Tampere University

RÉKA HAJDU-RAHKAMA

Potential of Biological Sulphur Recovery Under Haloalkaline Conditions

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ACADEMIC DISSERTATION
To be presented, with the permission of
the Faculty of Engineering and Natural Sciences
of Tampere University,
for public discussion in the Pieni Sali 2 (FA133)
of the Festia Building, Korkeakoulunkatu 8, Tampere,
on 9 December 2022, at 12 o'clock.

ACADEMIC DISSERTATION
Tampere University, Faculty of Engineering and Natural Sciences
Finland

Responsible supervisor and Custos	Professor Jaakko Puhakka Tampere University Finland	
Supervisors	Professor Bestami Özkaya Yildiz Technical University Turkey	Doctor Aino-Maija Lakaniemi Tampere University Finland
Pre-examiners	Professor Domingo Cantero University of Cádiz Spain	Assistant Professor Stefano Papirio University of Naples Federico II Italy
Opponent	Professor David Gabriel Buguña Autonomous University of Barcelona Spain	

Cover design: Roihu Inc.

ISBN 978-952-03-2656-2 (print)
ISBN 978-952-03-2657-9 (pdf)
ISSN 2489-9860 (print)
ISSN 2490-0028 (pdf)
http://urn.fi/URN:ISBN:978-952-03-2657-9

Carbon dioxide emissions from printing Tampere University dissertations have been compensated.

PunaMusta Oy – Yliopistopaino
Joensuu 2022

PREFACE

This thesis work was carried out at the Faculty of Engineering and Natural Sciences and founded by the Doctoral School of Tampere University. Also, the partial funding received from the Faculty of Engineering and Natural Sciences is acknowledged.

I am very grateful to my responsible supervisor Prof. Jaakko Puhakka for giving me the chance to do my doctoral studies at Tampere University and for organizing the funding for me. I am also thankful for his guidance, positive attitude and being available for my questions. I also want to show my gratitude to my co-supervisor Prof. Bestami Özkaya for his help during the first two papers of this thesis. I really appreciate your guidance with the kinetics and modelling. I would like to sincerely thank my other co-supervisor Dr. Aino-Maija Lakaniemi for her support during this work and her comments on the first two papers. I would like to thank also the pre-reviewers Prof. Cantero Domingo and Asst. Prof. Stefano Papirio for giving their valuable comments on the thesis. Moreover, special thanks to Prof. David Gabriel Buguña for kindly agreeing to serve as an opponent during the public examination of my thesis.

I would like to express my gratitude to Alessio D´Aquino, the co-author of the Paper III, for helping me with the bioreactor experiments and writing the manuscript draft. I am also grateful to Dr. Ramita Khanongnuch, Payam Rasoulnia, Dr. Donya Kamravamanesh, Dr. Anubhuti Bhatnagar, Erkki Halme, Dr. Johanna Rinta-Kanto, Veera Koskue and Elina Yli-Rantala who have supported me during my thesis work. I appreciate your help in the laboratory and the conversations we had. Thanks also to my other co-workers who were around to talk about our works or just to have a chat. I also want to acknowledge the help I have received from the laboratory staff, Antti Nuottajärvi, Tarja Ylijoki-Kaiste and Mika Karttunen.

Finally, I would like to thank my friends and family for their support. Special thanks to my husband Jaakko for believing in me and supporting me throughout these years.

ABSTRACT

Process industries, such as pulp and paper (P&P) and petrochemical, generate concentrated sulphurous process streams and wastewaters. The process streams, which are saline and alkaline (haloalkaline), require careful management as they potentially increase operational costs, related to chemical balancing and corrosion. Sulphur recovery by haloalkaliphilic sulphur oxidizing bacteria (SOB) instead of physicochemical methods would be a cost-efficient approach as it works at ambient pressure and temperature and produces separable elemental sulphur (S^0). However, the wastewaters of organic raw-material processing industries can contain organic compounds that may harm chemolithoautotrophic SOB.

The aim of this work was to study the biological S^0 recovery potential by chemolithoautotrophic SOB from haloalkaline sulphurous solutions for possible use in industrial process streams and wastewaters. Therefore, the kinetics of thiosulphate ($S_2O_3^{2-}$) biotransformation and growth of model haloalkaliphilic SOB (*Thioalkalivibrio versutus* and *T. denitrificans*) were delineated. Also, the S^0 recovery by *T. denitrificans* under anoxic conditions was investigated. Third, the potential of continuous bioprocess with increasing $S_2O_3^{2-}$ loading rates was studied in a *T. versutus* amended fluidized bed bioreactor (FBBR). Finally, the effects of organic compounds and P&P wastewaters on $S_2O_3^{2-}$ biotransformation were delineated.

The kinetic studies showed high-rate $S_2O_3^{2-}$ biotransformation by *T. versutus* (q_m=0.083 h^{-1}) and somewhat lower by *T. denitrificans* (q_m=0.024 h^{-1}) at high initial substrate concentrations under aerobic conditions. S^0 was formed by both bacteria in the aerobic batch assays whilst it was not formed by denitrification by *T. denitrificans*. In the FBBR, 100% $S_2O_3^{2-}$ removal efficiency and 27±2% S^0 yield were achieved at loading rates of 19 g S/L/d and 22 g S/L/d, respectively. The non-aseptic FBBR system was suitable to maintain the pure culture but was not suitable for S^0 settling. *T. versutus* showed high tolerance towards P&P mill wastewaters (primary filtrate of bleaching, composite wastewater) and the constituents studied in this work. Yeast extract (2.5-5 g/L) enhanced biotransformation and growth.

This work demonstrates efficient $S_2O_3^{2-}$ biotransformation from synthetic solutions (pH 10, 14-26 g/L Na^+) and P&P wastewaters under aerobic conditions. The outcomes of this thesis can be used for future bioprocess development.

TIIVISTELMÄ

Massa- ja paperiteollisuuden (P&P) sekä petrokemian teollisuuden prosesseissa esiintyy rikkipitoisia virtoja ja rikkiyhdisteitä sisältäviä jätevesiä. Ylimäärärikin talteenotto mekaanisesti erotettavana alkuainerikkinä emäksisistä ja suolaisesta liuoksesta haloalkalifiilisiä rikinhapettajabakteereita käyttäen ei edellytä korkeaa painetta ja lämpötilaa kuten fysikaalis-kemialliset menetelmät. Orgaanisten raaka-aineiden jalostamisen jätevedet sisältävät kuitenkin orgaanisia yhdisteitä, jotka saattavat olla haitallisia kemolitoautotrofisille bakteereille.

Tämän työssä tutkittiin biologista alkuainerikin tuottoa tiosulfaattia sisältävistä liuoksista. Kemolitoautotrofisten ja haloalkalifiilisten rikinhapettajabakteerien (*Thioalkalivibrio versutus* ja *T. denitrificans*) tiosulfaatin ($S_2O_3^{2-}$) biotransformaatioiden ja kasvun kinetiikkaa tutkittiin ja mallinnettiin. Jatkuvatoimista bioprosessia rikin tuottoon tiosulfaatista tutkittiin leijupetibioreaktoria (FBBR) ja *T. versutus* -puhdasviljelmää käyttäen. Lopuksi tutkittiin orgaanisten yhdisteiden ja P&P-jätevesien vaikutuksia $S_2O_3^{2-}$ biotransformaatioihin.

Aerobinen S^0 tuottava tiosulfaatin biotransformaationopeus *T. versutus* bakteerilla oli suurempi (q_m=0,083 h^{-1}) kuin *T. denitrificans -bakteerilla* (q_m=0,024 h^{-1}) suurilla substraattipitoisuuksilla. *T. denitrificans* ei tuottanut alkuainerikkiä dentirifikaatiolla. FBBR:ssä saavutettiin lähes 100 % $S_2O_3^{2-}$ biotransformaatio kuormituksella 19 g $S_2O_3^{2-}$-S/L/d ja suurin S^0 -saanto (27±2 %) kuormituksella 22 g $S_2O_3^{2-}$-S/L/d. Ei-aseptinen FBBR-järjestelmä soveltui puhdasviljelmän ylläpitoon ja tiosulfaatin biotransformaatioon. Tuotetun S^0:n erotukseen sentrifikaatio ja $FeCl_2$ -koagulaatio olivat tehokkaammat menetelmät. *T. versutus* kasvoi ja tuotti S^0:a tiosulfaatista valkaisun primäärisuodoksen ja sellutehtaan komposiittijäteveden sekä niiden orgaanisten aineosien läsnäollessä. Hiivauute (2,5-5 g/l) tehosti $S_2O_3^{2-}$ biotransformaatioita ja kasvua.

Täässä työssä osoitetaan $S_2O_3^{2-}$ biotransformaatio alkuainerikiksi synteettisissä emäksisissä (pH 10) ja suolaisissa (14-26 g/L Na^+) panosviljeilyissä ja jatkuvatoimisissa reaktoreissa käyttäen *T. versutus* -puhdasviljelmää aerobisissa olosuhteissa. *T. versutus* sieti massa- ja paperiteollisuuden jätevesien orgaanisia aineosia, mikä viittaa mahdollisuuteen kehittää bioprosessia erityisesti massa- ja paperiteollisuuden jätevesien rikin talteenottoon.

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ABBREVIATIONS

AC	activated carbon
AD	anaerobic digestion
AFBR	anaerobic fluidized bed reactor
APS	adenosine phosphosulphate
AR	airlift reactor
BEC	bioelectrochemical systems
BR	biotransformation rate
BSB	bioreactor with suspended biomass
BTF	biotrickling filter
Ca^{+}	calcium ion
CE	conversion efficiency
Cl^{-}	chloride ion
CO	carbon monoxide
COD	chemical oxygen demand
CO_2	carbon dioxide
CO_3^{2-}	carbonate
CH_4	methane
CSTR	continuous stirring tank reactor
DF	dark fermentation
DSMZ	Microorganisms and Cell Cultures GmbH
DO	dissolved oxygen
DOC	dissolved organic carbon
d.w.	dry weight
EBR	expanded bed reactor
FBR	fluidized bed reactor
FBBR	fluidized bed bioreactor
FCSD	flavocytochrome c sulphide dehydrogenase
GSB	green sulphur bacteria
HCO_3^{-}	bicarbonate
HRT	hydraulic retention time

HS^-	bisulphide ion
H^+	proton
H_2	hydrogen
H_2S	hydrogen sulphide
IC50	concentration that causes 50% inhibition
K_s	half saturation constant
LR	loading rate
MBfR	membrane biofilm reactor
MEC	microbial electrolysis cell
MFC	microbial fuel cell
Mg^+	magnesium ion
NaCl	sodium chloride
NH_3^+	ammonia
NaOH	sodium hydroxide
NO_2^-	nitrite ion
NO_3^-	nitrate ion
N_2O	nitrous oxide
ORP	oxidation-reduction potential
PFB	primary filtrate of bleaching
PHA	polyhydroxyalkanoates
pK_a	acid-base dissociation constant
PFB	primary filtrate of bleaching
PSB	purple sulphur bacteria
P&P	pulp and paper
PAC	polyaluminium chloride
PAM	polyacrylamide
qPCR	quantitative polymerase chain reaction
q_m	maximum, specific substrate biotransformation rate
RE	removal efficiency
RFLR	reverse fluidised loop reactor
RU	recirculation unit
SEM	scanning electron microscopy
SOB	sulphur oxidizing bacteria
S^{2-}	sulphide ion
S^0	elemental sulphur
S_n^{2-}	polysulphide

$S_n(SO_3)_2^{2-}$	polythionate
SO_3^{2-}	sulphite ion
SO_4^{2-}	sulphate ion
$S_2O_3^{2-}$	thiosulphate ion
SRB	sulphate reducing bacteria
SRSB	supernatant-recycling settler
SQR	sulphide: quinone oxidoreductase
TOC	total organic carbon
UASB	anaerobic sludge blanket reactor
UBRFF	upflow bioreactor with fixed film
VFAs	volatile fatty acids
V_{fb}	volume of fluidized bed
VOC	volatile organic carbon
VSCs	volatile organic sulphurous compounds
WW	composite wastewater of pulping
WWTP	wastewater treatment plant
Y	growth yield
μ_m	specific growth rate
-COOH	carboxylic group
$-NH_2$	amino group

ORIGINAL PUBLICATIONS

Publication I **Hajdu-Rahkama, R.**, Özkaya, B., Lakaniemi, A. M., & Puhakka, J. A. Kinetics and modelling of thiosulphate biotransformations by haloalkaliphilic *Thioalkalivibrio versutus*. Chemical Engineering Journal (2020), 401, 126047.

Publication II **Hajdu-Rahkama, R.**, Özkaya, B., Lakaniemi, A. M., & Puhakka, J. A. Potential of biological sulphur recovery from thiosulphate by haloalkaliphilic *Thioalkalivibrio denitrificans*. Environmental Technology (2021) 1-13.

Publication III D'Aquino, A., **Hajdu-Rahkama, R.**, & Puhakka, J. A. Elemental sulphur production from thiosulphate under haloalkaline conditions in a *Thioalkalivibrio versutus* amended fluidized bed bioreactor. Biochemical Engineering Journal, 172 (2021) 108062.

Publication IV **Hajdu-Rahkama, R.** & Puhakka, J.A. High tolerance of chemolithoautotrophic sulphur oxidizing bacteria towards pulp and paper mill wastewaters and their organic constituents supporting sulphur recovery in alkaline conditions. Chemical Engineering Journal (2022) 137972.

AUTHOR CONTRIBUTIONS

Publication I RHR performed the experimental work, did the data analysis, and wrote the first draft of the manuscript. BÖ did the kinetic calculations and modelling, and interpreted the results. BÖ, AML and JP participated in the planning, supervision, and revision of the manuscript. JP arranged financial support for the work.

Publication II RHR planned and implemented the laboratory experiments and analysed the results. RHR wrote the manuscript draft, did the kinetic calculations and is the corresponding author. BÖ, AML and JP participated in the planning, supervision and revision of the manuscript. JP arranged financial support for the work.

Publication III RHR planned the experiments. RHR and AD carried out the experiments and did the data analysis. AD wrote most of the manuscript draft and is the corresponding author. JP and AD participated in the planning, and RHR and JP did the supervision and revision of the manuscript. JP arranged financial support for the work.

Publication IV RHR planned and implemented the laboratory experiments and analysed the results. RHR wrote the manuscript draft and is the corresponding author. JP did the supervision, participated in the planning, and revised the manuscript. JP also arranged financial support for the work.

1 INTRODUCTION

According to the United Nations, the world´s population has more than tripled between 1950 and 2020 and will reach 8 billion in 2022 (United Nations Department of Economic and Social Affairs, 2021). Fulfilling the need of the world´s increasing population is becoming more challenging and already resulted in limitations of some non-renewable resources. To decrease the scarcity of resource availability and to support the circular economy, efficient recovery and recycling of non-renewable resources have become a must.

To meet stricter legislations, many industries, such as pulp and paper (P&P), have enormously reduced their water consumption since the 20th century (Ordóñez et al., 2014). As a result of efficient water recirculation and capture of off gases, chemical constituents (e.g., sulphurous compounds) accumulate in the process streams. The excess sulphur (e.g., sulphide, thiosulphate) needs to be removed from these streams as its presence increases the need for water and chemical addition (sodium based) to maintain chemical balance (Tikka, 2008). Moreover, sulphide and thiosulphate are toxic, odorous and cause corrosion (Guidotti, 1996; Wu et al., 2020). Therefore, the excess of these sulphurous compounds increases operational costs.

The traditional physico-chemical methods used to remove sulphide from the process streams are energy intensive and produce chemical side-streams. Moreover, at low sulphide concentration, they are not economically viable (Lagas, 2000). Therefore, alternative biological sulphur recovery is gaining increasing attention (Pokorna and Zabranska, 2015). Reduced inorganic sulphurous compounds can be biotransformed into e.g., elemental sulphur (S^0) and sulphate (SO_4^{2-}) by chemolithoautotrophic sulphur oxidizing bacteria (SOB). However, S^0 is preferred as it has lower activation energy, as a solid it can be separated from the liquid streams, and can be further used as raw material in various applications (pulping, metallurgy, agriculture, etc.) (Florentino et al., 2015). Moreover, biological S^0 has several advantages compared to chemical S^0. For example, due to its higher bioavailability, it is a better alternative for

fertilizer production and autotrophic denitrification (Di Capua et al., 2016; Janssen et al., 1994).

The main concern for biological sulphur oxidation is the harsh conditions present in the process streams. For example, many of the sulphurous streams of Kraft pulping and petrochemical industries have high salinity (Na^+) and are strongly alkaline (Tikka, 2008), and therefore, their treatment requires special SOB that can tolerate these conditions. Haloalkaliphilic SOB are naturally present in soda lakes which are unique habitats as they are rich in Na^+ (up to saturation) and highly alkaline (up to pH 11) (Sorokin et al., 2011; Sorokin and Kuenen, 2005). For example, haloalkaliphilic bacteria belonging to the genus *Thioalkalivibrio* tolerate 4.3 M Na^+, pH up to 10.6 and can use a wide range of reduced sulphurous compounds (e.g., HS^-, S^{2-}, $S_2O_3^{2-}$, S_8, $S_3O_6^{2-}$) as an energy source (Sorokin et al., 2011). In particular, under aerobic conditions, *Thioalkalivibrio versutus* utilizes reduced sulphurous compounds and oxygen as an electron acceptor, and produces elemental sulphur as a metabolic intermediate (Sorokin et al., 2001b; Sorokin and Kuenen, 2005). Sometimes supplying sufficient concentration of dissolved oxygen (DO) to the industrial streams can be challenging, and therefore microaerophilic *T. denitrificans* that can utilize both oxygen and nitrogenous compounds could be the preferred type of SOB (Sorokin et al., 2001a). Both *T. versutus* and *T. denitrificans* produce extracellular S^0 (Sorokin et al., 2001a and 2001b), hence they can enable sulphur recovery from haloalkaline process streams and wastewaters.

Another concern with the process and wastewaters of organic raw-material processing industries is that they potentially contain organic compounds that may be toxic for chemolithoautotrophic SOB. For example, some of the common organics present in the P&P mill wastewaters are methanol, acetic acid, furfural and wood sugars (e.g., xylose) (Badshah et al., 2012; Sharma et al., 2020; Toczyłowska-Mamińska, 2017), whereas the sulphidic spent caustic of petrochemical industry may contain organosulphur compounds, phenol, benzene and toluene (Kolhatkar and Sublette, 1996; Park et al., 2009; Sipma et al., 2004). The sensitivity of acidophilic chemolithoautotrophic bacteria belonging to the genus of *Acidithiobacillus* towards organic compounds has been already documented (Fang and Zhou, 2006; Gu and Wong, 2004; Määttä et al., 2022), but for haloalkaliphilic chemolithoautotrophic SOB, the tolerance has not been comprehensively investigated (de Graaff, 2012).

This is the first study reporting thorough kinetics of thiosulphate biotransformation by *T. versutus* and *T. denitrificans* and providing a tool to estimate their S^0 production yields. To our knowledge, studying the thiosulphate biotransformation by *T. versutus* in a fluidized bed bioreactor (FBBR) with activated carbon (AC) as carrier material has not been done before. Moreover, the effects of various organic compounds (methanol, acetate, xylose, phenol, benzene) commonly present in P&P wastewaters and two selected wastewaters (primary filtrate of bleaching and composite wastewater) have not been reported.

2 BIORESOURCE RECOVERY FROM INDUSTRIAL STREAMS

Extensive use of non-renewable resources, such as fossil fuels, metals and nutrients, has resulted in limitations of resource availability. Therefore, closing the production cycles and recovery of resources from waste and side streams is increasing. Recovery of resources by using physicochemical methods is oftentimes expensive due to high investment and operational costs. Moreover, these methods can have negative impact on the environment as many of them produce chemical side streams. Therefore, biological resource (bioresource) recovery processes, which as a potentially lower-cost approach are gaining increasing attention. Bioresources can be either recovered as energy/energy carriers or other valuable products (e.g., nutrients, chemicals, metals).

2.1 Recovery of energy carriers

Many industrial wastewaters are rich in organics, i.e. have a high energy content, that can be recovered as energy carriers by microorganisms (Table 1). These energy carriers can be gaseous (biogas, biohydrogen), liquid (e.g., bioethanol, biodiesel, renewable diesel) or electricity.

Anaerobic digestion (AD) is a well-known commercialized biological process that converts organic compounds from industrial (e.g., P&P manufacturing, food industry, slaughterhouse, agriculture) and municipal wastewaters into biogas containing mainly methane (CH_4), carbon dioxide (CO_2) and low concentrations of impurities (e.g., H_2S) (for reviews, see (Puyol et al., 2017; van Lier et al., 2015)). Using AD to recover energy from organic matter has several advantages compared to physicochemical approaches. For example, it can efficiently remove high organic loads, produce less excess sludge, and requires less space for operation (Puyol et al., 2017).

Another commercially used bioprocess is bioethanol production. Second generation bioethanol is the product of non-edible feedstock, such as lignocellulosic and starchy materials. Although petrol has 68% higher energy content, bioethanol has

higher oxygen content that results in lower emission of toxic substances. For example, by using bioethanol, the CO_2 emissions can be reduced by 80% compared to petrol (for a review, see (Aditiya et al., 2016)).

