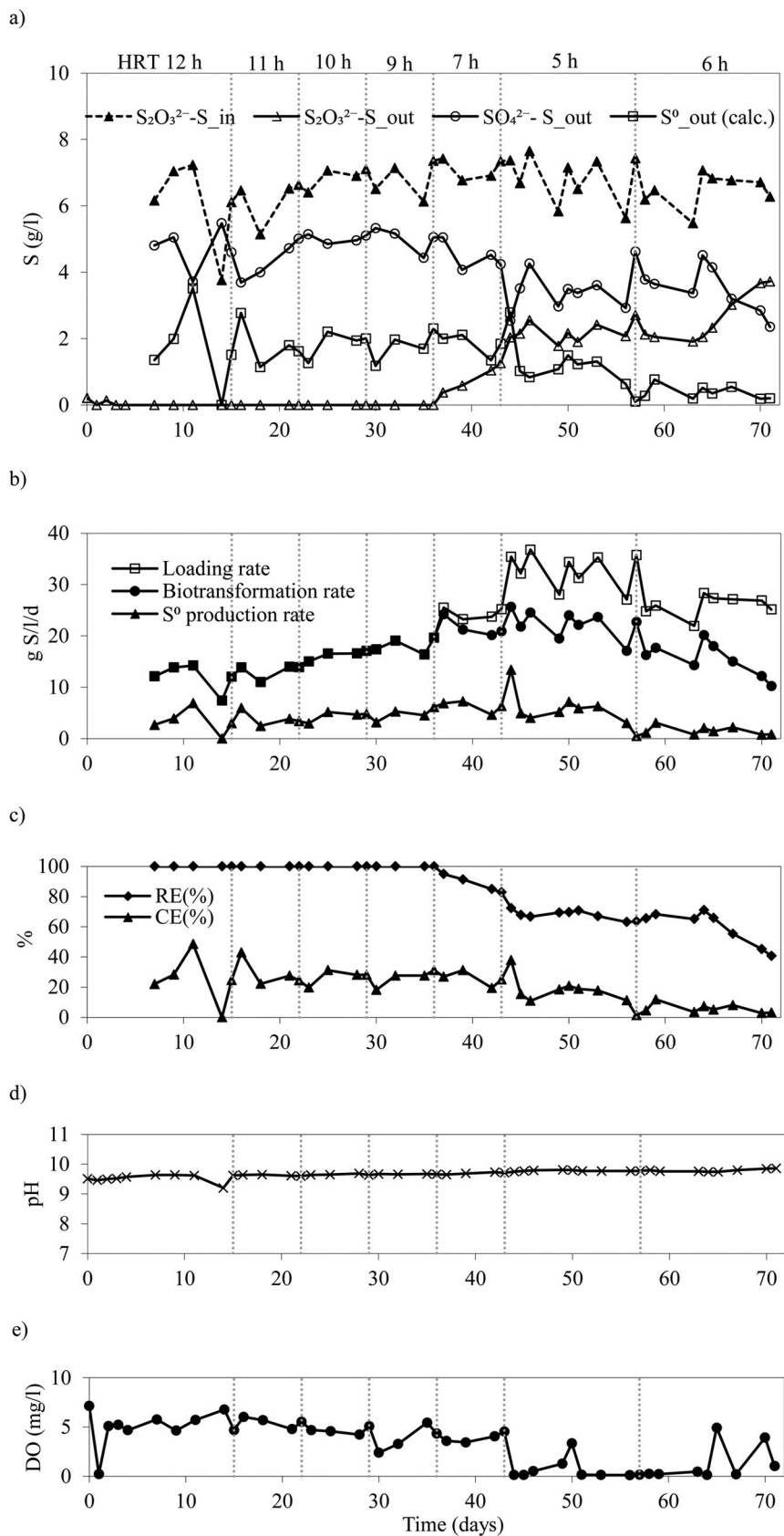


Fig. 2. Fluidized bed bioreactor performances during the continuous operation. Time course profiles of sulphur compounds concentration (a); loading rate, biotransformation rate, elemental sulphur production rate (b); removal efficiency (RE), conversion efficiency (CE) (c); pH and dissolved oxygen (DO) (d and e). S^0 formation was based on mass balance calculation (Eq. 1). The inlet sulphate had an average value throughout the continuous operation of 0.29 ± 0.04 g S/l.