Another energy carrier that can be biologically produced is biohydrogen. Biohydrogen can be produced by biophotolysis, photofermentation and dark fermentation (DF) (Puyol et al., 2017). DF has several challenges, such as low yields, difficulty to control the process and end product, the presence of other microorganisms (e.g., methanogens) reducing efficiency and often the substrates requiring pre-treatment. (Noike and Mizuno, 2000; Puyol et al., 2017; Roy and Das, 2016) However, the bioresource recovery can be enhanced by combining DF with other biogenic processes such as microbial electrolysis cell (MEC), photofermentation, sulphate reduction, microalgal cultivation and bioplastic production (Puyol et al., 2017).

AD is a more robust and economically viable process than DF. For example, when using anaerobic digestion of microalgal biomass, the potential yields of biomethane and biohydrogen are 14.4 and 1.2 kJ/g biomass. The lower heating value of methane and hydrogen are around 35.9 MJ/m^3 and 10.8 MJ/m^3, respectively, and therefore, currently, more energy can be obtained from biomethane production (for a review, see (Lakaniemi et al., 2013)). Although both biomethane and biohydrogen are good energy carriers, they need to be converted into liquid fuel to be able to use with existing gasoline and diesel engines. Biodiesel (produced from oleaginous microorganisms, e.g., algae) is already a liquid fuel and therefore, it can be used with little or no modifications, especially as additive fuel for diesel engines (Zhang et al., 2016). Although biodiesel is a smooth alternative to fossil fuels, it has still limitations when using wastewater as a substrate (Puyol et al., 2017).

Another approach to biologically recover energy from organic-containing industrial wastewaters is the conversion of organic compounds into electricity by bioelectrochemical systems (BECs). In microbial fuel cells (MFCs, one type of BES), electrochemically active anaerobic microorganisms (exoelectrogens) generate current from organic compounds at the anode electrode (Kokko et al., 2016). Although BES is an attractive approach, it still has technical challenges and its bioelectricity has low value, thus needs further optimization to make it economically viable. (for reviews, see (Kokko et al., 2016; Puyol et al., 2017)

Table 1. Bioenergy carrier production from different organic-rich industrial wastes.

Biotechnology	Microorganism	Product	Exemplary process designs	Reference
Anaerobic digestion	Methanogenic bacteria and archaea	Biogas (CH_4)	Continuously stirred tank reactor (CSTR), high-rate bed reactors, membrane bioreactor	For a review, see Puyol et al., 2017
Dark fermentation	Anaerobic and facultative bacteria	Biohydrogen	CSTR, anaerobic fluidized bed reactor (AFBR), membrane bioreactors, upflow anaerobic sludge blanket reactor (UASB) reactors, leaching bed reactors, plug flow reactors, anaerobic baffled reactors	For reviews, see Puyol et al., 2017; Roy and Das, 2016
Fermentation	Yeast or fermenting bacteria	Bioethanol	Fermenter	For a review, see Aditiya et al., 2016
Transesterification of oil extracted from microorganisms	Oleaginous* microorganisms (microalgae, yeast, fungi, bacteria)	Biodiesel	Photobioreactors, open ponds, CSTR, tank reactor	For a review, see Puyol et al., 2017(Puyol et al., 2017)
Bioelectrochemical system (BES)	Exoelectrogens (microorganisms able to donate electrons to anode electrode)	Bioelectricity	Bioelectrochemical system (BES) - microbial fuel cell (MFC)	For a review, see Kokko et al., 2016

* microorganisms that accumulates > 20% oil/d.w.

2.2 Recovery of other valuable resources

Besides the biological production of energy carriers, various other resources can be produced and/or recovered from industrial wastewaters via biological processes (Table 2). Metal recovery by bioleaching is widely applied with low-grade ores. Bioleaching with metallic waste streams (e.g., electric waste, mining side streams) is gaining increasing attention as there is a surge for new metal resources (Guezennec et al., 2015). Another way to recover metals from wastewaters is by bioprecipitation. Wastewaters from mining processes (e.g., acid mine drainage) are not just rich in metals but sulphate, and therefore suitable environment for sulphate reducing microorganisms. These microorganisms biologically reduce sulphate to sulphide that reacts with metals and consequently forms precipitates (Sánchez-Andrea et al., 2015). Metals can be also recovered by immobilization onto biomass by biosorption or bioaccumulation (Puyol et al., 2017).

Industrial (pulp and paper, petrochemical, dairy, etc.) wastewaters are potent streams for bioplastic production. For instance, certain bacteria and haloarchea can ferment sugars and lipids present in industrial wastewaters and produce polyhydroxyalkanoates (PHAs) under unfavourable conditions (e.g., limited nutrient availability and electron donor/acceptor) (Puyol et al., 2017). PHAs are biocompatible non-toxic polyesters and therefore they are suitable for various applications, such as packaging, pharmaceuticals and medical, personal hygiene, coatings etc. (for a review, see (Tarrahi et al., 2020)).

Besides generating energy, exoelectrogens can also convert different substrates into valuable products (e.g., organic acids and alcohols). However, they cannot utilize complex fermentable substrates and therefore, they require the help of electrochemically inactive microbes to convert these substrates (Kokko et al., 2016). Microbial electrosynthesis (MES) has the potential to recover chemicals, nutrients (e.g., nitrogen and phosphorus) and metals (Puyol et al., 2017).

Industrial processes often generate off gases such as syngas, volatile organic compounds (VOCs) and sulphur-containing gases. Syngas, a gas mixture of CO, CO_2, H_2, CH_4 and other gases, is a valuable waste gas for bioresource recovery. Syngas is a side-product of the thermochemical processes of fossil fuels (Abubackar et al., 2011).

Fermenting syngas is used to produce ethanol, butanol, hexanol and other acids. Although syngas fermentation has several advantages (e.g., high product selectivity, and low sensitivity of biocatalysts), it still requires process development due to high production costs and mass transfer limitations (Khanongnuch et al., 2022; Abubackar et al., 2011). Various VOCs present in wastewaters like pulp and paper and paint industries can be biodegraded to volatile fatty acids (VFAs) in anaerobic conditions. For instance, methanol can be degraded with electron acceptors such as thiosulphate, sulphate, or selenate (for a review, see (Khanongnuch et al., 2022)).

Biological sulphur recovery from off gases, process streams and wastewaters as alternatives for physicochemical treatment methods is gaining increasing attention. Both inorganic (containing reduced sulphur compounds) and organic sulphurous streams are odorous, toxic, and corrosive and therefore, their concentrations allowed by legislations in effluents are very low (for a review, see (Pokorna and Zabranska, 2015)). Biological recovery of elemental sulphur (S^0) from odorous/sulphurous gases offers several advantages compared to conventional recovery methods (for more information, see section 4.2). As many industries, e.g., pulp and paper, use sulphurous process chemicals, reduced sulphurous compounds (e.g., HS^-, $S_2O_3^{2-}$, SO_3^{2-}) are also present in the process and wastewaters of these industries (Tikka, 2008). Conventional applications of sulphur oxidizing bacteria (SOB) have mainly taken place in acidic and neutral conditions (Paques, 2022a; Veolia, 2018), but as many industrial streams have high pH and are saline, the recovery process applicable in alkaline pH and saline condition have recently gained more attention (D'Aquino et al., 2021; de Graaff et al., 2011; Hajdu-Rahkama et al., 2021). In addition to reduced sulphurous compounds, sulphate (SO_4^{2-}), the most oxidized form of sulphur is often present in wastewaters of industries using sulphurous chemicals or sulphur-rich resources (Costa et al., 2020). SO_4^{2-} can serve as an electron acceptor for the recovery of valuables (e.g., nutrients) during anaerobic processes (for more information, see section 4.1). The H_2S formed as a product of SO_4^{2-} reduction can be further biotransformed to S^0 as mentioned above.

The biological recovery of different resources from industrial process and waste streams supports environmental sustainability, in addition to the circular economy. Moreover, the increasing demand for resources such as energy, chemicals and nutrients can be partially fulfilled by biological resource recovery.

Table 2. p. 1/2. Bioresource recovery from various waste streams.

Biotechnology	Microorganism	Substrate	Product	Exemplary process design	Reference
Bioleaching	Iron and sulphur oxidizing bacteria	Solid metal-containing wastes	Metals	CSTR, fluidized bed reactor	Guezennec et al., 2015 For a review, see Puyol et al., 2017
Biosorption	Various	Metal-containing wastewaters	Metals		For a review, see Puyol et al., 2017
Bioaccumulation	Various	Metal-containing wastewaters	Metals		For a review, see Puyol et al., 2017
Metal precipitation	Sulphate reducing microorganisms	Metal and sulphate containing wastewaters	Metals	Permeable reactive barriers/ infiltration beds	Younger et al., 2003
Aerobic and anoxic sulphur oxidation	Sulphur oxidizing bacteria (SOB)	Gaseous and dissolved sulphurous compounds (e.g., hydrogen sulphide, polysulphide, thiosulphate, sulphite, sulphide, mercaptans, carbon disulphide, dimethyl sulphide, nitrogen compounds)	Elemental sulphur	Biofilter, biotrickling filter (BTF), gas-lift reactor, CSTR, upflow sludge anaerobic blanket	For a review, see Khanongnuch et al., 2022
Anaerobic biodegradation	anaerobic bacteria	volatile organic compounds (e.g., benzene, methanol, chloroform, xylene)	volatile fatty acids (VFAs)	BTF, UASB	For a review, see Khanongnuch et al., 2022
Syngas fermentation	acetogenic carboxydotrophs	CO, CO_2, H_2, CH_4, and other gases	acids (e.g., ethanol, butanol, hexanol)	stirred tank fermenter	For a review, see Khanongnuch et al., 2022

Table 2. p.2/2 Continued.

Biotechnology	Microorganism	Substrate	Product	Exemplary process design	Reference
Batch bioreactor operation (polyhydroxyalkanoates (PHA) accumulation)	bacteria, haloarchea	sugars and lipids	biopolymers (e.g., PHA)	three stage bioreactor operation	For reviews, see Puyol et al., 2017; Tarrahi et al., 2020
Bioelectrochemical system (BES) - microbial electrolysis (MES)	exoelectrogens	various wastewaters	nutrients, chemicals, metals	MES reactor	For a review, see Kokko et al., 2016; Puyol et al., 2017

3 MICROBIOLOGY OF SULPHUR RECOVERY

Sulphur, one of the most abundant elements on Earth, occurs in nine different oxidation states, ranging from -2 (sulphide) to +6 (sulphate) (Guo et al., 2022). As part of the biogeochemical cycle, numerous biological and chemical oxidation-reduction reactions of sulphur can take place (Figure 1). Depending on the reaction, sulphur can be either an electron acceptor (reductive process) or a donor (oxidative process). Two groups of microorganisms, sulphate reducing microorganisms (e.g., sulphate reducing bacteria (SRB)) and sulphur oxidizing bacteria (SOB), are responsible for the biological transformation (biotransformation) of sulphurous compounds (Lin et al., 2018). Both sulphur reducers and SOB gain energy from the biotransformation of sulphurous compounds and can use CO_2 as a carbon source for the construction of cells (Pokorna and Zabranska, 2015). Based on the source of carbon, sulphur bacteria can be classified as autotrophic, mixotrophic or heterotrophic, meaning that they can obtain their carbon requirement from CO_2, from CO_2 and organic compounds or only from organic compounds, respectively (Madigan et al., 2018a). SRB use oxidized sulphur compounds as electron acceptor, while SOB utilize reduced sulphur compounds as electron donor (Lin et al., 2018). Besides catalysis strictly the reduction or oxidation of sulphurous compounds, some sulphur bacteria can also disproportionate sulphur, meaning that they can catalyse both processes simultaneously. For instance, during the disproportionation of elemental sulphur (S^0), sulphate (SO_4^{2-}) and hydrogen sulphide (in the form of H_2S, HS^- or S^{2-}) are formed while thiosulphate ($S_2O_3^{2-}$) is disproportionated to S^0, sulphite (SO_3^{2-}) and SO_4^{2-} (Madigan et al., 2006). Also, some microorganisms like *Desulfovibrio sulfodismutans sp.* can disproportionate SO_3^- to HS^- and SO_4^{2-} (Bak and Pfennig, 1987).

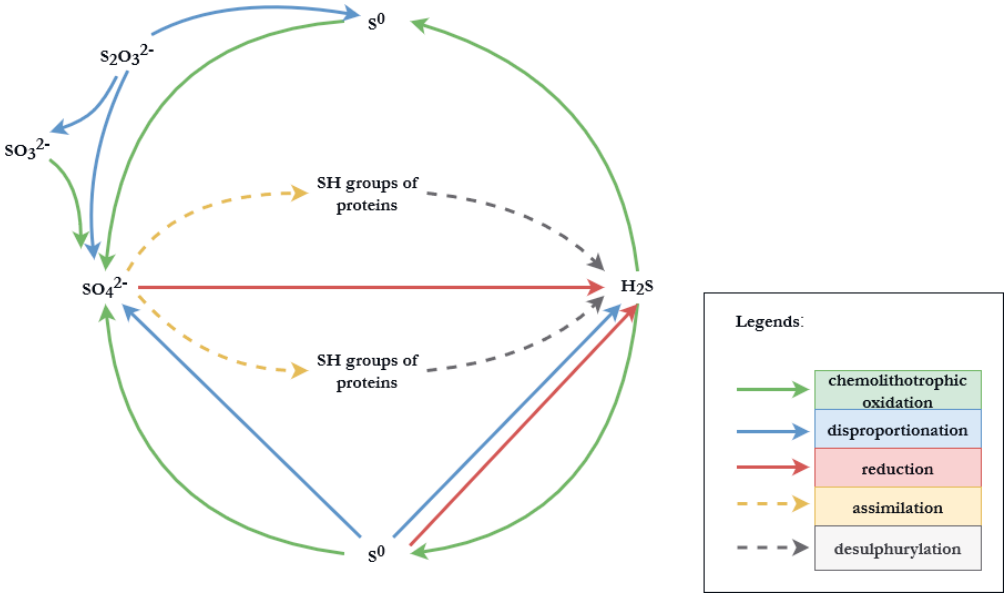

Figure 1. Most significant states of the biological sulphur cycle. Thiosulphate disproportionation was also added to the cycle. Modified from (Madigan et al., 2006).

3.1 Reduction of sulphurous compounds

Various oxidized sulphurous compounds can be used as electron acceptors by SRB. Sulphate is the most oxidized form of sulphur, and sulphate ion (SO_4^{2-}) is one of the most abundant anions in seawater. Although most of the microorganisms can assimilate sulphate to make cysteine and methionine (organic sulphur compounds), only SRB and *Archeoglobus* (genus of *Archaea*) can use it for energy conservation (Madigan et al., 2006). Besides SRB, also non-sulphate reducing microorganisms exist that can reduce less oxidized forms of sulphur but not SO_4^{2-}. These sulphur reducers cannot activate SO_4^{2-} to adenosine phosphosulphate (APS), which separates them from SRB (Madigan et al., 2018a). Sulphate reduction can proceed by dissimilative and assimilative pathways. SO_4^{2-} is reduced to SO_3^{2-} and then to H_2S by both pathways, but H_2S is only excreted from the cell by the dissimilative pathway. The end products of the assimilative pathway are organic compounds (e.g., cysteine and methionine) (Madigan et al., 2006).

SRB thrive in anaerobic environments that are rich in sulphate and organics. Organic compounds serve both as electron donors and carbon sources, while SRB can also use

H_2 as an electron donor instead of organic compounds but then, CO_2 or some organic compound is needed to be supplied as a carbon source (e.g., acetate) (Tang et al., 2009). Some of the organic compounds that SRB can utilize as electron donors include formate, propionate, lactate and alcohols (e.g., methanol, ethanol, 1-butanol etc.). For a detailed review of the possible electron donors, see Tang et al. (2009).

Besides SO_4^{2-}, SO_3^{2-} and $S_2O_3^{2-}$, some SRB of the genera *Desulphohalobium*, *Desulphoromusa*, *Desulphofustis*, and *Desulphospirs* can also utilize S^0 as sulphurous electron acceptor. Also, non-sulphurous electron acceptors such as nitrate, nitrite and some heavy metals have been reported as electron acceptors of SRB (for a review, see (Tang et al., 2009)).

3.2 Oxidation of sulphurous compounds

SOB can be categorised based on their growth conditions, energy and carbon source, physiology etc. (Table 3). The biological oxidation of sulphurous compounds (e.g., HS^-, S^0, $S_2O_3^{2-}$, SO_3^{2-}) is carried out by phototrophic or chemolithotrophic SOB. The main difference between these two groups of SOB are the source of energy they use for their metabolism. Phototrophs capture light energy while chemolithotrophs gain energy from the oxidation of inorganic compounds (for a review, see (Pokorna and Zabranska, 2015)). Phototrophic SOB are present in anoxic layers of aquatic environments to which light penetrates. These SOB are classified as green sulphur bacteria (GSB) or purple sulphur bacteria (PSB). While GSB are obligately photoautotrophic and anaerobic, PSB can also grow photoheterotrophically (for a review, see (Lin et al., 2018). Phototrophic SOB grow slowly and supplying sufficient light for their growth is often challenging due to limited light penetration (Madigan et al., 2006). Chemolithotrophic SOB have high capacity for the redox transformation of inorganic sulphur compounds (Sorokin and Kuenen, 2005). They can use oxygen (aerobic species) and/or nitrous compounds (anoxic species) as electron acceptors (Pokorna and Zabranska, 2015). Besides NO_2^- and NO_3^-, some SOB like microaerophilic (can utilize oxygen only at low concentrations) denitrifying *Thioalkalivibrio denitrificans* can also utilize N_2O (Sorokin et al., 2001a).

Physiologically, chemolithotrophic SOB can be categorized into four groups. All four groups can oxidize inorganic reduced sulphurous compounds, 1) obligate

chemolithotrophic SOB, which fix CO_2 as carbon source. 2) facultative chemolithotrophs, that can utilize both inorganic and organic carbon. 3) chemolithoheterotrophs, using organic carbon, and 4) chemoorganoheterotrophs which are heterotrophs but do not derive energy from sulphur oxidation, only use the process to detoxify its metabolically produced hydrogen peroxide (e.g., some species of *Beggiatoa*, *Thiothrix*) (Lin et al., 2018; Muyzer et al., 2013; Pokorna and Zabranska, 2015).

Table 3. Classifications of SOB.

Growth condition					
	pH	Acidophiles (pH< 5.5)	Neutrophiles (5.5< pH< 7.9)	Alkaliphiles (pH> 7.9)	
	temperature	Psychrophiles (< 20°C)	Mesophiles (20-45°C)	Thermophiles (> 45°C)	
Energy source					
		Phototrophs	Chemotrophs		
Physiology					
	phototrophs	Green sulphur bacteria	Purple sulphur bacteria		
	chemotrophs	Obligate chemolithotrophs	Facultative chemolithotroph	Chemolitho-heterotrophs	Chemoorgano-heterotrophs

For reviews, see Lin et al., 2018; Pokorna and Zabranska, 2015.

3.2.1 Oxidation mechanisms of sulphurous compounds

Various mechanisms of sulphur oxidation have been reported for chemolithotrophic SOB, GSB and PSB (for reviews, see (Ghosh and Dam, 2009; Lin et al., 2018)). One of them is the traditional sulphur oxidation (Sox) pathway which includes complete sulphur oxidation, and it is found in diverse sulphur chemolithoautotrophs (Figure 2). In the Sox pathway, four periplasmic proteins (SoxAX, SoxYZ, SoxB and SoxCD) of the multienzyme complex are responsible for the oxidation of reduced sulphurous compounds (Madigan et al., 2018a). The second pathway is HS^- oxidation to S^0 by enzymes flavocytochrome c sulphide dehydrogenase (FCSD) or sulphide: quinone oxidoreductase (SQR) and their subunits. During the oxidation, electrons are transported to the cytochrome c pool or quinone pool that are bounded to the membrane, respectively (Lin et al., 2018). The oxidation-reduction state of the cytochrome c and quinone determines the final oxidation product. For example, at low oxidation levels, S^0 is the main product (Klok et al., 2013). The third pathway involves the oxidation of S^0 to SO_4^{2-} with intermediate SO_3^{2-} formation. In this pathway, first sulphur oxygenase reductase (SOR) oxidizes S^0 to SO_3^{2-} and SO_3^{2-} is subsequently oxidized to SO_4^{2-} by sulphite oxidase enzyme or reverse activity of enzyme adenosine phosphosulphate (APS) reductase. During the oxidation by the sulphite oxidase enzyme, electrons are transferred to cytochrome c, while energy-rich phosphate bond is formed from the conversion of adenosine monophosphate (AMP) to adenosine diphosphate (ADP) by the reverse activity of the enzyme adenosine (for a review, see (Lin et al., 2018)).