Table 1

Average biotransformation rate (BR) and elemental sulphur production rate in the fluidized bed bioreactor for HRT 11-6 h. The S^0 concentration was calculated by using the sulphur mass balance (Eq. 1).

HRT	11 h	10 h	9 h	7 h	5 h	5–6 h
Days	16–22	23–29	30–36	37–43	44–53	54–71
\overline{BR} (g S/l/d)	13.0 ± 0.6	16.3 ± 0.4	18.1 ± 0.7	21.6 ± 0.9	23.0 ± 0.8	16.6 ± 1.1
S^0 production rate (g S/l/d)	3.7 ± 0.6	4.4 ± 0.5	4.8 ± 0.6	6.3 ± 0.6	6.7 ± 1.2	1.6 ± 0.3

0.25 g/l and less than 1 ml with 0.1 g/l. The pH after 30 min settling remained around 9.8 at each $FeCl_2$ concentration. In summary, centrifugation showed better removal efficiency than coagulation.

3.2.1. Effect of $FeCl_2$ on biotransformation of thiosulphate

The effect of ferrous chloride was evaluated by monitoring thiosulphate biotransformation and pH changes during incubation of *T. versutus*. The pH in the flask with $FeCl_2$ decreased from 10 to approximately 9 (day 4) and had final value of 9.3, while the pH of the control flask decreased to 9.8 (see supplementary materials, Fig. S8). The thiosulphate removal (Fig. 6) was increased with the addition of ferrous chloride (0.1, 0.25 and 0.5 g/l initial concentrations). All thiosulphate was completely removed after 100 h. Sulphate was produced from thiosulphate, with only a minor elemental sulphur production. The results showed that all $FeCl_2$ concentrations enhanced the thiosulphate removal and the highest rate of 0.1 g $S_2O_3^{2-}$ -S /l/h was with 0.1 g/l $FeCl_2$. These results suggested that the use of ferrous chloride coagulant in the FBBR system could stimulate the biotransformation rates.

4. Discussion

This study revealed that thiosulphate bioconversion to elemental sulphur was obtained with an average efficiency of $27 \pm 2\%$ with *T. versutus* in an FBBR system at alkaline pH and $[Na^+]$ of 17.5 g/l and that the biotransformation capacity was controlled by HRT (loading rate). This study also revealed that solid-liquid separation efficiency of the sulphurous effluent was 93 % by centrifugation.

4.1. Performance of FBBR in continuous operation

Table 2 compares the average results obtained at HRT 9 and 7 h with other studies on the conversion of thiosulphate or hydrogen sulphide to elemental sulphur. In this study, the biotransformation rate was one of the highest among the reported. Only Krishnakumar et al. [21] reported a higher sulphur (as sulphide) biotransformation rate of 26 g S/l/d (estimated from their results) than in this study at HRT 7 h. The obtained biotransformation rates can potentially be further increased by more efficient aeration and more efficient biomass retainment than reported in this study. At pH 10, Baquerizo et al. [22] reported no sulphur production in biotrickling film reactor at low loading rates of 3.4 g $S_2O_3^{2-}$ -S/l/d, whereas our study showed an average rate of 6.3 ± 0.6 g S^0 /l/d at HRT of 7 h. This difference was probably due to the higher thiosulphate loading rate in our study. However, the CE to elemental sulphur in our study ($27 \pm 2\%$) was the second lowest (Table 2), which was likely because O_2/S ratio was not optimized. By comparing the studies where thiosulphate was used, the initial concentrations of substrate were consistently higher than those using sulphide. This was because SOB tolerate dissociated sulphide forms only up to 0.8 g/l (for a review, see [23]). To overcome the sulphide inhibition, HS^- can be first chemically oxidized to $S_2O_3^{2-}$ followed by biotransformation by SOB, as reported by de Graaff et al. [24].