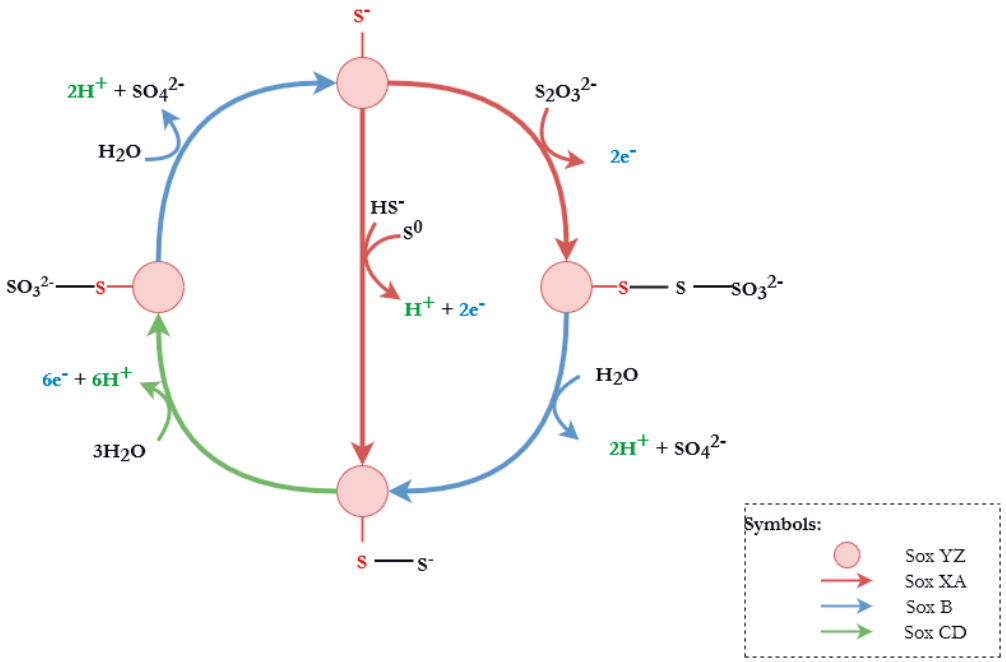

Figure 2. Sox pathway of reduced sulphur compounds (HS^-, S^0, $S_2O_3^{2-}$) oxidation by chemolithoautotrophic microorganisms. The four key proteins that are presented in the periplasm are Sox YZ, Sox XA, Sox B and Sox CD. Modified from (Madigan et al., 2018a).

3.2.2 Electron acceptors for oxidation of reduced sulphur

As aforementioned, SOB can utilize oxygen and/or nitrous compounds as electron acceptors of sulphur oxidation. As it is shown in Equations 1-12, the end product of the oxidation of different reduced sulphurous compounds depends on the ratio of electron acceptor and electron donor concentrations. For instance, when biologically oxidizing H_2S, the end product will be S^0 or SO_4^{2-} with 0.5 mol or 2 mol of oxygen, respectively (Equations 1-2) (Ang et al., 2017; Hajdu-Rahkama et al., 2020; Tang et al., 2009). Generally, a slightly higher concentration of oxygen needs to be supplied to obtain the end product the stoichiometry suggests. For example, when the molar O/S ratio is 0.7, S^0 dominates but >1, mainly SO_4^{2-} is formed. At the O/S ratio <0.6, mainly chemical oxidation of HS^- to $S_2O_3^{2-}$ takes place (de Graaff et al., 2012; Janssen et al., 1995; Lin et al., 2018; Van Den Bosch et al., 2007). The results of Janssen et al. (Janssen et al., 2009) suggest maintaining the oxygen: sulphide ratio between 0.6-1 to have S^0 as the main product of biotransformation. The actual dissolved oxygen (DO) concentration that favours S^0 formation should be between 0.1-0.15 mg/L (Janssen et

al., 1997; Lohwacharin and Annachhatre, 2010). Oftentimes, it is hard to control the DO concentration due to continuous consumption, and therefore controlling the oxidation-redox potential (ORP) is a more feasible option. The ORP requirement differs by SOB but below -420 mV, the high concentration of HS^- typically inhibits the biotransformation processes (de Graaff et al., 2012). When multiple substrates are available, the one that is energetically more favourable will be preferred by the SOB. For example, the Gibbs-free energy changes of H_2S and $S_2O_3^{2-}$ (Pokorna and Zabranska, 2015) bioconversion (Equations 2 and 4) to SO_4^{2-} are higher than of S^0 (Equation 4) and therefore, SOB will prefer them as energy sources.

$$H_2S+\frac{1}{2}O_2\rightarrow S^0+H_2O \qquad \Delta G^0=\text{-}209.4\ kJ\cdot(mol\ S\text{-}substrate)^{-1} \qquad (1)$$

$$H_2S+2O_2\rightarrow SO_4^{2-}+2H^+ \qquad \Delta G^0=\text{-}798.2\ kJ\cdot(mol\ S\text{-}substrate)^{-1} \qquad (2)$$

$$S^0+O_2+H_2O\rightarrow SO_3^{2-}+2H^+ \qquad \Delta G^0=\text{-}249.4\ kJ\cdot(mol\ S\text{-}substrate)^{-1} \qquad (3)$$

$$S^0+\frac{3}{2}O_2+H_2O\rightarrow SO_4^{2-}+2H^+ \qquad \Delta G^0=\text{-}507.4\ kJ\cdot(mol\ S\text{-}substrate)^{-1} \qquad (4)$$

$$S_2O_3^{2-}+\frac{1}{2}O_2\rightarrow S^0+SO_4^{2-} \qquad \Delta G^0=\text{-}231.6\ kJ\cdot(mol\ S\text{-}substrate)^{-1} \qquad (5)$$

$$S_2O_3^{2-}+2O_2+H_2O\rightarrow 2SO_4^{2-}+2H^+ \qquad \Delta G^0=\text{-}738.7\ kJ\cdot(mol\ S\text{-}substrate)^{-1} \qquad (6)$$

$$SO_3^{2-}+\frac{1}{2}O_2\rightarrow SO_4^{2-} \qquad \Delta G^0=\text{-}258.0\ kJ\cdot(mol\ S\text{-}substrate)^{-1} \qquad (7)$$

In anoxic conditionss, the concentration of the nitrogenous compounds need to be also controlled to have selectivity towards the required product(s) (Equations 8-12) (Kelly, 1999; Pokorna and Zabranska, 2015). For example, at N/S=1.6, complete oxidation of HS^- to SO_4^{2-} and reduction of NO_3^- to N_2 gas takes place (Equation 10). Also, to favour S^0 formation from sulphide, the molar ratio of N/S should be around 1. However, at N/S=1, nitrate will be only oxidized to nitrite (Reyes-Avila et al., 2004).

$$HS^-+0.4NO_3^-+1.4H^+\rightarrow S^0+0.2N_2+1.2H_2O \qquad (8)$$
$$\Delta G^0=\text{-}253\ kJ\cdot(mol\ S\text{-}substrate)^{-1}$$

$$HS^- + NO_3^- + H^+ \rightarrow 0.5S^0 + 0.5SO_4^{2-} + 0.5N_2 + H_2O \quad (9)$$
$$\Delta G^0 = -513 \text{ kJ} \cdot (\text{mol S-substrate})^{-1}$$

$$HS^- + 1.6NO_3^- + 0.6H^+ \rightarrow SO_4^{2-} + 0.8N_2 + 0.8H_2O \quad (10)$$
$$\Delta G^0 = -767 \text{ kJ} \cdot (\text{mol S-substrate})^{-1}$$

$$HS^- + 0.67NO_2^- + 1.67H^+ \rightarrow S^0 + 0.33N_2 + 1.33H_2O \quad (11)$$
$$\Delta G^0 = -310 \text{ kJ} \cdot (\text{mol S-substrate})^{-1}$$

$$HS^- + 2.67NO_2^- + 1.67H^+ \rightarrow SO_4^{2-} + 1.33N_2 + 1.33H_2O \quad (12)$$
$$\Delta G^0 = -949 \text{ kJ} \cdot (\text{mol S-substrate})^{-1}$$

3.3 Elemental sulphur recovery by extremophiles

Some sulphur oxidizing and reducing microorganisms can tolerate harsh environmental conditions. Therefore, they are abundant even in high salt environments, volcanic hot springs, deep seas and glaciers (Madigan et al., 2018b). These environments can have extreme temperatures, pH levels, salt concentration and/or pressure. The microorganisms that can tolerate these extreme conditions are called extremophiles. Extremophiles can, for example, have the unique ability to maintain the pH of their cytosol within the optimal range (around neutral) even in acidic or alkaline environments by alkalinizing or acidifying their cytoplasm, respectively, relative to the environment (Padan et al., 2005).

3.3.1 Haloalkaliphilic sulphur recovery

Depending on the pH of the environment, the extremophiles can be acidophiles or alkaliphiles. Alkaliphiles have pH optimum > pH 9 and have slow growth at neutral pH (Horikoshi, 2004). In environments with increasing salt (NaCl) concentration, halophiles, microorganisms that tolerates and requires NaCl for optimal growth, are present. These microorganisms can grow in saline (1-4% NaCl), hypersaline (3-12% NaCl) or extremely saline (15-30% NaCl) environments (Madigan et al., 2018b). Some extreme environments can also be saline (up to 33% NaCl) and alkaline (haloalkaline) at the same time. For example, soda and saline lakes that are found in the arid regions

of the world are haloalkaline, and therefore they are natural and unique habitats for salt and alkalinity tolerating/preferring (haloalkaliphilic) microorganisms (Horikoshi, 2004). These haloalkaliphilic microorganisms have several strategies to adapt to saline and alkaline environments, including adjusting the cell wall structure, lipid composition, membrane transport system, bioenergetics and osmoregulation (Banciu and Muntyan, 2015). For example, haloalkaliphiles can deal with high osmotic pressure and low H^+ concentration (due to alkaline pH) by decreasing their membrane permeability for both Na^+ and H^+.

Soda lakes are saline lakes that have high salt and carbonate concentration due to the accumulation of ions as a result of higher evaporation than inflow rate. The main anions present in these lakes are carbonate (CO_3^{2-}), sodium bicarbonate ($NaHCO_3$), chloride (Cl^-), SO_4^{2-} and cations Na^+, magnesium (Mg^+) and calcium (Ca^+) (Sorokin et al., 2011). Although the concentration of sodium carbonate can vary over time, it makes the soda lakes extremely well buffered (pH 9.5-10), pH stable environments. Therefore, these lakes have fully functioning microbial systems and have highly active microbial sulphur cycling. (Sorokin et al., 2011; Sorokin and Kuenen, 2005) Although the major biogeochemical process in soda lakes is sulphidogenesis (reduction of oxidized sulphurous compounds to HS^-), sulphur oxidation is also known to occur (Sorokin et al., 2015). Having alkaline pH has several advantages for microbial sulphur cycling. First, at pH≥10, H_2S is present in its ionic form (HS^-) which is less toxic for SOB and SRB as it cannot freely enter through the cell membrane. Therefore, the inhibitory substrate/product concentrations are higher than in neutral or acidic conditions. Second, the sulphuric acid that is produced during sulphur oxidation in the periplasm would be buffered (Sorokin et al., 2011). However, at high pH (~pH10), CO_2 fixation is challenging as CO_3^{2-} dominates over HCO_3^-, and therefore, the growth of autotrophic alkaliphiles is limited. (Sorokin et al., 2011)

Four genera of chemolithotrophic SOB belonging to *Gammaproteobacteria* have been classified in soda lakes (for a review, see (Sorokin et al., 2015)). Two genera, *Thioalkalimicrobium* and *Thioalkalispira*, are moderately salt tolerant and the other two, *Thioalkalivibrio* and *Thioalkalibacter*, are high-salt tolerant. All of them use reduced sulphur compounds (e.g., HS^-, S_n^{2-}, $S_2O_3^{2-}$, $S_n(SO_3)_2^{2-}$, S^0) as electron donors and can fix inorganic carbon. Of these genera, *Thioalkalivibrio* is the metabolically most flexible genus and can tolerate even 4M Na^+. Besides using oxygen as an electron acceptor, some of its species, e.g., *T. denitrificans*, can utilize nitrogenous compounds. Moreover,

some species (e.g., *T. thiocyanoxidans*) can use thiocyanate as sole energy, sulphur, and nitrogen source.

4 BIOPROCESSING OF SULPHUROUS STREAMS

Different forms of sulphur can be present in industrial process streams and wastewaters. Depending on the oxidation-reduction state of the sulphurous compound, it might be used as an energy source for oxidative or reductive bioprocesses. For example, wastewaters from mining, fermentative industries, seafood processing and landfills are rich in SO_4^{2-}, while process streams and wastewaters of pulping, tanneries and petrochemical industries are rich in H_2S (Costa et al., 2020). Bioprocessing for sulphur recovery can involve several process steps. First, sulphur oxyanions can be reduced to hydrogen sulphide by SRB, and then, biological oxidation of H_2S to S^0 can take place (de Graaff et al., 2012). The final product S^0 is desired as it is non-toxic, separable and can be further used e.g., in agriculture, denitrification and sulphuric acid production.

4.1 Sulphur recovery via reduction

Sulphate containing wastewaters are generally rich in organic compounds but some, such as the wastewaters from sulphur-rich ore or waste leaching and scrubbing of sulphurous off-gases are organics-deficient (Costa et al., 2020). Under anaerobic conditions, SRB and methanogens compete for organic compounds present in the wastewaters. To overcome the competition, the process conditions can be adjusted to enhance sulphate reduction. For example, the organic intermediates can be chosen based on the thermodynamics of the reactions. In sulphate-rich wastewaters, anaerobic degradation of propionate, acetate and hydrogen is energetically more favourable for SRB than methanogens and therefore, will favour sulphate reduction (Thauer et al., 1977). Theoretically at $(COD)/SO_4^{2-}=0.67$, there is a sufficient amount of COD to reduce the sulphate content of the wastewater whereas, under this ratio, some sulphate will remain (Rinzema and Lettinga, 1988). Moreover, the concentration of SO_4^{2-} also affects the competition. Also, when using hydrogen as an electron donor, maintaining low partial pressure is advantageous for SRB (Elferink Oude et al., 1994). Furthermore, operational conditions, such as pH, salinity and temperature can also

affect the outcome of the competition between SRB and methanogens (for review, see (Costa et al., 2020)).

Depending on the electron donors (Fig. 3), SRB can be complete or incomplete oxidizers of organic compounds. The majority of SRB are incomplete oxidizers, meaning that they partially oxidize organic compounds and generates acetate by-product, that they are unable to further oxidize, while complete oxidizers mineralize organics to CO_2 (Muyzer and Stams, 2008). Table 4. shows various electron acceptors and donors and different biotechnological applications for sulphate. The selection of applicable electron donors and acceptors depends on the sulphur reducing microorganism (Costa et al., 2020; Hao et al., 2014).

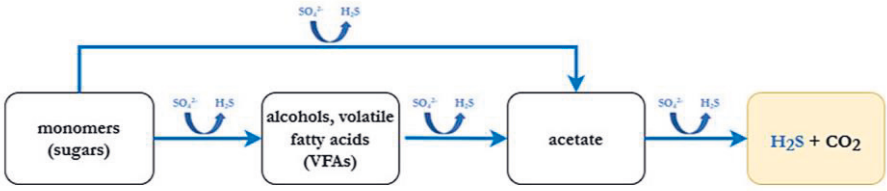

Figure 3. Pathway of sulphate reduction and organic compound oxidation by SRB. Sulphate reducers that are incomplete oxidizers of organic compounds can only produce acetate but not further utilize it, while complete oxidizers can also use acetate and mineralize organics to CO_2.

Table 4. Electron donors and acceptors for sulphur reducers, and biotechnologies targeting sulphur reduction.

e-donor/ e-acceptor/ biotechnology		Reference
e-donors:	Hydrogen, methane, amino acids, methanol, ethanol, formate, acetate, lactate, propionate, butyrate, sugars, aromatic compounds, carbon monoxide, long-chain fatty acids, aliphatic compounds, alkenes	For reviews, see Costa et al., 2020; Hao et al., 2014
e-acceptors:	Sulphate, sulphite, elemental sulphur, thiosulphate, nitrite, nitrate, manganese dioxide, fumarate, ferric iron, oxygen, carbon dioxide	For a review, see Hao et al., 2014
Biotechnologies:		For a review, see Hao et al., 2014
	THIOTEQ™: used with acid mine drainage (AMD) for metal precipitation	Muyzer and Stams, 2008
	Permeable reactive barriers/ infiltration beds: used with AMD	Younger et al., 2003
	Biotechnological flue-gas desulphurization (Bio-FGD)	Muyzer and Stams, 2008
	Two-stage anaerobic digestion: SO_4^{2-} conversion to HS^- (bioreactor 1) → HS^- removal →methane production (bioreactor 2)	Wei et al., 2007

The final product of SO_4^{2-} reduction is H_2S which is toxic for microorganisms already at low (IC50 around 200 mg/L) concentrations (O´Flathery et al., 1998). O´Flathery et al. (1998) reported decrease of sulphide toxicity above pH 7.2. As sulphide, it highly toxic for SRB, it is crucial to remove from the bioreactor during operation (Parkin et al., 1990). The inhibition can be either due to diffusion of undissociated H_2S through the cell membrane causing denaturation of proteins, or by metal precipitate formation that results to decrease bioavailability of micronutrients essential for the functioning of the cell (for a review, see (Costa et al., 2020)).

4.2 Sulphur recovery via oxidation

The most reduced sulphurous compound, H_2S, is a toxic and odorous gas that is present in gaseous streams of different industrial activities (e.g., Kraft pulping, petrochemical production, wastewater treatment, mineral processing etc.) (for a review, see (Lin et al., 2018)). Due to its hazardous nature, H_2S needs to be removed from the gaseous streams, and that is generally done by physicochemical methods such as scrubbing, *in-situ* precipitation by Fe^{2+} or Fe^{3+} based compounds, adsorption or membrane separation (Andreides et al., 2020; Dahl, 2008). During scrubbing, H_2S is dissolved in the scrubbing solution (caustic solution), that can be further treated (Lagas, 2000). Depending on the pH of the solution (Figure 4), sulphide is present as H_2S gas, HS^- or S^{2-} ions. To have all sulphide dissolved, the pH of the solution needs to be at least > pH 9. Therefore, the caustic solution used for example, in pulping and petrochemical industries has alkaline pH (even > pH 12) through NaOH addition. Another alternative of capturing H_2S from off-gases is by biological gas desulphurization (e.g., using biofilters or biotrickling filters, Table 5).

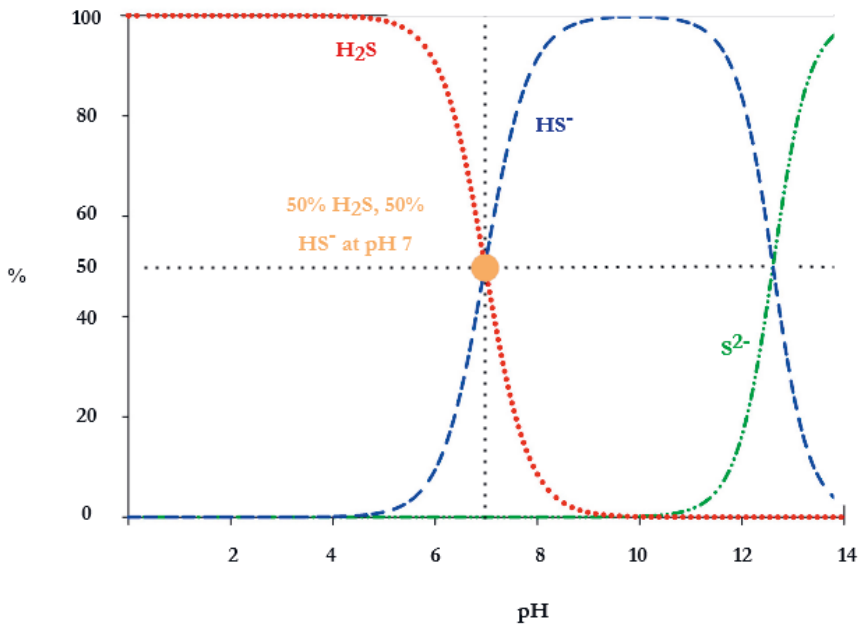

Figure 4. Shares of H_2S, HS^- and S^{2-} at different pH levels. Modified from (Minier-Matar et al., 2017).

The traditional physicochemical ways to recover sulphur from the scrubbing liquors are by the Claus process and amine treatment which generate chemical side-streams, are energy intensive and often increase maintenance costs related to corrosion (Lagas, 2000). Therefore, the recovery of dissolved sulphurous compounds via biological oxidation is a promising alternative that has recently gained increasing attention. Different bioreactor designs, such as air-lift reactor, up-flow reactor, continuously stirred tank reactor (CSTR), fluidized bed bioreactor (FBBR), photobioreactors, microbial fuel cells etc. have been used for the recovery of sulphur in its elemental form (Bosch et al., 2007; de Graaff et al., 2012; Janssen et al., 2009; Kijlstra et al., 2001; Lohwacharin and Annachhatre, 2010; Peh et al., 2022; Sulonen et al., 2014). The commercially available technologies treating dissolved sulphurous compounds (e.g., sulphide) are THIOPAQ® (Paques, Netherland) and Sulfothane™ (Veolia, France) (Table 5). Both technologies offer high sulphide removal (>99%) and S^0 recovery yield, work with high sulphide loads and the process conditions (feed, dissolved oxygen concentration, temperature, pH etc.) can be carefully controlled. As the pH of the process/ wastewaters are alkaline and often saline, alkaliphilic or haloalkaliphilic SOB are the suitable microorganisms for sulphur recovery.

Table 5. Biotechnologies applying SOB for the recovery of sulphur from H_2S gas.

Biotechnology	Process description	Pros and cons	Current commercial application(s)	Reference
Biofilter	H_2S is passed through a media bed where SOB biotransfer the gas to other sulphurous compounds. The bed is occasionally irrigated with nutrient solution. Suitable bed materials contain also indigenous microorganisms (e.g., compost and peat).	+ works with low loading rates + cheap bed material - limited buffering - difficulties with moisture control - long residence time	Municipal wastewater treatment	Omri et al., 2011; Shareefdeen et al., 2003 For a review, see Lin et al., 2018
Biotrickling filter	Similar to biofilters but here the nutrient solution is continuously fed and inert packing bed material is used (e.g., granular activated carbon, porous ceramics).	+ good buffering + works with high load - increasing back pressure - clogging due to biomass growth and sulphur accumulation	SulfurexBF® Biopuric™ BiogasCleaner®	Baquerizo et al., 2013 For reviews, see Andreides et al., 2020; Lin et al., 2018
Bioscrubber	Consists of two reactors. First, H_2S gas is dissolved in solution (e.g., alkaline solution containing HCO_3^- and CO_3^{2-}) and then the dissolved sulphide is biotransformed in a separate bioreactor.	+ can treat high loads + high conversion efficiency (>90%) + high S^0 recovery potential - requires precise process control - high investment costs	THIOPAQ® SULFOTHANE™	Paques, 2022a; Veolia, 2018 For reviews, see Andreides et al., 2020; Lin et al., 2018

Sulphide concentration in the feed can be inhibitory for the microorganisms. For instance, González-Sánchez and Revah (González-Sánchez and Revah, 2007) reported that sulphide is at least six times more inhibitory than thiosulphate for alkaliphilic SOB. Therefore, the high sulphide concentration needs to be reduced, for example by abiotic pre-treatment by forced aeration to thiosulphate (Figure 5). de Graaf et al. (de Graaff et al., 2012) studied a two-step process for the recovery of sulphur from sulphide. They applied two reactors, a bubble-column reactor for chemical oxidation of sulphide to thiosulphate by aeration, and a gas-lift (or air-lift) bioreactor for biological oxidation of thiosulphate by haloalkaliphilic SOB. The drawback of using abiotic pre-treatment of sulphide is that sulphate will be partially formed. Another way to reduce toxic concentrations of sulphide can be by reducing the feeding rate and therefore, the sulphide toxicity in the aerobic bioreactor.

Using SOB has multiple benefits. Besides biotransforming toxic sulphurous compounds into non-hazardous S^0, they also generate treated process water with NaOH content, which reduces water requirements and the addition of NaOH to the processes (Driessen et al., 2011). Moreover, corrosion of process equipment can be reduced by the removal of sulphurous compounds such as HS^- and $S_2O_3^{2-}$ which also enables the selection of cheaper construction materials. Therefore, the application of SOB is both an environental and cost-friendly option.

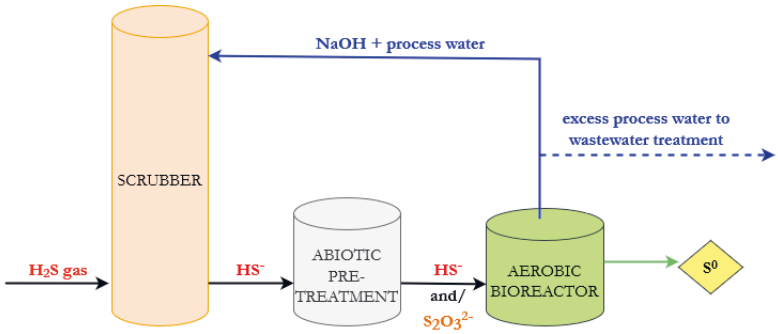

Figure 5. Elemental sulphur recovery from reduced sulphurous streams. In the scrubber, H_2S gas is dissolved in the scrubbing solution, which is fed to the bioreactor. In the bioreactor, sulphur oxidizers biotransform HS^- and other reduced sulphurous compounds (e.g., $S_2O_3^{2-}$) to S^0 and partially to SO_4^{2-}. After the S^0 removal, the process water can be regenerated. Using abiotic pre-treatment (shown with grey) to partially convert HS^- to less toxic $S_2O_3^{2-}$ is also an option.

4.3 Sequential reduction and oxidation processes for sulphur recovery

The biological sulphur recovery via reduction and oxidation can be integrated into one bioprocess (Figure 6). First, the reduction of sulphur oxyanions to H_2S can be implemented which is followed by the oxidation of H_2S to S^0. Application of this combined bioprocess enables the prevention of sulphurous emissions, reduces organic loads (COD), and creates valuable material (S^0) as a product. SULFATEQ™ (Paques, Netherlands) is one conventional example of this type of integrated bioprocess (Paques, 2022b). It has been applied with sulphate (influent concentration of 1000-25000 mg SO_4^{2-}/L) and metal-containing wastewaters. Moreover, it can also be used to convert nitrogenous compounds to N_2 gas. A demo-scale example of sulphur-cycle based organics and nutrient removal is the SANI® process (Wu et al., 2016). This process integrates sulphate reduction, denitrification and nitrification processes to treat saline municipal wastewaters. Similarly, as with SULFATEQ™, in the first bioreactor, organics are reduced and SO_4^{2-} is converted into sulphide, and in the second bioreactor, sulphide is converted into S^0 and NO_2^- and NO_3^- are converted to N_2 gas. As an addition to the SULFATEQ™ process, a third bioreactor for the biological conversion of ammonia to NO_2^- and NO_3^- is also applied.

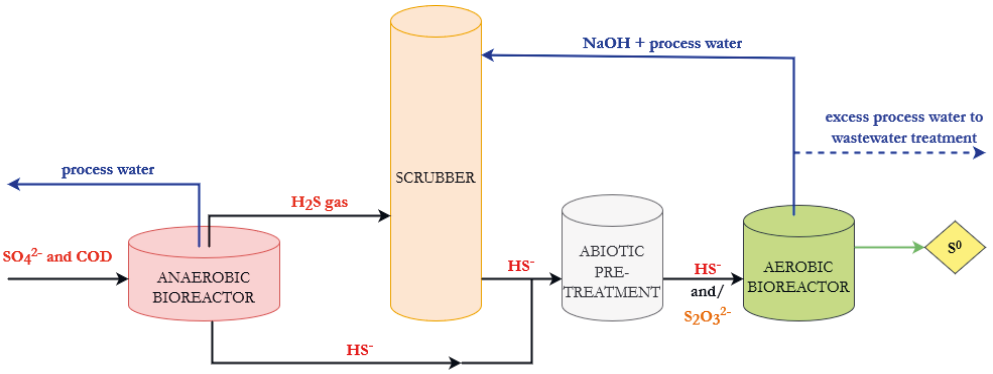

Figure 6. Combination of sulphate reduction and sulphide oxidation processes for recovery of sulphur as S^0. In the anaerobic bioreactor, sulphate reducing bacteria reduce SO_4^{2-} to H_2S gas and dissolved HS^-, and oxidize organic compounds. Thereafter, H_2S gas is dissolved in the scrubbing solution and together with the HS^- from the anaerobic bioreactor, forwarded to the aerobic bioreactor. In the aerobic bioreactor, sulphur oxidizing bacteria biotransform the reduced sulphurous compounds to S^0. The S^0 is then separated from the liquid phase and the process water can be regenerated.

4.4 Sulphurous streams containing organic compounds

In addition to sulphurous and inorganic process chemicals, multiple dissolved organic compounds can be present in the process and wastewaters of industries that process organic raw materials. For example, wastewaters of pulp and paper (P&P) mills can be rich in organics such as methanol, sugars (e.g., xylose), acetic acid and furfural (Sharma et al., 2020; Toczyłowska-Mamińska, 2017). Although at low concentrations, organohalogens are also present e.g., in bleaching effluents (Sharma et al., 2020). Two of the most organic-rich P&P wastewaters are bleaching wastewater (0.3-4-3 g COD/L) and condensate of evaporator (0.6-6.5 g COD/L) (Meyer and Edwards, 2014). In the petrochemical industry, sulphurous organics (e.g., methanethiol, ethanethiol, disulphides), phenol, benzene and toluene are of concern, especially in sulphidic spent caustics (Kolhatkar and Sublette, 1996; Park et al., 2010; Sipma et al., 2004). Organic sulphurous compounds, which are hazardous and toxic (Calbry-Muzyka et al., 2019) can be also present together with H_2S gas in the biogas coming from anaerobic bioreactor (Fisher et al., 2018).

High sensitivity of acidophilic chemolithoautotrophic SOB (e.g., species belonging to the genus *Acidithiobacillus*) towards various organic compounds has been reported (Fang and Zhou, 2006; Gu and Wong, 2004; Määttä et al., 2022a; Vardanyan and Vyrides, 2019) while for haloalkaliphilic chemolithoautotrophic SOB have been mainly for organosulphur compounds documented (de Graaff, 2012). The toxicity of organic compounds depends on their acid-base dissociation constant (pK_a), concentration, protonation, and the pH of the surrounding environment (Alexander et al., 1987). The cell membrane of SOB is negatively charged and therefore, protons can freely enter. The protonation of an organic compound depends on the relationship between its pK_a value and the pH of its environment (Alexander et al., 1987). For instance, when increasing the $pK_a > pH_{environment}$, the organic compound becomes more protonated. After diffusion into the cell, the protonated organic acid dissociates to protons and ions which accumulate within the cytoplasm. This accumulation acidifies the neutral cytoplasm, thus resulting in metabolic disorder of the cell (Alexander et al., 1987; Frattini et al., 2000; Vardanyan and Vyrides, 2019). Moreover, high concentrations of weak acids ($pK_a > 1$) or anions can also penetrate the cell membrane and accumulate in the cytosol (Vardanyan and Vyrides, 2019).

5 BIOGENIC ELEMENTAL SULPHUR

Sulphur is one of the most abundant elements on earth and it is present in all organisms e.g., in the form of lipids, proteins, amino acids, poly(peptides) and enzyme cofactors (Bruser et al., 2000). Elemental sulphur, with oxidation state zero, is the most versatile form of sulphur as it can be used for both oxidation and reduction processes (Guo et al., 2022).

5.1 Physicochemical properties of elemental sulphur

The most common types of elemental sulphur are chemical S^0, biological (or biogenic) S^0 and mineral S^0. Generally, in bioprocess applications, chemical S^0 or biological S^0 are used, as the mineral S^0 have low purity (Guo et al., 2022; Zhang et al., 2021). Chemical S^0 is orthorhombic, which means higher density compared to sulphur rings of biogenic S^0 (Kleinjan et al., 2003). Recently, the application of biogenic S^0 is gaining more attention as it is hydrophilic, has lower density and has smaller particle size and therefore, its availability for microorganisms is higher than that of chemical S^0 (Sun et al., 2021). The hydrophilic nature of biogenic S^0 is due to (bio)polymers that are present on the hydrophobic sulphur nucleus (Janssen et al., 1996; Steudel and Holdt, 1988).

Biogenic S^0 can be produced either internally or externally. Internal biogenic S^0 accumulates within the cell membrane of SOB, while external is excreted from the cell. The latter is the desired form during bioprocesses as it does not require additional extraction from the cell. The structure and surface properties of biogenic S^0 depend on the microorganism that has deposited it. In most cases, biogenic S^0 is granular (Cai et al., 2017; Kleinjan et al., 2003), but filamentous shape has been also reported (Sievert et al., 2007). Biogenic S^0 has size up to 1 μm and therefore, it is considered colloidal (Janssen et al., 1994). The stability of colloids depends on attractive (e.g., van der Waals), repulsive and structural forces (e.g., hydrogen bonding) (Kleinjan et al., 2003). While the van der Waals attraction is size and distance dependent, the repulsive electrostatic force depends on the surface charge of the colloids. This charge is due to

the presence of amino groups and carboxylic acid on the surface of the particle. In alkaline environments, the surface of S^0 colloid would have an overall negative charge due to the presence of $-COO^-$ and $-NH_2$ groups. On contrary, in acidic pH, the surface is covered with -COOH and NH_3^+ groups, which will result in a positive charge. The particles that have similar charge will be repelled and with opposite will be appealed (Kleinjan et al., 2003).

5.2 Separation of biogenic sulphur

To reduce operational costs related to S^0 separation, externally produced sulphur is desired. Although the separation of biogenic elemental sulphur from solutions is easier than other forms of sulphur, it can be still a challenge due to its colloidal properties (Janssen et al., 1994). The separation of S^0 can be done by various physicochemical methods, such as centrifugation, sedimentation, filtration, flotation and coagulation-flocculation (Table 6). Centrifugation is the most used method as it is fast and has high efficiency. For instance, the THIOPAQ® process, which is a commercially used biological sulphur recovery method, applies decanter centrifuge with up to 99% separation efficiency (Janssen et al., 2000). The main drawback of centrifugation is that its operational cost increases with the decrease in particle size. Another more commonly used separation method is gravity sedimentation which is a simple and cheap way to separate S^0 from the liquid phase. However, it requires a large area and has low efficiency at small particle sizes (Cai et al., 2017). When the settling of the colloids is challenging due to repulsive electrostatic force, the addition of chemical agent (coagulant) to enhance settling is an option (Chen et al., 2016). Fast separation of S^0 can be done by different filtration methods. For example, membrane filtration uses low pressure and results in easily collectable S^0 (Zhang et al., 2006). The main drawback of this method is membrane fouling (Chen and Liu, 2012). Flotation has been applied, for example, during Paques´s biological gas desulphurisation process (Cao et al., 2002). This separation method applies rising gas bubbles which transport the recoverable matter to the surface of the medium, where it can be then collected. Generally, it requires the addition of chemical agents to improve the separation efficiency, and therefore, resulting increase in operational costs and secondary waste generation (Deliyanni et al., 2017). Controlling process conditions during bioreactor operation may also help to enhance S^0 settleability. For example, increasing the sulphur loading rate can enhance the aggregation of biogenic S^0 and therefore, the settleability

(D'Aquino et al., 2021; Janssen et al., 1996, 1995; Velasco et al., 2004). Also, reducing the salt concentration can improve the aggregation of S^0 particles (Janssen et al., 1996).

Table 6. Efficiency of different methods used to separate S^0 from the liquid phase.

Method	Configuration	Separation efficiency (%)	Reference
Sedimentation	Expanded bed bioreactor	90	Janssen et al., 1999
Centrifugation	Decanter centrifuge	60-65*	Janssen et al., 2000
Extraction	Sand filtration, extraction and distillation with carbon disulphide	76-80	Li et al., 2000
Coagulation with polyaluminium chloride (PAC)	Jar Test	90-98	Annachhatre and Suktrakoolvait, 2001; Chen et al., 2016
Flocculation extracellular polymeric substance (EPS)	Airlift-loop reactor	28	Feng et al., 2018

* dry solid content, 95-99% purity of sulphur

5.3 Potential uses of biogenic sulphur

Compared to other forms of reduced sulphur compounds, biogenic S^0 is safe to handle and non-corrosive and therefore, it can be used in a wide range of applications (Table 7) (Cai et al., 2017; Di Capua et al., 2016; Johnson, 2014; Liu et al., 2008; Zhang et al., 2021). For example, S^0 can be used for high-purity sulphuric acid production, and this approach has been already applied in the P&P industry. Internal conversion of elemental sulphur to sulphuric acid reduces operational costs related to sulphuric acid storage and corrosion (Cai et al., 2017; Martikainen, 2022). S^0 has been applied in bioleaching applications to generate sulphuric acid by SOB to initiate metal dissolution. Using hydrophilic biogenic S^0 instead of chemical S^0 would provide better bioavailability for bioleaching microorganisms and therefore, accelerate sulphuric acid production (Lin et al., 2018). Within the mining sector, biogenic S^0 can be also used to remove organic content from metal-laden wastewaters. Generally, the COD of these wastewaters is too low to have efficient removal through SO_4^{2-} reduction.

Therefore, using biogenic S^0 that requires only two electrons per sulphide formed compared to SO_4^{2-} that requires eight is a better option (Zhang et al., 2021). Furthermore, biogenic S^0 can be also used for metal precipitation by forming metal sulphide (Lin et al., 2018). Due to the bioavailability of biogenic S^0, it is a more suitable raw material for fertilizer and pesticide production than chemical sulphur (Chung et al., 2013). Moreover, biogenic S^0 can be used as an adsorbent for heavy metals, such as zinc (Chen et al., 2012); and used as an electron donor during autotrophic denitrification (Du et al., 2019; Y. Wang et al., 2016). Although further optimization is needed, biogenic S^0 can be also used to produce sulphur-based cathode material for Li-S batteries (Manthiram et al., 2014) and to enhance concrete strength (Lin et al., 2018).

Table 7. Different applications of biogenic S^0 by fields.

Application	Field	Reference
Sulphuric acid production	P&P manufacturing processes, bioleaching	Cai et al., 2017; Lin et al., 2018; Martikainen, 2022
Adsorption metals	Treatment of mining wastewaters	Chen et al., 2012
Precipitation of heavy metals	Treatment of mining and industrial wastewaters, aquatic ecosystems	Lin et al., 2018; Zhang et al., 2021
COD removal	Treatment of mining wastewaters	Lin et al., 2018; Zhang et al., 2021
Denitrification	Treatment of municipal wastewater, drinking water, ground water, runoff water	Cui et al., 2019; Zhang et al., 2021
Fertilizer and pesticide production	Agriculture	Chung et al., 2013
Li-S couple of Li/S fuel cell	Energy storage	Manthiram et al., 2014
denitrification	Wastewater treatment	Lin et al., 2018
Partial denitrification ($NO_3^- \rightarrow NO_2^-$) prior to anammox process	Energy intensive-industrial and domestic wastewater treatment	Du et al., 2019; Lin et al., 2018
Sulphur concrete production	Construction	Lin et al., 2018

6 RESEARCH OBJECTIVES

The main objective of this thesis was to investigate the potential of biological S^0 recovery by chemolithoautotrophic SOB from haloalkaline solutions for possible use in industrial process streams and wastewaters. The objective was to investigate the thiosulphate biotransformation in selected solutions by haloalkaliphilic SOB. The specific objectives were as follows:

1) **Investigating the thiosulphate biotransformation kinetics by two selected species of haloalkaliphilic SOB under aerobic conditions (Paper I and II)**

When designing a bioprocess, thorough understanding of the kinetics and substrate limitation is required. Therefore, the kinetics of thiosulphate biotransformation and biomass growth of two species of haloalkaliphilic SOB, *T. versutus* and *T. denitrificans*, were investigated at different initial thiosulphate concentrations in batch assays. Moreover, the S^0 production potential at different initial thiosulphate concentrations was also estimated. Finally, the kinetics and S^0 recovery potential by *T. versutus* and *T. denitrificans* were compared.

2) **Assessing the possibility of S^0 recovery using anoxic thiosulphate biotransformation (Paper II)**

Supplying gaseous oxygen as electron acceptor can be challenging to certain industrial process streams/ wastewaters, thus, providing oxygen in dissolved, nitrogen bounded form (N_2O, NO_2^- or NO_3^-) might be more suitable. Therefore, the potential of S^0 recovery by *T. denitrificans* under anoxic conditions with NO_2^- was investigated and the results were compared to aerobic biotransformation.

3) **Determining the thiosulphate biotransformation rate and S^0 yield in continuous biofilm-based bioreactor operation (Paper III)**

As the sulphurous streams of process industries are generated in large volumes, they require continuous biological treatment. Therefore, the aim was to study the suitability of FBBR for continuous biotransformation of thiosulphate by *T. versutus*. Special

attention was paid to process limiting factors. Moreover, additional methods to optimize S^0 settling were implemented. In addition, one of the main points was to look if the amendment with pure culture to a non-aseptic system could be feasible.

4) Investigating the effects of organic compounds and P&P wastewaters on thiosulphate biotransformation and biomass growth (Paper IV)

The process streams and wastewaters of organic raw-material processing industries contain organic compounds that can be potentially inhibitory for the biotransformation and/or biomass growth. Therefore, the aim was to study the effects of organics commonly present in these wastewaters on thiosulphate biotransformation. Also, the possible mechanisms of inhibition by organic compounds were investigated based on the available literature. Moreover, the sensitivity towards two selected organic-rich P&P mill wastewaters was determined.

7 MATERIALS AND METHODS

Summary of the Materials and Methods of the thesis is presented in this chapter. Detailed information can be found from papers I-IV.

7.1 Model microorganisms and growth medium

Two haloalkaliphilic sulphur oxidizing bacteria *Thioalkalivibrio versutus* and *T. denitrificans* were used as model organisms in papers I, III and IV, and paper II, respectively. *T. versutus* is an obligate aerophilic bacterium while *T. denitrificans* is microaerophilic and biotransfers sulphurous compounds in denitrifying condition. Both model microorganisms were obtained from German Collection of Microorganisms and Cell Cultures GmbH (DSMZ) and originally isolated from soda lakes by Sorokin et al. (Sorokin et al., 2001b). The stock cultures were maintained in 925 alkaliphilic sulphur respiring strain medium (Feso and Gmbh, 2009).