In summary, the FBBR amended with *T. versutus* reached higher thiosulphate biotransformation rates than earlier reported and demonstrated the potential of elemental sulphur production at haloalkaline conditions (pH 10, $[Na^+]$ 17.5 g/l).

4.2. Biotransformation limiting factors

Results of this study showed that the thiosulphate loading rate and the oxygen availability played important roles in biotransformation.

The results show that the bioconversion capacity of the biomass retained in the FBBR was reached at the average $S_2O_3^{2-}$ -S feed rate of 24.4 ± 0.5 g S/l/d. Janssen et al. [15] and Velasco et al. [25] when using HS^- and $S_2O_3^{2-}$, respectively, reported increasing elemental sulphur production rate with increasing LR, as also seen in our study. In our study, the low S^0 conversion yield at HRTs higher than 7 h can be partially attributed to the complete removal of thiosulphate favouring bio-oxidation of the produced elemental sulphur to sulphate (see Eq. (c), Table S1 of Supplementary materials), as also reported by Janssen et al. [29].

Other reactor studies [14,25,29] reported that elemental sulphur formation was favoured by controlling the O_2/S_{in} ratio and maintaining DO below 0.1 mg/l, while at higher DO mainly sulphate was produced. For example, Janssen et al. [29] reported that the molar ratio of oxygen to sulphide of 0.6–1.0 favoured elemental sulphur formation. Annachatre and Suktrakoolvait [26] observed S^0 as the main end product of sulphide oxidation below 0.1 mg/l DO concentration. These suggest that the DO concentration (4.5 ± 0.2 mg/l) in our study before HRT 5 h promoted sulphate as the main product. Velasco et al. [25], Janssen et al. [15] and Annachatre and Suktrakoolvait [26] reported different concentrations of DO in the aeration unit (>4 mg/l) and the bioreactor (<0.1 mg/l). However, DO conditions in our FBBR and RU were likely the same and elevated (>3 mg/l) due to fully mixed FBBR conditions and were not optimal for S^0 production. On the other hand, higher conversion yields to S^0 for *T. versutus* have been reported by Hajdu-Rahkama et al. [17] in batch assays with $S_2O_3^{2-}$ and by Mu et al. [16] in a reactor system with HS^- (Table 2), suggesting that the sulphur yield could also be improved in the FBBR by improved oxygen supply control. Afterwards, due to clogging of the glass sinter at the bottom of aeration unit, the air supply became compromised and the process became oxygen limited. This also resulted in further decline in biotransformation performance after day 53. Fig. 2b shows that decreasing the loading rate did not result in recovery of the system. On the contrary, the thiosulphate biotransformation rate and elemental sulphur production rate (Fig. 2b) as well as the elemental sulphur recovery efficiency (Fig. 2c) further declined.

In summary, the FBBR demonstrated high thiosulphate biotransformation rates whilst the elemental sulphur production remained partial. For the optimization of elemental sulphur production from thiosulphate, substrate limitation should be avoided in order to prevent further oxidation of the produced S^0 . Therefore, high loading rates together with low DO concentration should be maintained to optimize elemental sulphur production. For example, DO should be monitored rather by the oxidation redox potential (ORP) than the less reliable DO probes. ORP monitoring was used by Mu et al. [16] and suggested by Janssen et al. [29], as the optimal DO for elemental sulphur production is below the detection limit of the DO probes.