7.2 Overview of the experiments

The experimental program is presented in Figure 7. Prior to the experiments, two haloalkaliphilic SOB (*T. versutus* and *T. denitrificans*) were selected based on their capability of producing elemental sulphur, high tolerance to Na^+ and alkaline pH (optimum ~pH 10). $S_2O_3^{2-}$ was used as model compound during this study due to its lower toxicity to SOB than HS^-. To distinguish between the thiosulphate biotransformation efficiencies by *T. versutus* and *T. denitrificans*, batch experiments in duplicate shake flasks and batch bottles (Figure 8) were implemented. The thiosulphate biotransformation to S^0 and/or SO_4^{2-}, and biomass growth in the presence of oxygen (Paper I and II) and nitrite (Paper II) were analysed. After the batch kinetic experiments with the two SOB, the biotransformation in continuously operated aerobic fluidized bed bioreactor (FBBR) with *T. versutus*, that showed the highest rate of thiosulphate biotransformation and high S^0 formation yield during the batch studies, was implemented (Paper III). The FBBR was operated with different

thiosulphate loading rates (LRs) for over 70 days. As industrial wastewaters such as of pulping can contain organics, the effects of common organic compounds (methanol, acetate, xylose, phenol, benzene), yeast extract and two pulping wastewaters (primary filtrate of bleaching and composite wastewater) on thiosulphate biotransformation by *T. versutus* and biomass formation were also investigated in batch assays (Figure 8b, Paper IV).

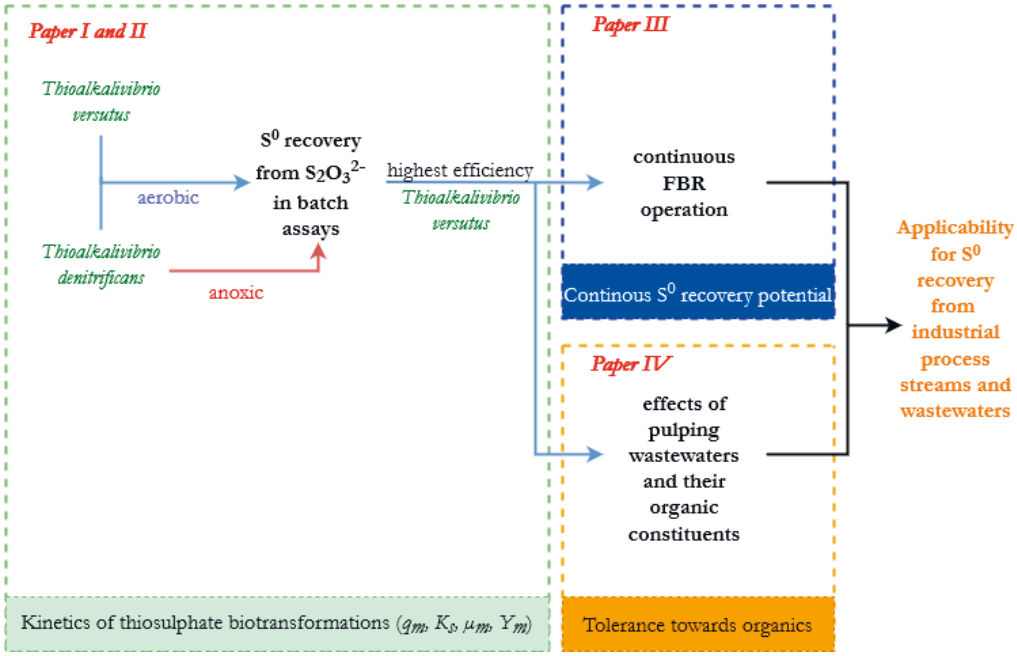

Figure 7. Overview of the experimental programs. First, the batch kinetics of thiosulphate biotransformation (Paper I and II) were studied with two selected sulphur oxidizing bacteria (SOB), *Thioalkalivibrio versutus* and *T. denitrificans*). Then, continuous fluidized bed bioreactor (FBBR) operation with SOB (*T. versutus*) that was more potent for S^0 recovery was implemented. Finally, the effects of pulping wastewaters and their organic constituents on thiosulphate biotransformation were investigated in batch assays. Thiosulphate was used as model sulphurous compound during the studies. Process streams and wastewaters refer to streams that are recirculated within the process and streams leaving to wastewater treatment, respectively.

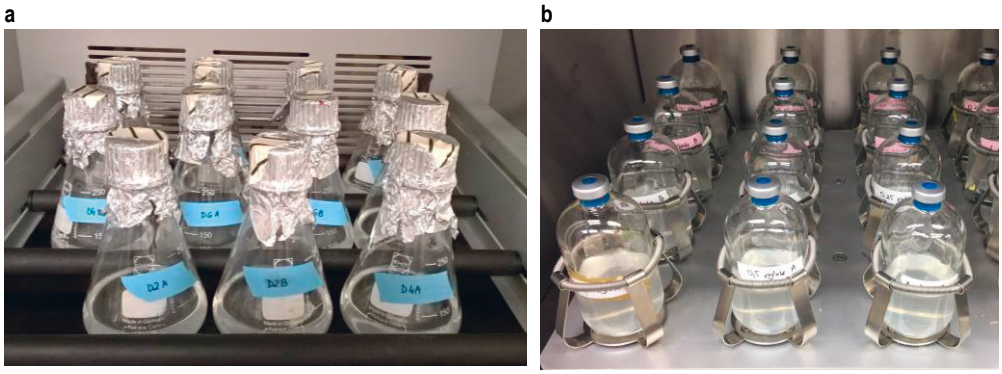

Figure 8. Batch assays used during the different studies. a) shake flasks used during the aerobic kinetic studies (Paper I and II); b) batch bottles used during the anoxic experiment with *T. denitrificans* (Paper II), and experiments with organic compounds and wastewaters (Paper IV).

7.3 Bioreactor design

Fluidized bed bioreactor (FBBR) system (Figure 9) consisting of FBBR unit (1 L), gravity settler (6 L total volume) and recirculation unit (1 L) were used during the continuous thiosulphate biotransformation experiment. In the FBBR unit, activated carbon (Filtrasorb 200) was used as biomass carrier material (Paper III). The volume of the activated carbon and its fluidization were 458 mL (without fluidization) and 17-21%, respectively. The settler had cone shape to enhance elemental sulphur settling that could be collected through the bottom valve. Air was supplied through the lower end of the recirculation unit (RU) and the treated effluent left the FBBR system from the top of the RU. The effluent was collected to perform different settling tests (coagulation and centrifugation) to further enhance the settleability of the formed elemental sulphur. Detailed description of the bioreactor design and its operation can be seen from Paper III.

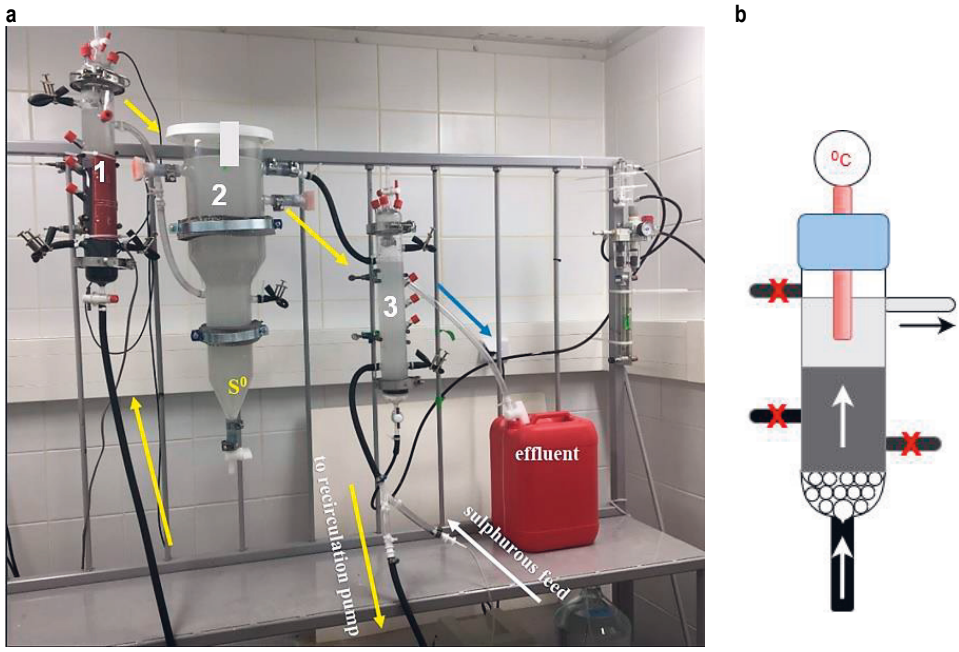

Figure 9. Fluidized bed bioreactor (FBBR) system used for continuous thiosulphate biotransformation. a) Part 1 is FBR unit, part 2 is settler and part 3 is recirculation/ aeration unit. b) schematic of the FBR unit without heating blanket.

7.4 Analytical methods

The different physicochemical analyses conducted in this study are summarized in Table 8. The biomass content was quantified by using either quantitative polymerase chain reaction (qPCR) (Paper I and II) or Bradford protein analysis (Paper III). Prior to the qPCR, the DNAs of the samples were extracted by using DNeasy Power Soil Kit (Qiagen) and then analysed for 16S rRNA gene copy numbers. The protein content was determined by using a microplate reader. Moreover, the biofilm on the activated carbon (Paper III) was visualized by scanning electron microscopy (SEM).

Table 8. Summary of physicochemical analyses conducted in this study.

Analyses	Instrument	Paper
Acetate, Formate, nitrate, nitrite, sulphate, phosphate, thiosulphate	anion-chromatograph	I, II, III, IV
Sodium, magnesium, ammonium, potassium, calcium	cation-chromatograph	IV
Elemental sulphur	CHNS elemental analyser	I, II
Dissolved organic carbon	total organic carbon (TOC) analyser	IV
Acetic acid, propionate, methanol, isobutyrate	gas chromatography–mass spectrometry	IV
Particle size analysis	scanning electron microscopy	IV
Total solids	oven, balance	I, II, III
Turbidity	turbidimeter	III
pH	pH electrode	I, II, III, IV
Dissolved oxygen (DO)	DO probe	IV

7.5 Calculations

The different kinetic constants and parameters, and their equations used in papers I-IV are summarized in Table 9.

Table 9. p. 1/2 The calculation of different parameters/constants present in the papers of this thesis.

Parameter/constant	Calculation	Paper
K_s (g/L), q_m (h^{-1})	$q = \frac{q_m [S_2O_3\text{-}S]}{K_s + [S_2O_3\text{-}S]}$	I, II
μ_m (h^{-1})	$\mu = \frac{\mu_m [S_2O_3\text{-}S]}{K_s + [S_2O_3\text{-}S]}$	I, II
SPR_1, SPR_2 (g/L/d)	$SPR_1 = \frac{d[SO_4^{2-}]}{dt} = \left([S_2O_3\text{-}S]_0 - \frac{d\,[S_2O_3\text{-}S]}{dt} \cdot f_1 \right)$ and	I, II

Table 9. p.2/2 Continued.

	$SPR_2 = \frac{\mathrm{d}[S^0]}{\mathrm{dt}} = \left([S_2O_3\text{-}S]_0 - \frac{d\,[S_2O_3\text{-}S]}{dt} \cdot f_2 \right)$	
Y (g cell/g $S_2O_3^{2-}$-S)	$Y = \frac{\mu_m}{q_m}$	I
HRT (h)	$HRT = \frac{V_{fb}}{Q_{in}}$	III
LR (g/L/d)	$LR = \frac{[S_2O_3\text{-}S]_{in} \cdot Q_{in}}{V_{fb} \cdot 24h}$	III
BR (g/L/d)	$BR = \frac{([S_2O_3\text{-}S]_{in} - [S_2O_3\text{-}S]_{out}) \cdot Q_{in}}{V_{fb} \cdot 24h}$	III
S^0 production rate (g/L/d)	$S^0 production\ rate = \frac{S^0 \cdot Q_{in}}{V_{fb} \cdot 24h}$	III
RE (%)	$RE = \frac{[S_2O_3\text{-}S]_{in} - [S_2O_3\text{-}S]_{out}}{[S_2O_3\text{-}S]_{in}} \cdot 100$	III
CE (%)	$CE = \frac{S^0}{[S_2O_3\text{-}S]_{in}} \cdot 100$	III
Share of S^0 (%)	$S^0 = \frac{[S_2O_3\text{-}S]_0 - [S_2O_3\text{-}S]_t - [SO_4^{2-}]_t + [SO_4^{2-}]_0}{[S_2O_3\text{-}S]_0}$	IV

K_s: half saturation constant, q_m: maximum, specific substrate biotransformation rate, μ_m: specific growth rate, $[S_2O_3^{2-}\text{-}S]_0$: initial $S_2O_3^{2-}$-S concentration, SPR_1: SO_4^{2-} production rate, SPR_2: S^0 production rate, *t*: time, f_1: conversion fraction of $S_2O_3^{2-}$-S to SO_4^{2-} (mol/mol), f_2: conversion fraction of $S_2O_3^{2-}$-S to S^0 (1-f_1), *Y*: growth yield, LR: loading rate, $[S_2O_3^{2-}\text{-}S]_{in}$: $S_2O_3^{2-}$-S concentration of feed, $[S_2O_3^{2-}\text{-}S]_{out}$: $S_2O_3^{2-}$-S concentration of effluent, Q_{in}: feed flow, V_{fb}: volume of fluidized bed, BR: biotransformation rate, RE: recovery efficiency, CE: conversion efficiency to S^0

8 RESULTS AND DISCUSSION

This chapter summarizes and discusses the main results and their significance. The main goal of this thesis was to show the potential of S^0 recovery by chemolithoautotrophic SOB from haloalkaline sulphurous process streams and wastewaters. $S_2O_3^{2-}$ instead of HS^- was used as the model compound due to its lower toxicity to SOB. Also, the toxic concentrations of HS^- in industrial streams can be chemically oxidized to $S_2O_3^{2-}$ by excess aeration. Therefore, there were less safety concerns during implementaion of the experiments and the kinetics could be studied with a wide range of substrate concentrations when using $S_2O_3^{2-}$.

8.1 Thiosulphate biotransformation in batch assays

There have been only limited studies on the kinetics of thiosulphate biotransformation by *T. versutus* (Ang et al., 2017; Banciu et al., 2004a; Makzum et al., 2016) and *T. denitrificans* (Sorokin et al., 2001a). For instance, these studies with *T. versutus* mainly focused on growth kinetics (Ang et al., 2017; Banciu et al., 2004a), thiosulphate removal (Makzum et al., 2016), effects of Na^+ concentrations (Banciu et al., 2004a; Makzum et al., 2016) and expression of sulphur oxidation genes(Ang et al., 2017); and with *T. denitrificans* on the growth kinetics (Sorokin et al., 2001a). Therefore, the kinetics of thiosulphate biotransformation and biomass growth of both *T. versutus* and *T. denitrificans* were thoroughly investigated at different initial thiosulphate concentrations (Paper I and II). The biotransformation by *T. versutus* was studied under aerobic conditions (Paper I) while by *T. denitrificans*, both under aerobic and anoxic conditionss (Paper II). Moreover, the S^0 and SO_4^{2-} formations yields by both SOB were reported. According to the stoichiometry of thiosulphate biotransformation with nitrite (Eq. 10, Paper II), only sulphate is formed and no elemental sulphur. However, if the substrate would be sulphide, instead of thiosulphate, elemental sulphur would be also formed (Eq. 11, section 3.2.2). Therefore, the tolerance of nitrite by *T. denitrificans* was investigated.

8.1.1 Kinetics of thiosulphate biotransformation and biomass growth

Table 10 shows a comparison of the results with other studies implemented in haloalkaline condition with thiosulphate as substrate. *T. versutus* and *T. denitrificans* biotransformed thiosulphate at initial concentrations up to 17.6 and 19.6 g $S_2O_3^{2-}$-S/L, respectively. The highest substrate concentration that *T. versutus* can tolerate is much above our highest studied one, as Makzum et al. (Makzum et al., 2016) reported biotransformation at 48 g $S_2O_3^{2-}$-S/L (750 mM $S_2O_3^{2-}$). The substrate consumption rate by *T. versutus* increased with increasing initial concentrations, while by *T. denitrificans*, it increased only up to initial 8.5 g $S_2O_3^{2-}$-S/L and thereafter the consumption rate decreased. The lag times of substrate utilization at different initial thiosulphate concentrations were shorter with *T. versutus* than with *T. denitrificans* (Table 10). Based on the Monod-based fitting of the results, the q_m by *T. versutus* was 3.5 times higher than by *T. denitrificans*. Also, the K_s was higher (~4 times) for *T. versutus*. The Haldane models did not fit the Monod models and the substrate inhibition constants (K_i) were high, and therefore, none of the studied initial thiosulphate concentrations were inhibitory to any of the species studied. Although *T. denitrificans* could use NO_2^- as electron acceptor, the $S_2O_3^{2-}$ biotransformation was slower than under aerobic conditions. As the measured SO_4^{2-} concentration was lower than the calculated one (based on stoichiometry), NO_2^- was possible reduced to gaseous N_2O and therefore, was not used as electron acceptor of thiosulphate oxidation.

The maximum specific growth rates (μ_m) of *T. versutus* and *T. denitrificans* under aerobic conditions were similar, but the growth yield (Y) of *T. versutus* was higher (Table 10). The μ_m (0.048 h^{-1} at 17,2 g $S_2O_3^{2-}$-S/L) of *T. versutus* was lower than 0.082 h^{-1} (at 2.6 g $S_2O_3^{2-}$-S/L) that was reported by Makzum et al. (Makzum et al., 2016). Under anoxic conditions with 2.5 g $S_2O_3^{2-}$-S/L, the specific growth rate of *T. denitrificans* was much lower (0.022 h^{-1} with K_s=0.42 g $S_2O_3^{2-}$-S/L) than under aerobic conditions.

Table 10. p.1/2 Summary of the experimental conditions and kinetic constants of thiosulphate biotransformation under haloalkaline condition reported in different batch studies. Modified from tables 2 of Papers I and II.

Experimental conditions								Kinetic results					Ref.
Microorg.	**Exp. system**	**Na^+ (g/L)**	**Initial $S_2O_3^{2-}$-S (g/L)**	**Initial NO_2^- (g/L)**	**e- acceptor**	**pH**	**Temp. (°C)**	**Lag time (h)**	**q_m (h^{-1})**	**K_s (g/L)**	**μ_m (h^{-1})**	**Y (g cell/g $S_2O_3^{2-}$-S)**	
T. versutus	BA	14-26	0.8-17.6	-	O_2	10	30	1-38	0.083	1.74	0.048	0.31	Paper I
T. versutus	BA	N.R.	2.56	-	O_2	10	30	25	0.049	N.D.	0.082	N.D.	Makzum et al., 2016
T. versutus	BA	N.R.	2.56	-	O_2	10	37	20	0.064	N.D.	0.095	N.D.	Makzum et al., 2016
T. versutus	LSF	13.8, 4 and 92	2.56	-	O_2	10	35	N.R.	N.D.	N.D.	0.27, 0.21 and 0.11	0.12,0.086 and 0.055	Banciu et al., 2004a
T. denitrificans	BA	14-26	0.8-19.6	-	O_2	10	30	8-70	0.024	0.42	0.046	0.22	Paper II
T. denitrificans	BA	N.R.	2.56	-	O_2	10	30	40-60	N.D.	N.D.	0.028	0.021 g protein/ g $S_2O_3^{2-}$-S[a]	Sorokin et al., 2001a
T. denitrificans	BA	N.R.	2.56	4.14	NO_2^-	10	30	60-70	N.D.	N.D.	0.022	0.024 g protein/ g $S_2O_3^{2-}$-S[b]	Sorokin et al., 2001a

Table 10. p.2/2 Continued.

Experimental conditions								Kinetic results					Ref.
Microorg.	**Exp. system**	**Na^+ (g/L)**	**Initial $S_2O_3^{2-}$-S (g/L)**	**Initial NO_2^- (g/L)**	**e- acceptor**	**pH**	**Temp. (°C)**	**Lag time (h)**	**q_m (h^{-1})**	**K_s (g/L)**	**μ_m (h^{-1})**	**Y (g cell/g $S_2O_3^{2-}$-S)**	
T. denitrificans	BA	15	2.5	0.2-0.58	NO_2^-	10	30	N.D.	N.D.	0.42	0.022[c]	N.D.	Paper II
T. denitrificans	LSF	N.R.	1.66	2.94	NO_2^-	10	30	N.R.	N.D.	N.D.	0.055	0.029 g protein/ g $S_2O_3^{2-}$-S[d]	Sorokin et al., 2001a

BA: batch assay, N.R.: not reported, N.D.: not determined, LSF: lab-scale fermenter, [a] reported as 4.2 mg protein/ mmol $S_2O_3^{2-}$, [b] reported as 4.7 mg protein/ mmol $S_2O_3^{2-}$, [c] calculated with $S_2O_3^{2-}$, [d] reported as (5.6 mg protein/ mmol $S_2O_3^{2-}$)

Potentially there was a biotransformation rate (BR) limiting factor other than substrate concentration or biomass growth that affected the thiosulphate biotransformation rates by *T. denitrificans*. Although our experimental design and aseptic conditions did not allow to monitor the concentration of DO, it can be assumed that elevated DO concentration/O_2 partial pressure was the rate limiting factor. *T. denitrificans* is a microaerophile, meaning that it prefers oxygen partial pressure below atmospheric which was not possible to maintain in the shake flasks. The limitation by oxygen partial pressure is in accordance with the finding of Sorokin et al. (Sorokin et al., 2001a), who reported severe growth inhibition of *T. denitrificans* by forced aeration in batch assay. They demonstrated that the activation speed of N_2O reducing activity is directly proportional to the air saturation applied during aerobic cultivation. For example, the activation time was 0, 40-60 and 90-120 min, with air saturation of 0, 30 and 80%, respectively. As the thiosulphate biotransformation and growth rates suggests, *T. denitrificans* should be grown in microaerophilic instead of anoxic conditions.

8.1.2 Elemental sulphur and sulphate production

Elemental sulphur formation was visual in all bioassays, except at 0.8 g $S_2O_3^{2-}$-S/L. At this lowest concentration, both species formed only SO_4^{2-} as product. Also, the formed S^0 started to disappear from some of the bioassays with *T. versutus* and *T. denitrificans* before the end of the 14 days incubation. Therefore, indicating S^0 utilization by the SOB. When $S_2O_3^{2-}$ gets limited, SOB start to utilize another available substrate, which was in our studies S^0. At initial 6.4 g $S_2O_3^{2-}$-S/L (100 mM $S_2O_3^{2-}$), Makzum et al. (Makzum et al., 2016) reported oxidation of S^0 to SO_4^{2-} by *T. versutus*, after depletion of $S_2O_3^{2-}$. Therefore, when S^0 is the wanted product, maintaining the thiosulphate concentration is crucial to prevent oxidation of S^0. Under anoxic conditions with 0.2-0.58 g NO_2^-/L, visually no S^0 was formed.

The highest S^0 recovery in the independent batch assays with initial 9.5 g $S_2O_3^{2-}$-S/L were 29 and 10% by *T. versutus* and *T. denitrificans*, respectively. Based on the models used to estimate S^0 formation by time, the highest S^0 accumulations of 45 and 61% would be at 17.6 g $S_2O_3^{2-}$-S/L and 16.5 g $S_2O_3^{2-}$-S/L (lag-phases were omitted from calculations) by *T. versutus* and *T. denitrificans*, respectively (Figure 10a). In case including the lag times to the calculations also with *T. denitrificans*, the maximum share of S^0 as product would be around 40% at initial 6 g $S_2O_3^{2-}$-S/L. The biogenic S^0

produced by both SOB looked similar (Figure 10b). Therefore, both species are potent for S^0 production under aerobic conditions. The S^0 recovery was not studied in independent batch assays under anoxic conditions with *T. denitrificans*. Figure 11 shows the calculated fractions of biogenic S^0 production in the independent batch assays.

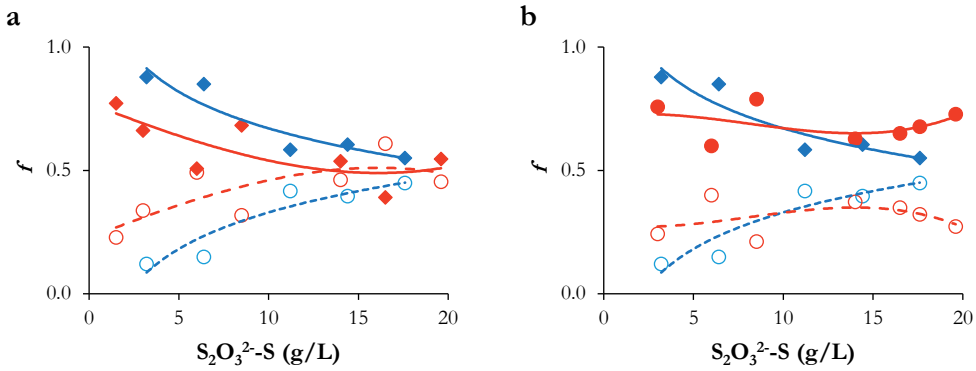

Figure 10. Fractions of biotransformation of [S_2O_3-S] to [SO_4^{2-}-S] (f_1, ♦) and [S_2O_3-S] to [S^0] (f_2, o) with *T. versutus* (blue) *and T. denitrificans* (red). The solid (—) and dashed (- - -) lines show the fitted curves for f_1 and the f_2. a) Lag times were omitted from the calculations with *T. denitrificans*. b) Lag times included in the calculations with *T. denitrificans*. In both figures, the lag times were not omitted from the calculations with *T. versutus*.

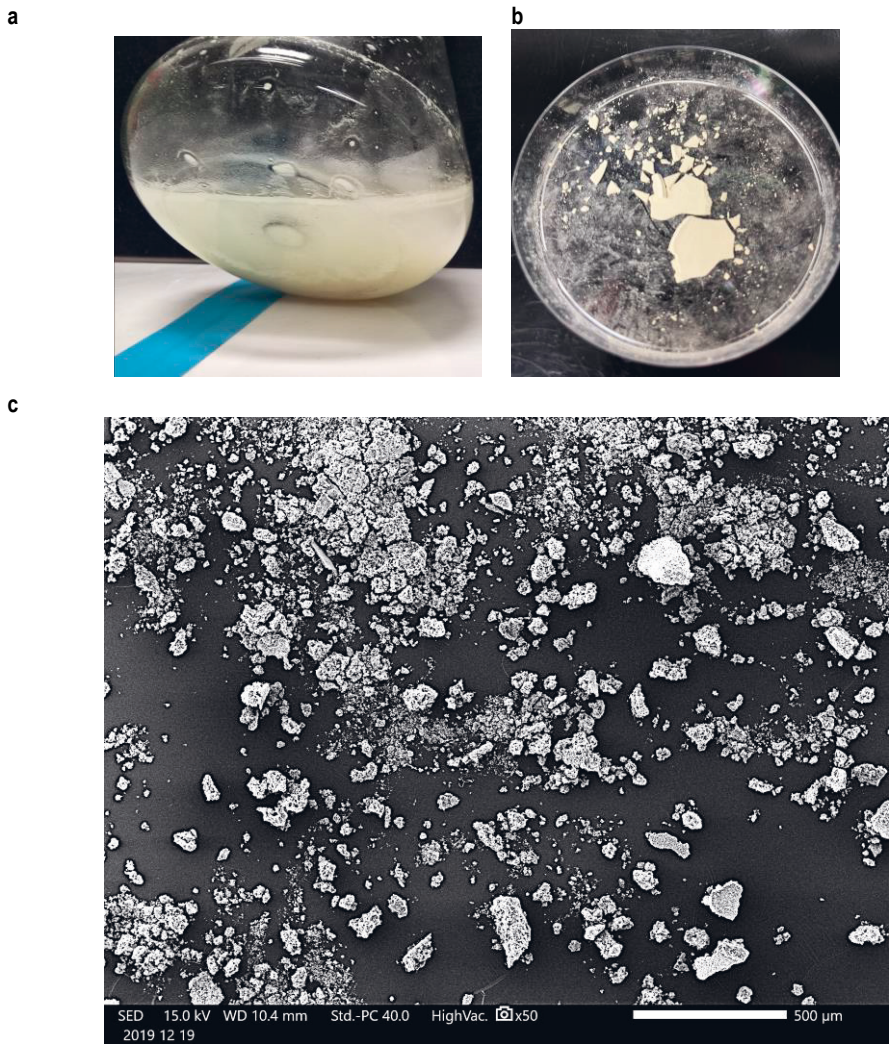

Figure 11. Biogenic sulphur formed in the batch bioassays. a) sulphur formation in the independent batch assay with *T. versutus*. b) S^0 collected from the batch assays with *T. versutus* and *T. denitrificans* by filtration (1.2 µm GF/C glass microfiber filter, Whatman). c) scanning electron microscope (SEM) image of the biogenic sulphur.

In summary, based on the results of thiosulphate biotransformation and biomass growth, the aerobic biotransformation was not limited by *T. versutus*, while potentially the high concentration of DO was the controlling/limiting factor for microaerophilic *T. denitrificans*. Under aerobic conditions with thiosulphate concentrations >8.5 g

$S_2O_3^{2-}$-S/L in batch bioassay, *T. versutus* is a more potent catalyst of thiosulphate biotransformation than *T. denitrificans* and therefore, the thiosulphate biotransformation was further investigated by *T. versutus* (Paper III and IV). However, in oxygen-limited conditions, e.g., in concentrated process streams, using *T. denitrificans* can be a better option. The model used to estimate the S^0 formation under aerobic conditions by both SOB was highly compatible (R^2>0.9) with the experimental data. Therefore, the kinetic constants obtained, and the model used to estimate the fractions of SO_4^{2-} and S^0 production can be used during designing bioreactor operation.

8.2 Thiosulphate biotransformation in continuous bioreactor operation

As high-rate thiosulphate biotransformation was achieved in batch bioassays (Paper I) by *T. versutus*, studying its applicability in continuous bioreactor operation under aerobic conditions was the next to be implemented. Mu et al. (Mu et al., 2021) has reported 86% conversion efficiency of HS^- (3.2 g S/L/d) to S^0 by *T. versutus* in a suspended biomass reactor. Having the microbial culture as cell suspension may result loss of biomass via the effluent. Therefore, maintaining the biomass within the bioreactor may further enhance S^0 recovery. To see the recovery potential of biofilm-based continuous process, FBBR operation with activated carbon (AC) as biofilm support material was implemented. Also, potentially higher sulphur loading rate can be achieved when having $S_2O_3^{2-}$ as substrate as it is less toxic for SOB than HS^- (González-Sánchez and Revah, 2007), and therefore, the FBBR operation was carried out with $S_2O_3^{2-}$. Prior to the continuous operation, the FBBR was operated in semi-batch mode for 14 days to enable biofilm formation on the AC. During this period, high aeration was supplied and half of the liquid phase was twice transferred with fresh medium including thiosulphate. To find the limitation of the thiosulphate biotransformation, increasing thiosulphate-S loading rates (12-33 g/L/d, corresponding HRT 11-5 h) were applied to the FBBR. The aeration was kept at high flow rate to enable sufficient oxygen concentration for thiosulphate bioconversion. Besides monitoring the process performance ($S_2O_3^{2-}$ consumption, SO_4^{2-} and S^0 formation, DO concentration), centrifugation and coagulation tests to enhance S^0 settling were implemented.

8.2.1 Process performance and biofilm formation

Thiosulphate was completely biotransformed by *T. versutus* at loading rates (LRs) 12-18 g S/L/d (HRT 12-9 h), but the average S^0 production rate was only 27% (Figure 12). As sulphide is more toxic to SOB than thiosulphate, these loading rates would be much lower with sulphide and therefore, the biotransformation to elemental sulphur slower if sulphide would be used as substrate (González-Sánchez and Revah, 2007). Increasing further the LRs resulted decrease of the process efficiency. Although the thiosulphate biotransformation rate (BR) increased between HRT 9-5 h (LR of 18-33 g S/L/d), its removal efficiency (RE) decreased (Figure 12 a and b). Therefore, further increase of the LRs resulted increasing concentrations of thiosulphate in the effluent. The S^0 production rate also increased between HRTs 9-5 h, but its conversion efficiency (CE) stabilized between LRs of 9-7 h and then started to decline. The DO concentration was considerably stable until HRT 7 h, and then, at HRT 5h, it decreased to close to 0 g/L, resulting further decrease of the shares of the BR and S^0 formation. Therefore, at HRT 5 h, the DO concentration became process limiting. Recovering the bioprocess by increasing the HRT above HRT 5 h was not possible. Although high thiosulphate removal efficiency (99.9-80%) could be achieved in the FBBR system, the CE to S^0 stayed well below the value reported by Mu et al (Mu et al., 2021) in suspended biomass reactor. Also, in slightly alkaline condition (pH 8.5 and 7.8), Buismann et al. (Buisman et al., 1990) and Lohwacharin and Annachatre. (Lohwacharin and Annachhatre, 2010) reported >90% CE in an upflow bioreactor with fixed film and >80% CE in an airlift reactor, respectively, with mixed cultures and sulphide as substrate. Moreover, in acidic condition (pH 5.5) González-Sánchez and Revah (González-Sánchez et al., 2008) achieved 77% CE by *Thiobacilli spp.* in a thiosulphate fed supernatant-recycling settler bioreactor with PVC packing. A comparison of the results obtained in this and other bioreactor studies with sulphide or thiosulphate are shown in Table 11.

Around 80% of the total biomass of *T. versutus* grew as biofilm on the AC particles. The biofilm was mainly formed until HRT 10 h (22 days) and thereafter, remained nearly constant. The scanning electron microscopy (SEM) images showed some biofilm coverage on the AC particles. Although biofilm growth was achieved on AC, the concentration of active biomass in the FBBR possibly remained below the concentration of cell suspension in the bioreactor of Mu et al. (Mu et al., 2021). Therefore, the properties of AC (e.g., surface charge) were possibly not favourable for

efficient biomass retainment. Moreover, the highest concentration of cell suspension was around 0.092 mg cell/mL which was considerably lower than the highest 4.25 mg cell/mL during the batch kinetic study (Paper I).

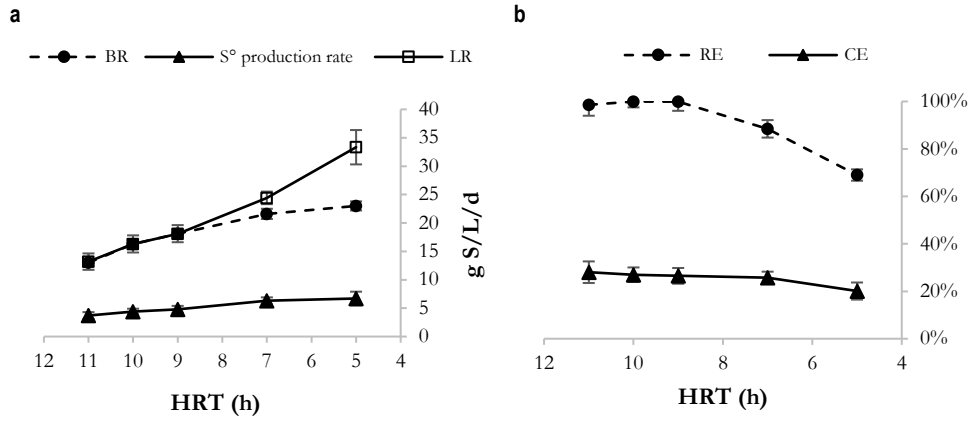

Figure 12. Fluidized bed bioreactor (FBBR) performance in continuous operation. a) Average biotransformation rates (BR), S^0 production rates and loading rates (LR) by hydraulic retention times (HRT). b) Thiosulphate removal efficiency (RE) and conversion efficiency to S^0 (CE) at different HRTs.

Table 11. p. 1/2 Comparison of the performances of different bioreactor designs used for sulphur recovery. Modified from the Table 2 of D´Aquino et al. (D'Aquino et al., 2021).

Experimental conditions						Performance*			Reference
Exp. design	**Inoculum**	**Substrate**	**Feed conc. (g S/L)**	**Temp. (°C)**	**pH**	**LR (g S/L/d)**	**highest RE (%)**	**highest CE (%)**	
FBBR	*T. versutus*	$S_2O_3^{2-}$	~6	30±2	10	18-22	100	27	Paper III
BSB	*T. versutus*	HS^-	N.R.	25	9.5	3.2	N.R.	86	Mu et al., 2021
UBSB	*Thiobacilli spp.*	$S_2O_3^{2-}$	6.8	30	5 5.5	N.R.	98	60	Velasco et al., 2004
SRSB	*Thiobacilli spp.*	$S_2O_3^{2-}$	6.4	30	5 5.5	8.7	100	77	González-Sánchez et al., 2008
BTF	*alkaliphilic SOB*	$S_2O_3^{2-}$	12.2	25	10	3.32	100	N.R.	Baquerizo et al., 2013
FBBR	tannery sludge	HS^-	~0.1	30±2	5.5 6.5	N.R.	92	75	Midha et al., 2012
FBBR	distillery sludge	HS^-	0.1-0.5	25-30	7.8	1.6	90	76	Annachhatre and Suktrakoolvait, 2001
RFLR	*Thiobacillus denitrificans*	HS^-	0.24	N.R.	8	7.5-30	90	65	Krishnakumar et al., 2005
AR	domestic WWTP sludge	HS^-	~0.3-0.5	ambient	7.8	4	93	90	Lohwacharin and Annachhatre, 2010
EBR	*Thiobacillus*-like bacteria	HS^-	0.24	22±2	7.2-7.6	~7	100	70	Janssen et al., 1997
UBRFF	SOB	S^{2-}	0.15	20	8.5	5 - ~29	95	90	Buisman et al., 1990

Table 11. p.2/2 Continued.

Experimental conditions						Performance*			Reference
Exp. design	Inoculum	Substrate	Feed conc. (g S/L)	Temp. (°C)	pH	LR (g S/L/d)	highest RE (%)	highest CE (%)	
EGSB	anaerobic sludge	HS^-	0.1	25	8 - 9	0.4	98	84	Sposob et al., 2017
MBfR	activated sludge	HS^-	0.52-0.81	32±3	7- 9	21-66*	99	89	Sahinkaya et al., 2011

FBBR: fluidized bed bioreactor, BSB: bioreactor with suspended biomass, UBSB: upflow bioreactor with suspended biomass, SRSB: supernatant-recycling settler bioreactor, BTF: biotrickling filter system, RFLR: reverse fluidised loop reactor, AR: airlift reactor, EBR: expanded bed reactor, MBfR: membrane biofilm reactor, WWTP: wastewater treatment plant, UBRFF: upflow bioreactor with fixed film, EGSB: expanded granular sludge reactor, N.R.: not reported, * in $g/m^2/d$. The colours of the different pH levels are the universally used colours for the indication of pH. The highest recovery efficiency (RE) and highest conversion efficiency (CE) are shown with blue and orange colours, respectively.

8.2.2 Limitations of the bioprocess

The concentration of DO was not possible to adjust in the laboratory-scale experimental system and therefore, it was either in excess or limited. Adjusting the DO concentration in large-scale bioreactors can be simply implemented by using DO sensors and online monitoring. However, when the aim is to maintain the DO at very low concentrations (often below the detection level of the DO probes/sensors), the oxygen/air supply can be adjusted based on the oxidation-reduction potential (ORP) (Janssen et al., 1995; Peh et al., 2022). Also, due the cloggage of the glass sinter in the recirculation unit of the FBBR, the aeration efficiency decreased, resulting insufficient O_2 supply at high thiosulphate LRs and hence, decrease of the BR. Several studies (González-Sánchez et al., 2008; Janssen et al., 1995; Lohwacharin and Annachhatre, 2010; Velasco et al., 2004) reported optimal DO concentration of <0.1 mg/L to favour S^0 formation instead of SO_4^{2-} when sulphide was the substrate. In case of using HS-, the molar ratio of O_2:HS^- resulting S^0 as major product would be 0.6-1.0 (Janssen et al., 1995). Therefore, maintaining the DO concentration low is crucial to have efficient S^0 recovery.

During the continuous bioreactor operation, most of the S^0 formed left the FBR with the effluent. This was partially due to the settler design and partially due to the physical properties of the biosulphur formed. The continuous turbulence and vertical up-flow in the gravity settler resulted decrease of the sulphur particles settling, and disruption of the S^0 aggregates. Although the difficulties with the gravity settler, it was possible to enhance the S^0 settling by increasing the LR to 24 g S/L/d. Similar improvement of S^0 settling was reported in other studies (Janssen et al., 1997; Velasco et al., 2004). Therefore, the optimization of the settler design and adjustment of process parameters (e.g., LR, DO concentration) are highly important to achieve efficient sulphur recovery and settling. Moreover, biosulphur is colloidal as it has negative charge (Janssen et al., 1999). It was reported by Janssen et al. (Janssen et al., 1999) that this negative charge even increased by the increase of pH and salinity, resulting decrease of settling efficiency. Therefore, separation by some additional mean is oftentimes required. To overcome the settling difficulty of S^0 in our FBBR system, centrifugation and coagulation with different chemicals were implemented. After testing several centrifugation speeds and durations, the highest separation achieved (based on removal of turbidity) was 93% at 3417 real centrifugation force

(rcf) in 5 min. As this speed may be costly, increasing of duration at a lower speed (214 rcf) was also implemented. The highest separation efficiency at 214 rcf was only 71% in 15 min. S^0 separation by centrifugation is a robust technique and it has been commercially applied as part of the THIOPAQ™ process (Driessen et al., 2011). Separation by coagulation was less efficient than centrifugation. From the tested coagulants (aluminium sulphate, iron sulphate, ferrous chloride, ferric chloride), the highest separation efficiency was 40% with 0.5 g/L ferrous chloride. Although, ferrous chloride was not separating S^0 efficiently, it enhanced thiosulphate biotransformation and was not inhibitory for *T. versutus*. However, it is important to mention that ferrous ion rapidly oxidizes to ferric ion in alkaline environment, resulting formation of ferric precipitates that can serve for biomass attainment (Zou et al., 2016). Using some alternative coagulant, such as organic flocculant, polyaluminium chloride (PAC) or polyacrylamide (PAM) may be potential to enhance S^0 settling (Chen et al., 2016). For instance, Chen et al. has reported enhancement of S^0 separation (90% efficiency) with 0.27±0.02 polyaluminium chloride (PAC) at pH 6. However, it is important to mention that the efficiency of PAC may reduce at high pH and Na^+ concentration as S^0 becomes more colloidal/ negatively charged. Moreover, it is also possible to enhance S^0 settling by addition of organic extracellular substance (EPS) that destroys the colloidal state of S^0 (Feng et al., 2018).