Although the findings of this study revealed the potential of *T. versutus* amended bioprocess for elemental sulphur production from thiosulphate under haloalkaline conditions, further studies with real process streams such as of pulping is essential. The process streams may contain chemical constituents inhibitory or competitive for this

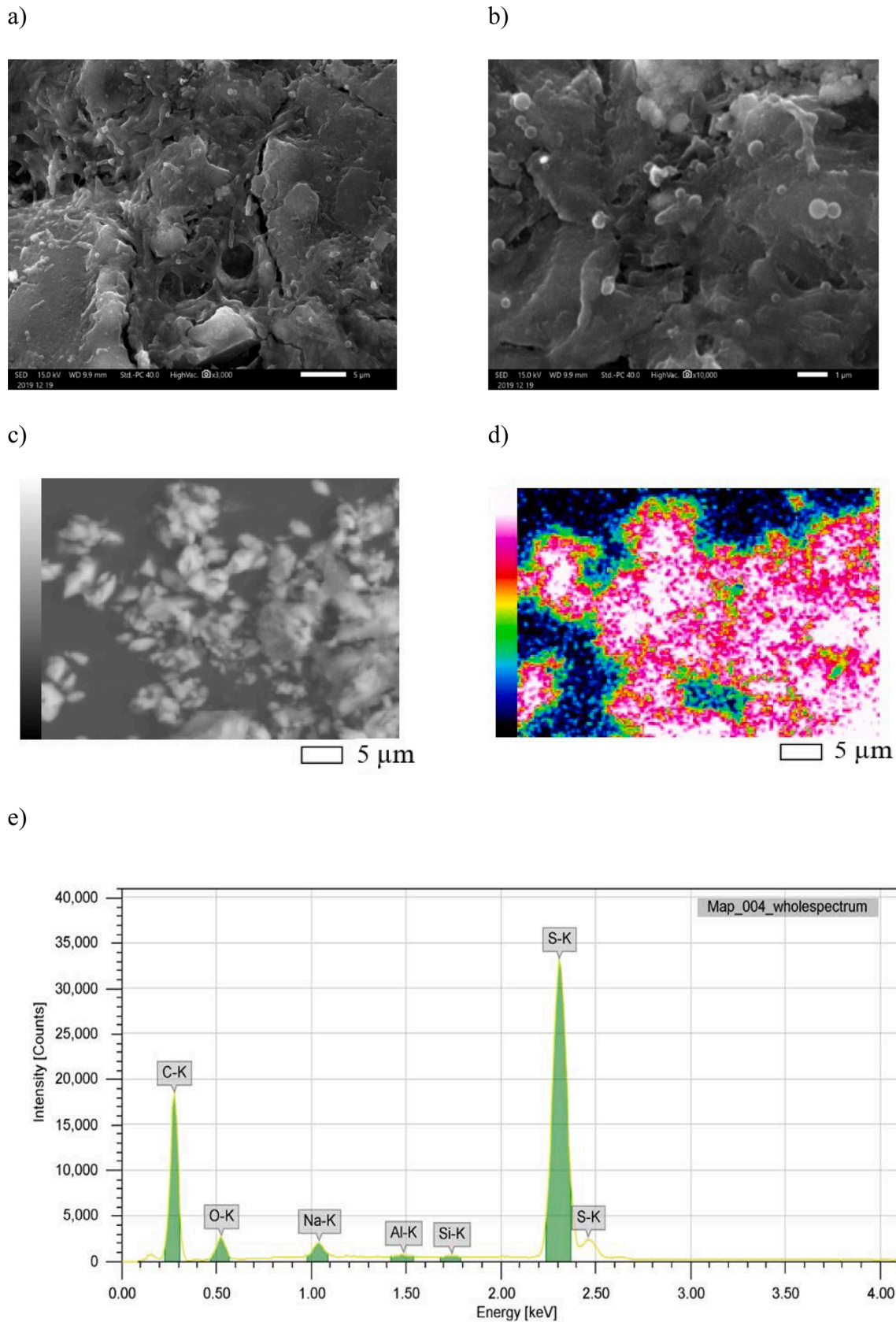


Fig. 3. Scanning electron microscope (SEM) images of the activated carbon (a-b) and sulphur from the settler (c-e) taken on day 69 of continuous bioreactor operation (HRT 6 h). a-b) biofilm formed on the surface of the activated carbon in the fluidized bed bioreactor; c) sulphur and d-e) SEM and energy-dispersive X-ray spectrometer (EDS) images. Fig. 3d) visual elemental sulphur distribution in the sample. The colour bar on the left-hand side indicates the concentration of the element. When going from down (black) to up (white), the concentration increases. Fig. 3e) quantitative analysis of the elements present in the sample. The K after the element indicates K(alpha)-radiation of a certain element.