In summary, high thiosulphate biotransformation could be achieved in our FBBR system, however the CE to S^0 stayed low. Therefore, further improvements such as better adjustment of DO concentration, changing the experimental design to decrease turbulence and vertical upflow, and applying further method to separate S^0 is still needed.

8.3 Effects of organic compounds on thiosulphate biotransformation

As many of the sulphurous industrial process streams and wastewaters, especially of wood-based industries, are rich in organic compounds, they might be inhibitory for chemolithoautotrophic SOB. The sensitivity of acidophilic chemolithoautotrophic

bacteria towards organics has been known (Fang and Zhou, 2006; Gu and Wong, 2004; Määttä et al., 2022; Vardanyan and Vyrides, 2019) whereas for haloalkaliphiles, it has not been comprehensively studied (de Graaff et al., 2012; Van Den Bosch et al., 2007). Therefore, the effects of different organic compounds (methanol, acetate, D-(+)-xylose, phenol, benzene) that can be present in industrial streams, such as of P&P industry, and a complex organic compound (yeast extract), on thiosulphate biotransformation and biomass growth were investigated in batch assays (Paper IV). Table 12 shows toxicity of organic compounds on chemolithoautotrophs found in the literature and this study.

The results showed low sensitivity of *T. versutus* towards acetate, xylose, methanol and yeast extract while lower concentrations of phenol (0.25 g/L) and benzene (1 g/L) already inhibited biotransformation and growth. The highest studied concentrations (20 g/L) of acetate and xylose were not inhibitory for thiosulphate biotransformation, while it was already limiting with methanol (90% reduction) and yeast extract (87%). Aston et al. (Aston et al., 2009) reported inhibition of biotransformation by chemolithoautotroph *Acidothiobacillus caldus* at pH 3 at 0.12 g/L acetate (Table 12), and therefore suggesting that tolerance of acetate is pH related. Xylose ≥5 g/L, 10 g/L yeast extract and 0.25 g/L phenol decreased the efficiency of thiosulphate biotransformation. Yeast extract (2.5-5 g/L) was the only studied organic compound that stimulated thiosulphate biotransformation and growth (OD_{600}). The stimulatory effect of yeast extract on biomass growth has been widely studied (Labrenz et al., 2013; Sorokin et al., 2001b; van Hille et al., 2009), and for instance, Sorokin et al. (Sorokin et al., 2002) has reported for the genus *Thioalkalivibrio*. S^0 was formed at all non-inhibitory concentrations of the studied organic compounds, however the S^0 production yield was stimulated only by 1, 2.5 and 10 g/L methanol, 0.1-2.5 g/L acetate and 20 g/L xylose.

Table 12. Toxicity of organic compounds on chemolithoautotrophs reported by different studies. Modified from Paper III.

Compound	Microorganism	pH	Exp. design	Lowest inhibitory conc. (g/L)	References
Glucose	*At.ferrooxidans*	2.1	SF	12.6	Frattini et al., 2000
	At. ferrooxidans	3	SF	1	Marchand et al., 2010
Xylose	*T. versutus*	10	SF	N.D.	Paper IV
Citric acid	*At. ferrooxidans*	2.1	SF	9.6	Frattini et al., 2000
Galacturonic acid	*At. ferrooxidans*	2.1	SF	8.5	Frattini et al., 2000
Acetic acid	*At. ferrooxidans*	3	SF	0.375	Fang and Zhou, 2006
Acetate	*At. caldus*	3	SF	0.12	Aston et al., 2009
	T.versutus	10	SF	N.D.	Paper IV
Oxaloacetate	*At. caldus*	3	SF	0.033	Aston et al., 2009
Butyric acid	*At. ferrooxidans*	3	SF	0.275	Fang and Zhou, 2006
Propionic acid	*At. ferrooxidans*	3	SF	0.308	Fang and Zhou, 2006
Formic acid	*At. ferrooxidans*	3	SF	0.077	Fang and Zhou, 2006
Formic acid	*At. thiooxidans*	3	SF	0.077	Fang and Zhou, 2006
Methanol	*T. versutus*	10	SF	20	Paper IV
Methanethiol	*Thioalkalivibrio*	9±2	CGC	0.031	Bosch et al., 2009
2-ketoglutarate	*At. caldus*	3	SF	0.73	Aston et al., 2009
Cellobiose	*At. ferrooxidans*	2.1	SF	2.6	Frattini et al., 2000
Phenol	*T. versutus*	10	SF	0.25	Paper IV
Benzene	*T. versutus*	10	SF	1	Paper IV
Yeast extract	*T. versutus*	10	SF	20	Paper IV

At.: *Acidothiobacillus*, *T.*: *Thioalkalivibrio*, SF: shake flask, CGC: chemostated glass chamber. The colours of the different pH levels are the universally used colours for the indication of pH.

Organic acids become inhibitory for microorganisms once they diffuse through the cytoplasmic membrane, where they acidifies the cytosol (Cobley and Cox, 1983; Guan and Liu, 2020; Padan et al., 2005; Pronk et al., 1991). Whether an acid diffuses depends on its pK_a constant, protonation, and the pH of the surrounding environment. When the pK_a constant increases (acid gets weaker), the dissociation in a solution decreases. Also, when pK_a of the acid increases above the pH of the solution, it becomes more protonated and therefore, it can easier enter the cytoplasmic membrane. As the pK_a constants of xylose, methanol and benzene were above pH 10, these compounds were protonated in the solution and therefore, they

caused inhibition at certain concentrations. As methanol was tolerated up to 10 g/L, the protonation was not an explaining factor for its non-toxicity. The pK_a of benzene (43) is way above of xylose (12) and methanol (16), meaning that it was more protonated than the other two organic acids. Although the pK_a constant of phenol (10) was the same as the pH of the solution (pH 10), it has been known to be highly toxic for microorganism (Lin et al., 2018), thus already at low concentrations can cause inhibition. However, the concentrations of phenol and benzene in P&P wastewaters (Meyer and Edwards, 2014), would possibly be well below the limits for *T. versutus* at pH 10. Acetate (pKa~5) was present in anionic form and therefore, did not enter the negatively charged cell membrane. Although yeast extract (pKa~5) was also dissociated in the solution, in high concentration (20 g/L) it may entered the cell membrane (Guan and Liu, 2020), and therefore limited the thiosulphate biotransformation.

8.4 Activity of SOB with P&P wastewaters

Two different P&P mill wastewaters (PFB and WW) were used to study the applicability of thiosulphate biotransformation and elemental sulphur production in organic-rich real wastewaters (Paper IV). Similar parameters were followed with these wastewaters as during the experiments with the organic compounds.

None of the 87% concentrated wastewaters were inhibitory for thiosulphate biotransformation. The biotransformation progressed similarly with WW than in the positive controls, while the rate with PFB was reduced approximately by -36%. Based on calculations, the S^0 formation was enhanced by WW but was reduced by PFB. The DOC of PFB was almost triple than of WW, which could be a reason for its more negative impact on thiosulphate biotransformation. Also, the pH of PFB decreased from pH 10 to 8.4, some of the constituents present in the wastewater became more dissociated, resulting increase of their diffusion through the cell membrane. The initial pH of PFB was close to the optimal for *T. versutus* and therefore, would not increase the operational costs related to chemical addition for pH adjustment, especially in continuous operation.

In summary, *T. versutus* has shown high tolerance towards organic compounds that can be present in industrial wastewaters, such as of P&P industry at pH 10.

Moreover, it tolerated the primary filtrate of bleaching (PFB) and composite wastewaters (WW) of selected P&P mill. Therefore, thiosulphate biotransformation by *T. versutus* is highly applicable with alkaline/haloalkaline organic-rich wastewaters when the pH is maintained at ~pH 10. Also, application of two-step process (Paques, 2022b) (similar as SULFATEQ®), where first SO_4^{2-} would be reduced and COD oxidized by SRB, followed by oxidation of HS^- and $S_2O_3^{2-}$ to S^0 by SOB, would be a potent application.

9 CONCLUSIONS

This work demonstrates efficient thiosulphate biotransformation from synthetic solutions and P&P wastewaters under aerobic conditions. In Paper I and II, it is demonstrated that under aerobic conditions, both *T. versutus* and *T. denitrificans* biotransform thiosulphate into S^0 and SO_4^{2-} at high initial substrate concentrations (17.6 and 19.6 g $S_2O_3^{2-}$-S/L, respectively). Based on calculations, the highest estimated S^0 production under aerobic conditions would be >40% by both SOB. However, *T. versutus* outperforms *T. denitrificans* in batch assays. The highest biotransformation rate (q_m=0.083 h^{-1}) by *T. versutus* is over triple the rate by *T. denitrificans* (q_m=0.024 h^{-1}). Also, at initial $S_2O_3^{2-}$-S/L the S^0 recovery by *T. versutus* (29%) is almost triple of the value by *T. denitrificans* (10%). Moreover, the highest specific growth rates of both species are similar (~0.047 h^{-1}), meaning that the cells of *T. versutus* more efficiently biotransforms thiosulphate than of *T. denitrificans*.

Under anoxic conditions (Paper II) with NO_2^- as electron acceptor, the $S_2O_3^{2-}$ biotransformation by *T. denitrificans* is slower than under aerobic conditions. Also, the growth rate is reduced (μ_m= 0.022 h^{-1}) compared to the rate under aerobic conditions.

In the FBBR (Paper III), high-rate thiosulphate biotransformation (up to 22 g $S_2O_3^{2-}$-S/L/d, 88% RE) by *T. versutus* can be achieved. This thiosulphate biotransformation rate in the FBBR was eleven times higher than in the batch bioassays (2 g/L/d). At LR of 18 g/L/d, 99.9% of the thiosulphate is biotransformed, but thereafter, the RE efficiency decreases possibly due to limitation of DO concentration. The maximum yield (27±2) of S^0 formation by the biomass (mainly present as biofilm) remains low. Besides supplying adequate concentration of DO, the design of the gravity settler also limits the efficiency of the bioprocess. The turbulence and vertical upflow in the settler disturb S^0 settling and therefore, resulting loss of S^0 through the effluent. The S^0 settling can be enhanced by 90% and 40% by using centrifugation and coagulation (using 0.5 g/L $FeCl_2$), respectively. Besides being more efficient, centrifugation does not require chemical addition and post separation of elemental sulphur as coagulation does. Although the FBBR

system was non-aseptic, the operational conditions (pH, salinity, high LR) were selective enough to maintain the pure culture amended system.

In Paper IV, high tolerance of *T. versutus* towards wastewaters of P&P mill (primary filtrate of bleaching, composite wastewater) and their constituents is demonstrated. Acetate up to 20 g/L and 87% concentrated PFB have no effect on thiosulphate biotransformation. Both thiosulphate biotransformation and biomass growth of *T. versutus* can be enhanced by addition of yeast extract (2.5-5 g/L). Although, xylose up to 20 g/L is not inhibitory but > 5g/L, it reduces the rate of biotransformation. Phenol (0.25 g/L), benzene (1 g/L), methanol (20 g/L) and yeast extract (20 g/L) causes 88, 94, 90 and 87% inhibition of thiosulphate biotransformation, respectively. Although the inhibitory concentrations of phenol and benzene are lower than of the other studied organic constituents, these remain below the concentrations present in e.g., P&P wastewaters.

Overall, the high-rate biotransformation in both bioassays and continuous FBBR operation, and modelling results show potent bioprocess application of *T. versutus* with haloalkaline sulphurous streams. The S^0 recovery efficiency by *T. versutus* can be enhanced by controlling the DO concentration and improving the settler design. Maintaining selective conditions (haloalkaline, high S load) possibly prevents competitive heterotrophic growth and biodegradation, and therefore, enables cost saving due to no need for sterilization. Using *T. versutus* to biologically recover S^0 from organic-rich sulphurous process streams and wastewaters has great future potential. Bioprocess application of *T. denitrificans* might be potent but only under microaerophilic condition.

10 RECOMMENDATIONS TO FURTHER RESEARCH

This thesis provides the proof-of-concepts for biological sulphur recovery by chemolithoautotrophic SOB from haloalkaline sulphurous streams. The outcomes of the conducted studies provide basic knowledge about thiosulphate biotransformation under haloalkaline condition by haloalkaliphilic SOB *T. versutus* and *T. denitrificans*. Further research to fulfil the knowledge gaps and enhance S^0 recovery should be implemented.

High thiosulphate biotransformation rates were achieved at high loading rates in the FBBR, but the S^0 recovery yield remained below 28%. Therefore, the FBBR should be optimized to enhance S^0 recovery. The supply of air/oxygen should be improved to maintain low oxygen partial pressure that is favoured for S^0 formation. Also, due to the limited oxygen supply to some parts of the FBBR system, and the settler design and shape, anaerobic zone could develop in the settler. Although, oxygen is primarily needed in the FBBR unit, as the thiosulphate biotransformation mainly takes place within the fluidized bed, it is important to improve the dissolution of oxygen throughout the system to prevent anaerobic processes. Once the oxygen supply is optimized, the oxygen limitation of S^0 formation should be studied by stepwise changing the DO concentration at constant substrate loading rate. Moreover, to improve S^0 recovery, further oxidation of elemental sulphur to sulphate should be prevented by providing sufficient amount of substrate (e.g., H_2S or $S_2O_3^{2-}$) and maintaining low oxygen partial pressure. Also, the produced S^0 sulphur should be promptly removed from the bioreactor to prevent its further oxidation.

In this thesis, the thiosulphate biotransformation by *T. versutus* was studied at ~pH 10 and 30°C that was recommended by DSMZ. Sorokin et al. (Sorokin et al., 2001b) reported that the genus *Thioalkalivibrio* can tolerate pH 7.5 to 10.65 and has optimum ~pH 10. Growing biomass as biofilm gives certain protection against unfavourable pH conditions compared to cell suspension (Kubota et al., 2009; Wang et al., 2016) and hence, the thiosulphate biotransformation should be investigated in the FBBR at pH below and above 10. However, it is important to keep in mind that lowering the pH can result higher risk of contamination especially when using

organic-rich wastewaters. The risk of contamination would be lower in saline industrial streams. As *T. versutus* tolerates high salinity (up to 4 M Na^+) (Banciu et al., 2004b), the Na^+ concentration could be kept high to prevent contamination at lower pH levels. Therefore, special attention should be paid on minimizing the risk of contamination when selecting the operational pH and salinity. Moreover, Makzum et al. (Makzum et al., 2016) reported increased biotransformation and biomass growth when increasing the temperature to 35 and 37°C. Also, the temperatures of different streams and wastewaters of process industries are oftentimes high, and therefore they require cooling prior their treatment. Therefore, studying the S^0 recovery from industrial process streams and wastewaters by *T. versutus* at 37°C should be implemented. Minimizing pH and temperature adjustments of industrial streams would enable cost-savings.

Settling of S^0 particles was challenging in the FBBR system and hence, future research should focus on the improvement of S^0 separation. For example, the gravity settler design could be modified to reduce turbulence and upflow velocity. Supplementation with some structure, e.g., lamella, could potentially enhance sulphur agglomeration. Moreover, as centrifugation is an efficient method to separate S^0 from the liquid, evaluation of its economic feasibility as an addition to gravity settling should be done. Although, separation of S^0 by coagulation, e.g., with $FeCl_2$, was less efficient than centrifugation, the conditions could be optimized to enhance sulphur settling when applying it with the effluent. Thereafter, its economic feasibility should be also evaluated.

Based on the results of protein analysis, the concentration of active biomass in the FBBR system could be further increased. Although, activated carbon has been widely used as biomass support material, under haloalkaline condition with haloalkaliphilic SOB, it may not be the most suitable material for biofilm formation. Therefore, the cell attachment of *T. versutus* on other support materials with large surface area should be investigated. Once the suitable support material is determined, carrier amended continuous FBBR operation should be implemented. Moreover, yeast extract (2.5-5 g/L) could be added to the feed to enhance biomass growth and thiosulphate biotransformation. In addition, the thiosulphate biotransformation in alternative bioreactor designs could be also studied. For instance, hybrid biofilm/suspended growth system could be a potent design. In this bioreactor, the quantity of active biomass can be enhanced as it grows both as immobile and mobile biofilm. The immobile biofilm is on surfaces (ribbons, plastic screens, etc.) that are

e.g., fixed to the wall of the aeration basin, whereas the mobile biofilm is on light-weight carriers (plastic mesh, pellets, sponges, etc.) that travel throughout the bioreactor with the liquid flow (Rittmann and McCarty, 2020).

In this thesis, thiosulphate was used as model compound due to its lower toxicity for microorganisms and less safety concerns of its use compared to sulphide. However, the haloalkaline sulphurous streams e.g., of P&P industry, is rather rich in sulphide. Once the bioreactor is optimized, the limitation of sulphide biotransformation in the FBBR should be also studied. Thereafter, the S^0 recovery potential by *T. versutus* with real sulphide containing industrial process streams and wastewaters, such as of P&P industry, should be implemented in continuous bioprocess. At high (toxic) HS^- concentrations, the liquid streams could be diluted with organic-rich wastewaters, such as of primary filtrate of bleaching and composite wastewater of P&P mills. Moreover, studying the sequential reduction and oxidation processes for S^0 recovery from SO_4^{2-} containing wastewaters should be also studied with both batch bioassays and continuous bioreactor operation.

Although the efficiency of thiosulphate biotransformation by *T. denitrificans* remained below of *T. versutus*, its real S^0 recovery potential under low O_2 partial pressure should be studied. As it is challenging to maintain low O_2 partial pressure in shake flasks and batch bottles, continuous-flow bioreactor experiment should be implemented. For example, studying the S^0 formation at low O_2 partial pressures could be done in the FBBR that was used with *T. versutus* (Paper III). However, it is important to consider that in case of bioreactor operation with gravity settling, prevention of anaerobic zone within the settler is even more challenging at low O_2 partial pressures.

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PUBLICATIONS

Publication I Hajdu-Rahkama, R., Özkaya, B., Lakaniemi, A. M., & Puhakka, J. A. Kinetics and modelling of thiosulphate biotransformations by haloalkaliphilic *Thioalkalivibrio versutus*. Chemical Engineering Journal (2020), *401*, 126047.

Publication II Hajdu-Rahkama, R., Özkaya, B., Lakaniemi, A. M., & Puhakka, J. A. Potential of biological sulphur recovery from thiosulphate by haloalkaliphilic *Thioalkalivibrio denitrificans*. Environmental Technology (2021) 1-13.

Publication III D'Aquino, A., Hajdu-Rahkama, R., & Puhakka, J. A. Elemental sulphur production from thiosulphate under haloalkaline conditions in a *Thioalkalivibrio versutus* amended fluidized bed bioreactor. Biochemical Engineering Journal, 172 (2021) 108062.

Publication IV Hajdu-Rahkama, R., Puhakka, J.A. High tolerance of chemolithoautotrophic sulphur oxidizing bacteria towards pulp and paper mill wastewaters and their organic constituents supporting sulphur recovery in alkaline conditions. Chemical Engineering Journal (2022) 137972.

PUBLICATION I

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

Hajdu-Rahkama, R., Özkaya, B., Lakaniemi, A. M., & Puhakka, J. A.

Chemical Engineering Journal, *401*, 126047.
DOI: 10.1016/j.cej.2020.126047

Chemical Engineering Journal 401 (2020) 126047

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

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

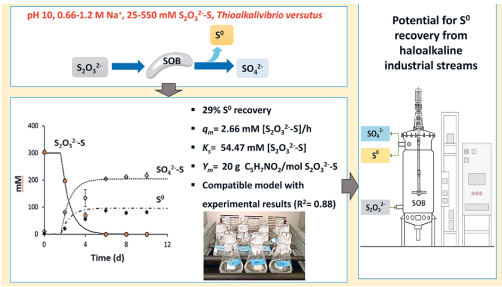
Réka Hajdu-Rahkama[a,*], Bestamin Özkaya[a,b], Aino-Maija Lakaniemi[a], Jaakko A. Puhakka[a]

[a] Tampere University, Faculty of Engineering and Natural Sciences, P.O. Box 541, FI-33104 Tampere University, Finland
[b] Yildiz Technical University, Department of Environmental Engineering, Davutpasa, Istanbul, Turkey

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.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Thioalkalivibrio versutus
Haloalkaliphilic sulfur oxidizing bacteria
Thiosulfate biotransformation
Sulfur disproportionation
Resource recovery
Kinetics

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 was produced with up to 29% efficiency. 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} (0.47 mM $C_5H_7NO_2$ h^{-1}) and the maximum growth yield 0.18 mM $C_5H_7NO_2$/mM $S_2O_3^{2-}$-S (20 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.

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

* Corresponding author.
E-mail address: reka.hajdu-rahkama@tuni.fi (R. Hajdu-Rahkama).

https://doi.org/10.1016/j.cej.2020.126047
Received 14 December 2019; Received in revised form 2 June 2020; Accepted 22 June 2020
Available online 27 June 2020
1385-8947/

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 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_{2max}) 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 0.5 mM $MgCl_2$. 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 3 × 2 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. [22]. The ion-chromatography was equipped with Dionex IonPac AS22 anion exchange column (Thermo Scientific). 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 m (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. [23]. For the estimation of the biomass concentration, the average 16S Gammaproteobacterial qPCR gene copy number (5.8) was used [24].