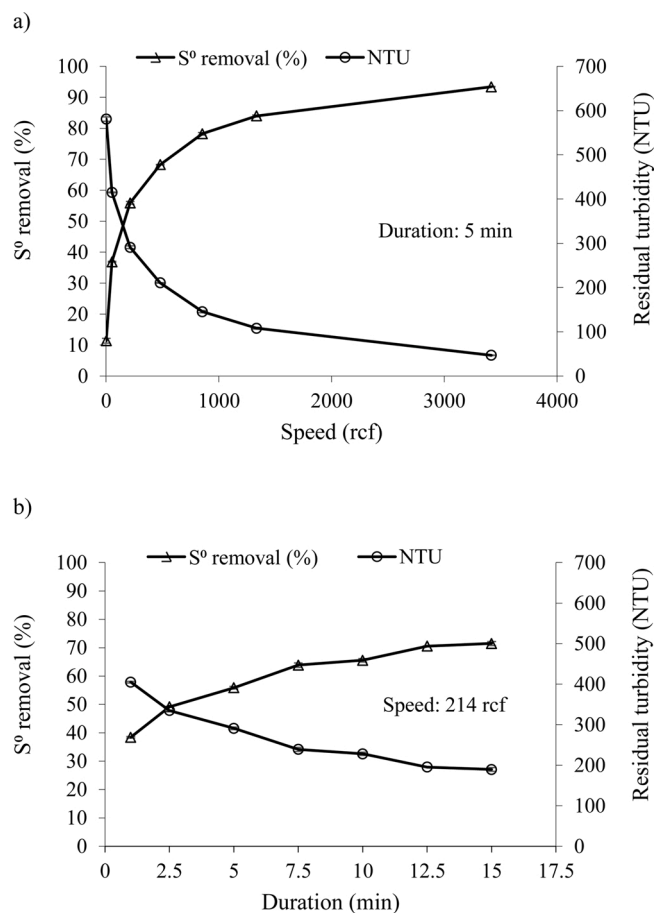


Fig. 4. Centrifugation tests results. Speed test (a) and duration test (b). Initial turbidity and TS in the effluent batch were 640 NTU and 58.1 g/l, respectively.

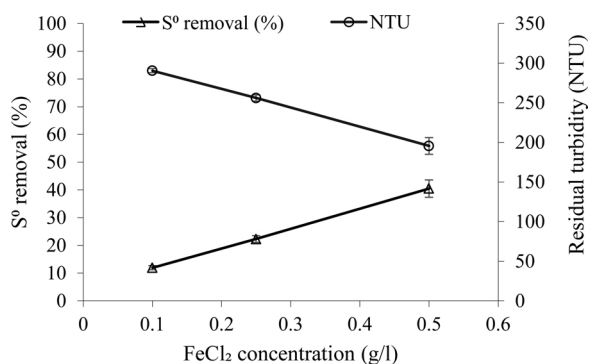


Fig. 5. Coagulation of sulphurous effluent from fluidized bed bioreactor after 30 min settling and as a function of $FeCl_2$ concentration. Initial turbidity and TS in the effluent batch were 320 NTU and 47.5 g/l, respectively.

biotransformation. For example, Janssen et al. [30] reported inhibition of sulphide oxidation by methanethiol present in sulphidic spent caustics of petrochemical industry. Moreover, the sulphurous process streams of several industries are rich in sulphide that can be inhibitory towards the oxidation of other sulphurous compounds [24].

4.3. Elemental sulphur separation

Scanning electron microscope confirmed that the majority of the whitish precipitates in the FBFR system consisted of sulphur. Removal of sulphur from liquid phase in elemental form can be easily done by solid/

liquid separation [17] making its re-use possible in various fields, such as agriculture, bioleaching processes and water treatment (for a review, see [1]). Gravity settling of this study was inefficient, and therefore, different methods to improve the elemental sulphur separation were revealed. The poor settling of sulphur particles in the FBFR system was likely due to the vertical up-flow in the settling tank. Besides, the consistent turbulence in the FBFR disrupted the sulphur aggregates which was also reported by Mu et al. [16] in a bio-desulphurizing system. However, based on visual observations, an increase in the LR (to approximately 24 g S/l/d) improved sulphur settleability. This was in accordance with the results of Janssen et al. [15,31] and Velasco et al. [25]. Regarding the morphology of S^0 particles in the FBFR system's settling tank, their dimensions of below 5 μm were similar to those reported by Mu et al. [16] for suspended *T. versutus* biotransforming sulphide (Table 2) and was likely affected by the shear stress caused by the up-flow in the FBFR. Janssen et al. [31] reported colloidal properties for the biologically produced elemental sulphur particles, presenting a negative charge increasing with pH and salinity. Hence, in this study, small dimension of elemental sulphur favoured a colloidal behaviour which limited their aggregation, together with the disruptive force of the vertical flow.

From the four coagulants tested, ferrous and ferric chloride were the most efficient. In alkaline environment, ferrous ions immediately oxidize to ferric ions, and simultaneously served as coagulant forming various hydroxyl precipitates [32].

Separation of biologically produced elemental sulphur by means of coagulation has been reported by Chen et al. [18] with polyaluminium chloride (PAC), polyacrylamide (PAM) and an organic flocculant (MBF). They obtained over 90 % coagulation efficiency of elemental sulphur at pH 6 with 0.27 ± 0.02 g/l of PAC, whereas our results in alkaline conditions with 0.5 g/l $FeCl_2$ resulted just in around 40 %. Lohwacharin and Annachatre [27] reported an optimal aggregation for 0.4 g/l biologically produced S^0 at pH 7.5 by using 0.71 g/l of PAC.

The possible effect of $FeCl_2$ coagulant on the FBFR efficiency was also investigated in this study with no adverse effects on biotransformation. On the contrary, all the ferrous chloride concentrations tested (0.1, 0.25 and 0.5 g/l) enhanced thiosulphate oxidation to sulphate. The ferric precipitates formed could help in retaining active biomass in the FBFR system, as reported by Ahoranta et al. [33]. However, possible effects of ferrous chloride on elemental sulphur production have not been investigated for haloalkaline applications.

Centrifugation turned out to be more efficient for sulphur removal than coagulation with ferrous chloride: at a speed of 214 rcf for 2.5 min duration, about 50 % of the sulphur was removed. Therefore, centrifugation is preferable as it is independent of the pH and of coagulants, and the separated sulphur is of better purity due to no extra added chemicals. Various speed and duration combinations should be optimized for higher than 90 % removal efficiency with particular attention to the energy consumption. The high performance of sulphur separation by centrifugation (decanter centrifuge) has been reported in the THIO-PAQ™ process, resulting in a slurry with 60–65 % dry solids content and above 95 % purity of the S^0 separated [11].

5. Conclusions

The conclusions of this study on elemental sulphur production from thiosulphate in haloalkaline conditions in a *T. versutus* amended FBFR during 71 days of operation are as follows:

- At stable condition, the 7 h HRT produces the highest average S^0 rate of 6.3 ± 0.6 g S/l/d with a yield of $27 \pm 2\%$.
- Thiosulphate biotransformation is complete at 9 h HRT but starts to decrease at 7 h HRT.
- Biomass is partially retained and S^0 is present on the activated carbon as demonstrated by SEM.

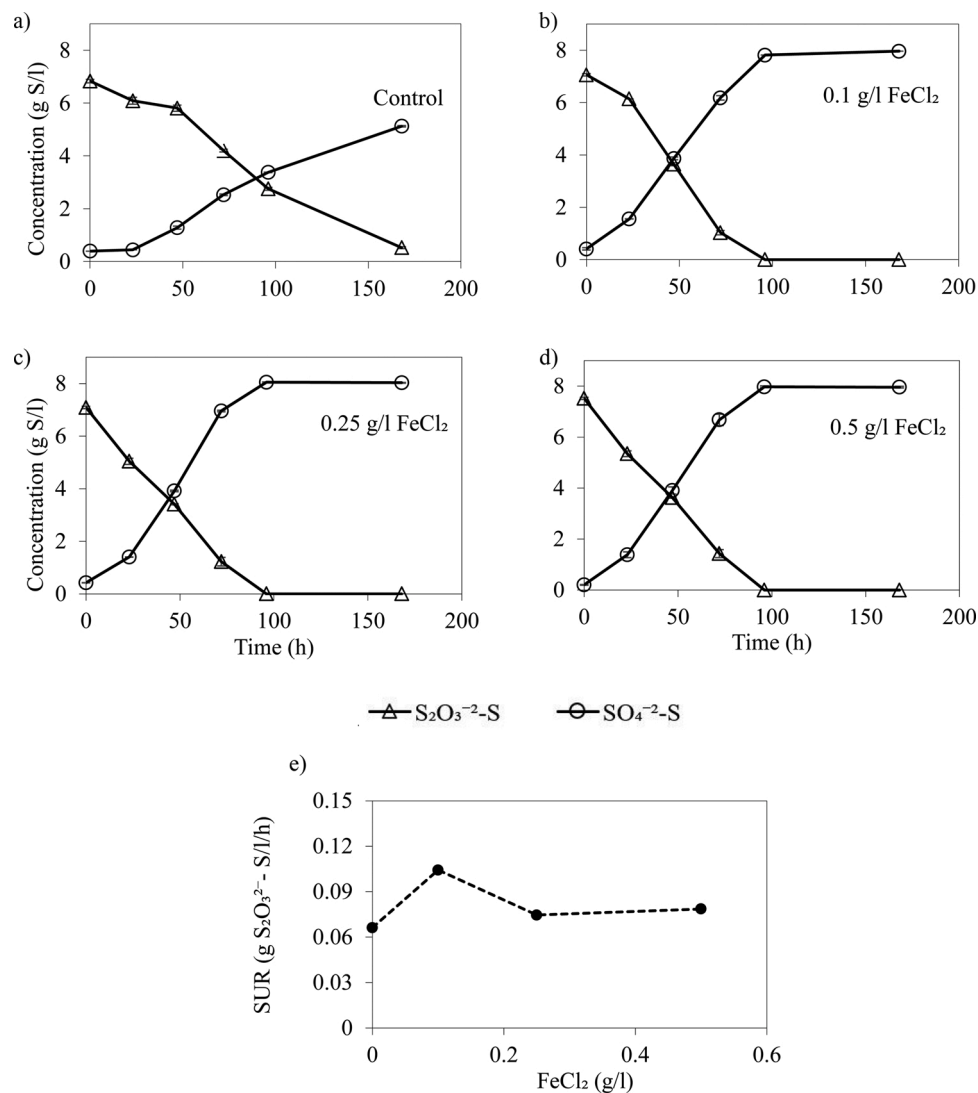


Fig. 6. Effect of FeCl_2 on thiosulphate biotransformation by *T. versutus*. SUR represents the substrate utilisation rate.

Table 2

Comparison of laboratory-scale studies using different SOBs and $\text{S}_2\text{O}_3^{2-}$ or HS^- as substrate. For this study average values for performance indicators are reported.

Experimental conditions								Performance indicators			Reference
Experimental design	Substrate	Initial conc. (g S/l)	T [°C]	pH	Microorganism	HRT (h)	LR (g S/l/d)	RR (g S/l/d)	RE (%)	CE (%)	
FBBR	$\text{S}_2\text{O}_3^{2-}$	~6	30 ± 2	~10	<i>T. versutus</i>	9–7	~18–24	~18–22	99.9–88	27 ± 2	This study
Batch assays	$\text{S}_2\text{O}_3^{2-}$	17.6	30	10	<i>T. versutus</i>	/	/	2	99.9	45	[17]
Upflow bioreactor with suspended biomass	$\text{S}_2\text{O}_3^{2-}$	6.8	30	5–5.5	<i>Thiobacilli spp.</i>	N.R.	3.4	N.R.	≥ 90	60	[25]
Supernatant-recycling settler bioreactor with PVC packing	$\text{S}_2\text{O}_3^{2-}$	6.4	30	5–5.5	<i>Thiobacilli spp.</i>	N.R.	< 8	N.R.	>90	77	[14]
Biotrickling filter system	$\text{S}_2\text{O}_3^{2-}$	12.2	25	10	Alkaliphilic SOBs	0.06	3.32	3.31	~100	0	[22]
Bioreactor with suspended biomass	HS^-	N.R.	25	9.5	<i>T. versutus</i>	0.25	3.2	N.R.	N.R.	~86	[16]
FBBR	HS^-	0.48	25–30	7.8	Distillery sludge	N.R.	1.6	N.R.	>90	76	[26]
Reverse fluidized loop reactor	HS^-	0.24	N.R.	8	<i>Thiobacillus demitrificans</i>	N.R.	29	N.R.	90	65	[21]
Airlift reactor (w/o recirculation)	HS^-	0.5	Ambient	7.8	Domestic WWTP sludge	3.5–3.7	4	4.3*	>93	>80	[27]
Expanded bed reactor	HS^-	0.24	22 ± 2	7.2–7.6	<i>Thiobacillus</i> -like bacteria	N.R.	~7	N.R.	~100	~70	[15]
Upflow bioreactor with fixed film	S^{2-}	~0.15	20	8.5	SOB	0.22	~17	16	95	>90	[28]

LR = Loading Rate, RR = Removal Rate (=Biotransformation rate), RE = Removal Efficiency, CE = Conversion Efficiency to S^0 , N.R.= Not Reported, * g S/gVSS/d.

- Gravity settling of our experimental system was inefficient, whilst separation can be enhanced from the effluent with efficiencies of over 90 % and 40 % by centrifugation and coagulation, respectively.
- Centrifugation is independent of the pH and thus, no chemical supply is needed.

In conclusion, the FBBR demonstrated potential for thiosulphate removal and sulphur production, representing a solution for biological S⁰ recovery under alkaline and saline conditions. However, further studies with real industrial sulphurous streams are needed prior to practical implementation.

CRedit authorship contribution statement

Alessio D'Aquino: participation in planning and implementation, running experiments, doing laboratory and data analysis, preparation of manuscript draft, editing manuscript. **Réka Hajdu-Rahkama:** participation in planning and implementation, supervision during experiments, doing some of the analysis, reviewing the manuscript and editing. **Jaakko A. Puhakka:** supervision of the project, participation in planning, reviewing the manuscript, arranging financial support for the project.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.bej.2021.108062>.

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