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 [$S_2O_3^{2-}$-S] h^{-1} from the batch assays conducted with varying initial thiosulphate-S 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[$S_2O_3^{2-}$-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 [$S_2O_3^{2-}$-S] to [SO_4^{2-}-S]) and f_2: (fraction of [$S_2O_3^{2-}$-S] to [S^0]) using (d[$S_2O_3^{2-}$-S]/dt) modelling data and measured [SO_4^{2-}-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 [$S_2O_3^{2-}$-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 [25]:

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

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 [26,27].

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:

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

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:

$$\mu_m = q_m \bullet Y \quad (3)$$

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) and (14). The μ_m is considered from growth kinetic as (h^{-1}), 1 cell = $6.25{\cdot}10^{-10}$ g [28], 113 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 [29]. First, substrate consumption was modelled as shown in the following equation:

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

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

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

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

and

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

In the current study, kinetic constants (q_m and K_s) shown in Eq. (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 Eq. (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 Eq. (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-S 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 $S_2O_3^{2-}$ by *T. versutus* follows the Eqs. (7)–(9):

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

$$S_2O_3^{2-} + \tfrac{1}{2}\,O_2 \rightarrow S^0 + SO_4^{2-} \quad (8)$$

$$S^0 + 1\tfrac{1}{2}\,O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+ \quad (9)$$

when *T. versutus* 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-}-S 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:

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

and the elemental sulphur production rate as:

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

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-S 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 Eq. (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:

$$rate\ \ of\ \ increase\ \ of\ \ cells = \mu{\bullet}number\ \ of\ \ cells \quad (12)$$

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) [24]. The dry weight (d.w.) of the cell mass was estimated based on the weight of one cell equalling to $6.25{\cdot}10^{-10}$ g [28]. Finally, the d.w. was converted to mM by using the simplified molecular cell formula of $C_5H_7NO_2$ [30].

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 [25] 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 results of $S_2O_3^{2-}$-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-S 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-S concentration of 25 mM. At 50 mM and higher thiosulphate-S concentrations, elemental sulphur production increased with the increasing initial thiosulphate concentration. Makzum et al. [6] also reported increase of elemental

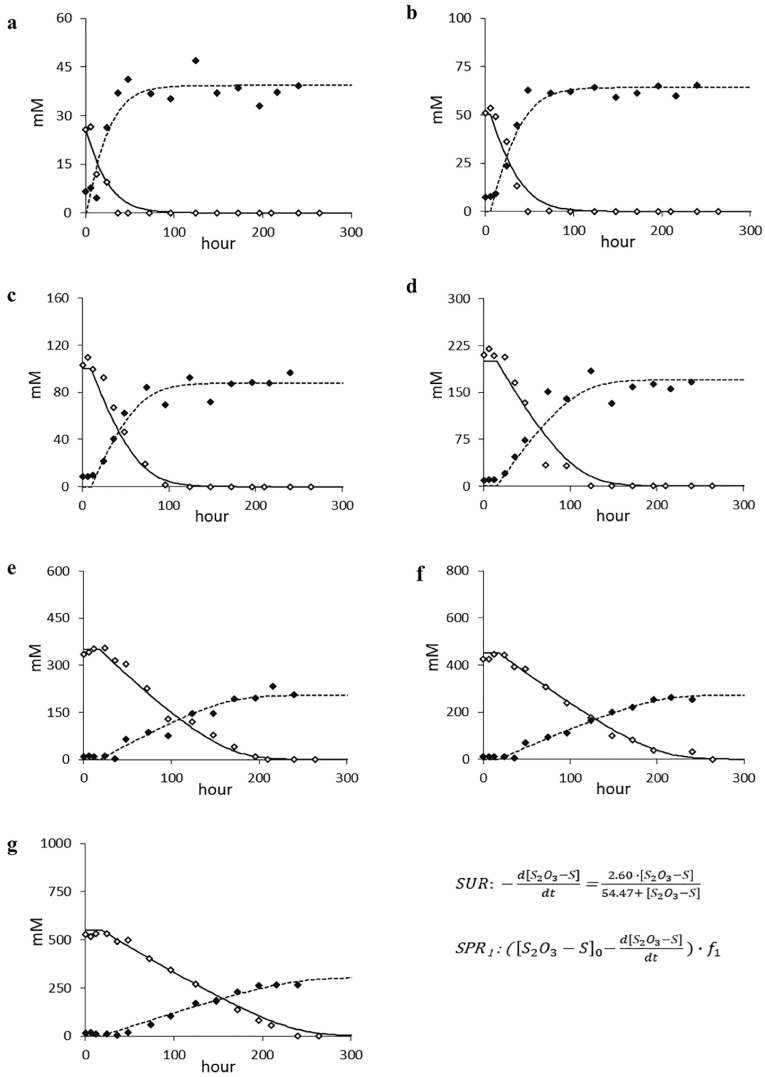


Fig. 1. Biotransformation kinetics of $S_2O_3^{2-}$-S and production kinetics of SO_4^{2-}-S for *T. versutus* at initial $S_2O_3^{2-}$-S concentrations of (a): 25 mM; (b): 50 mM; (c) 100 mM; (d) 200 mM; (e): 350 mM; (f): 450 mM; (g): 550 mM. (◊): $S_2O_3^{2-}$-S data from batch assays; (♦): SO_4^{2-}-S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

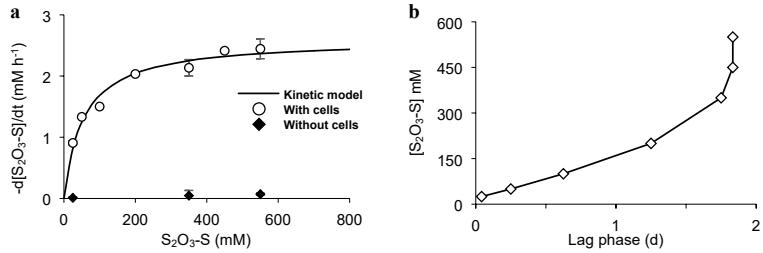


Fig. 2. (a) Monod based thiosulphate utilization kinetics (q_m = 2.66 mM $[S_2O_3^{2-}\text{-S}]$ h^{-1}; K_s = 54.5 mM $[S_2O_3^{2-}\text{-S}]$ for *T. versutus* and (b) lag phases of thiosulphate utilization at different initial thiosulphate-S concentrations.

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.

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 h (1.25 days) to 240 h (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-S concentrations (Fig. 2b). The lag phase varied from approximately 1 h at 25 mM, to 38 h at 300–550 mM $S_2O_3^{2-}$-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 obtained 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 concentration, thus indicating that the oxygen mass transfer did not control the rate of consumption with a possible exception of the highest initial thiosulphate-S 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].

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 [29]. 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.

In the shake flask study by Ang. et al. [7], the highest reported S^0 concentration was 3.5 mM from the initial 80 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.

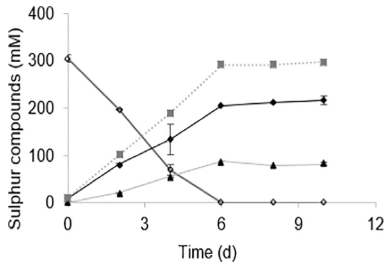


Fig. 3. Thiosulphate disproportionation to elemental sulphur and sulphate. The symbols are (◊): $S_2O_3^{2-}$-S, (▲) S^0, (♦): SO_4^{2-}-S and (■) S^0 + SO_4^{2-}-S.

3.3. Modeling of the experimental data

Fig. 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. Fig. 4 (b) shows elemental sulphur yield (mM [S^0] h^{-1}) as a funtion of the initial thiosulphate-S concentration. The results show that elemental sulphur production rate and the initial thiosulphate-S concentration had a linear correlation.

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-S 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.

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.

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.

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}

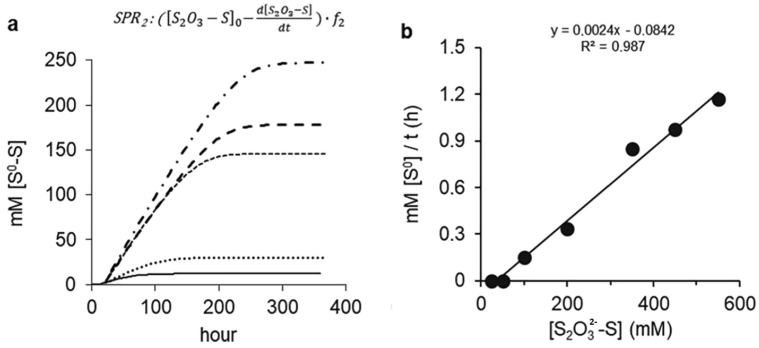


Fig. 4. (a) Elemental sulphur production from thiosulphate as a function of time ; (a) the initial $S_2O_3^{2-}$-S concentration from bottom to top is 100 mM, 200 mM, 350 mM, 450 mM and 550 mM and (b) elemental sulphur yield (mM [S^0] h^{-1}) as function of intial thiosulphate concentration.

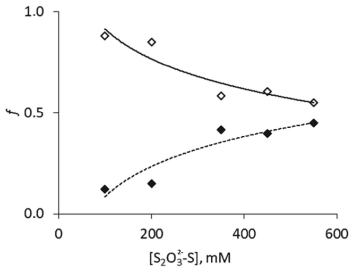


Fig. 5. Calculated fractions of $S_2O_3^{2-}$-S to SO_4^{2-}-S (f_1) and to S^0 (f_2) ratio during biotransformation in batch reactors depending on substrate concentration. These fractions were calculated from experimental and substrate biotransformation kinetics model data (for a detail see Eq. (7) 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.

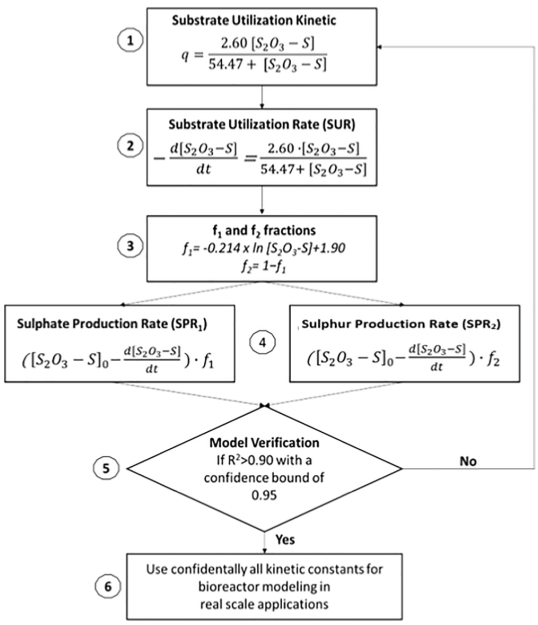


Fig. 6. The pathway for bioreactor modelling including substrate utilization rate and sulphur production rates and equations obtained in this study.

(0.47 mM $C_5H_7NO_2$ h^{-1}), corresponding a maximum yield of 0.18 mM $C_5H_7NO_2$/mM $S_2O_3^{2-}$-S (20 g $C_5H_7NO_2$/mol $S_2O_3^{2-}$-S or 35 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.

In this study, the kinetics of sulphate and elemental sulphur production were reported at intial thiosulphate-S 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 [31–34], 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

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}	2.66	Eqs. (1) and (4)
K_s, mM	54.47	Eqs. (1) and . (4)
f_1 from Fig. 5 $f_1 = -0.214xln\,[S_2O_3\text{-}S] + 1.90$	0.68	Eq. (7) for SPR_1
$f_2 = 1\text{-}f_1$	0.32	Eq. (8) for SPR_2

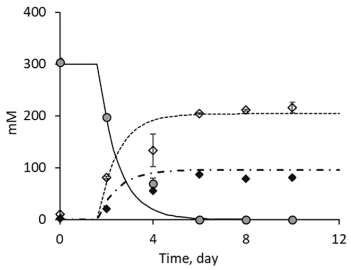


Fig. 7. Model validation for thiosulphate biotransformation to sulphur and sulphate. (Constants in Table 1 were used for calculations (SUR and SPR_1 and SPR_2), orange circle: SUR, transparent diamond: SPR1, black diamond: SPR2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

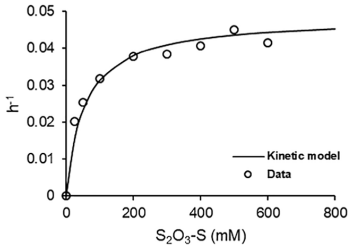


Fig. 8. Monod based growth kinetics μ_m = 0.048 h^{-1}; K_s = 54.47 mM [$S_2O_3^{2-}$-S], Yield (Y) = 0.18 mM cell/mM [$S_2O_3^{2-}$-S] (20 g cell/mol [$S_2O_3^{2-}$-S] or 35 g cell/mol [$S_2O_3^{2-}$] for *T. versutus*). Yield value was calculated by Eq. (13) considering μ_m 0.048 h^{-1}, 1 cell = $6.25{\cdot}10^{-10}$ g, 113 g/mol cell, q_m = 2.66 mM S_2O_3-S/h. From here maximum growth rate was calculated as 0.47 mM cell h^{-1}, which corresponds to Y value of 0.47/2.66 = 0.18 mM cell/mM $S_2O_3^{2-}$-S considering $C_5H_7NO_2$ molecular formula of cell.

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 $S_2O_3^{2-}$-S 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} (0.47 mM $C_5H_7NO_2$ h^{-1}) and 20 g $C_5H_7NO_2$/mol $S_2O_3^{2-}$-S, respectively. Elemental sulphur accumulation was observed at initial concentration of ≥ 50 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

Table 2
Summary of kinetic constants from different thiosulphate biotransformation studies conducted under aerobic conditions.

Experimental conditions								Kinetic parameters				Reference
Microorganism	Experimental system	Temp. (°C)	pH	rpm	Salinity (M Na^+)	Initial $S_2O_3^{2-}$ (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 (0.47 mM cell h^{-1})	0.18 mM $C_5H_7NO_2$/mM $S_2O_3^{2-}$-S (20 g cell/mol $S_2O_3^{2-}$-S or 35 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 $S_2O_3^{2-}$	[35]
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 $S_2O_3^{2-}$	[36]
Thiobacillus neapolitanus strain C	chemostat	30	6.8	750	N.R	10–14	N.D.	N.D.	N.D.	8.6 g cell/mol $S_2O_3^{2-}$	[36]
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 $S_2O_3^{2-}$	[36]

* as $S_2O_3^{2-}$-S.
N.D.: not determined.
N.R.: not reported.

haloalkaline industrial process streams.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Dr. Johanna Rinta-Kanto and Mr. Erkki Halme for their assistance with the qPCR. This study was funded by the Doctoral School of Tampere University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2020.126047.

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Supplementary Material

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

Réka Hajdu-Rahkama[a]*, Bestamin Özkaya[a,b], Aino-Maija Lakaniemi[a], Jaakko A. Puhakka[a]

[a] Tampere University, Faculty of Engineering and Natural Sciences, P.O. Box 541, FI-33104 Tampere University, Finland

[b] Yildiz Technical University, Department of Environmental Engineering, Davutpasa, Istanbul, Turkey

Supplementary information: 4 pages, 1, Figure, 1 Table,

* Corresponding author. Tel.: +358 50432 7061. Email address: reka.hajdu-rahkama@tuni.fi

S1: Calculating the fractions expressing SO_4^{2-}-S (f_1) and S^0 (f_2) production from $S_2O_3^{2-}$S biotransformations was done stepwise as follows:

(i) In the shake flasks with initial concentration of 200 mM [$S_2O_3^{2-}$S], $S_2O_3^{2-}$ and SO_4^{2-} concentrations were measured until thiosulphate was completely removed.

(ii) The thiosulphate was transformed into either sulphate (f_1) or sulphur (f_2). In this step, previously calculated substrate (thiosulphate) removal rates are taken into account.

(iii) For the prediction of the sulphate production, firstly f_1 was defined (the ratio of the substrate converted to SO_4^{2-}-S), which indicates how much of the removed $S_2O_3^{2-}$-S was converted to SO_4^{2-}-S. This was expressed as:

$$\text{Predicted } SO_4^{2-} - \text{S production at time t} = (S_2O_2^{2-} - S_{initial} - S_2O_2^{2-} - S_{at\ time\ t}) \cdot f_1 \quad (1)$$

The f_1 value was considered constant for each initial substrate concentrations, and this fraction was calculated as an objective cell in Microsoft Excel program in the decision to minimize sum of squared error (SSE) between measured and predicted data (see Fig. S1). The calculation of SSE was as follows:

$$SSE = Sum\ [(measured\ SO_4^{2-} - \text{S} - \text{predicted}\ SO_4^{2-} - \text{S})]^2 \quad (2)$$

The method used with Excel program was as shown in Fig. S1. First, the sum of squares (Column-F) of the differences (Column-G) between the measured (Column-D) and the predicted SO_4^{2-} − S (Column-C) concentrations was calculated. Second, the *f1* value (Column-E), which minimizes the sum of the squares of the errors, was calculated using the MS Excel add-in Solver program. During the calculation of the 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 measured values. The model results were expressed with 95% confidence interval.

The f_1 represents the sulphate production fraction for a given initial substrate concentration. Therefore, it is a coefficient expressing the ratio or fraction of how much of the biotransformed substrate (200 mM $S_2O_3^{2-}$-S - Column B) was converted to sulphate for the given initial substrate concentration. Another product formed during thiosulphate biotransformation was elemental sulphur. The sum of f_1 and f_2, expressing the sulphate and sulphur fractions produced

from the substrate, respectively, is 1. Therefore, f_2 value was calculated as 1-f_1. Then, the sulphur production rate was calculated in the next step with the help of this fraction for each initial thiosulphate-S concentration.

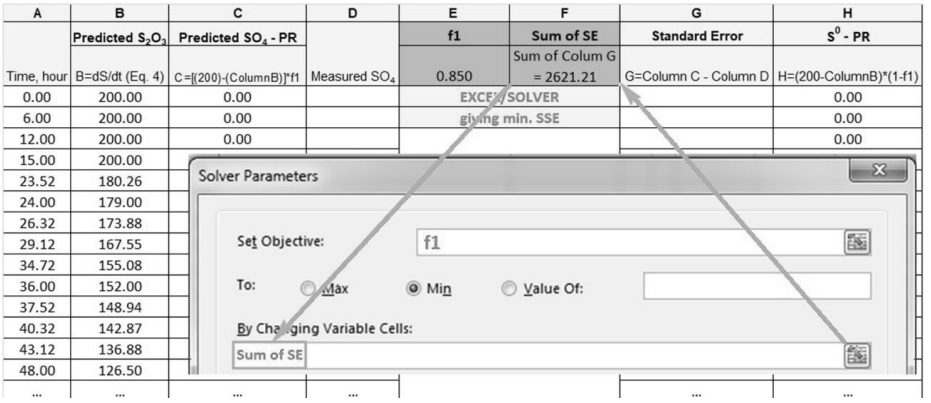


Fig. S1: Calculation of f_1, f_2 and SPRs production using Microsoft Excel Solver

(iv) The fraction of sulpur (f_2) was calculated as 1-f_1.

(v) The sulphur production rate was calculated (Column-H, Fig. S1) by multiplying the biotransformed substrate (200 mM $S_2O_3^{2-}$-S - Column B) with (1-f_1).

(vi) At the end of all experimentations, f_1 and f_2 were verified with independent experimental data.

Table S1 16S rRNA gene copy numbers of *Thioalkalivibrio versutus* at different thiosulphate concentrations (after 10 days of incubation) [1]

Initial S_2O_3-S (mM)	Copy number/ mL	Change (copy number/ml)	Change (%)
inoculum for 25-300	483807		
25	4498731	4014924	830
50	6088311	5604503	1158
100	8489951	8006144	1655
200	9884940	9401133	1943
300	10461970	9978162	2062
inoculum for 450	305399		
450	11252134	10946735	3584

References:

[1] J.M. Rinta-Kanto, H. Sinkko, T. Rajala, W.A. Al-Soud, S.J. SØrensen, M. V. Tamminen, S. Timonen, Natural decay process affects the abundance and community structure of Bacteria and Archaea in Picea abies, FEMS Microbiol. Ecol. 92 (2016) 1–10. doi:10.1093/femsec/fiw087.

PUBLICATION II

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

Hajdu-Rahkama, R., Özkaya, B., Lakaniemi, A. M., & Puhakka, J. A.

Environmental Technology, 1-13.
DOI: 10.1080/09593330.2021.1985620

ENVIRONMENTAL TECHNOLOGY
https://doi.org/10.1080/09593330.2021.1985620

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

Réka Hajdu-Rahkama [a], Bestamin Özkaya [a,b], Aino-Maija Lakaniemi [a] and Jaakko A. Puhakka [a]

[a]Faculty of Engineering and Natural Sciences, Bio- and Circular Economy Research Group, Tampere University, Tampere, Finland; [b]Department of Environmental Engineering, Yildiz Technical University, Istanbul, Turkey

ABSTRACT

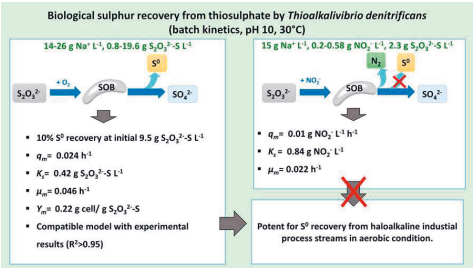
The aim of this study was to investigate the potential for elemental sulphur recovery from sulphurous solutions under aerobic and anoxic conditions by haloalkalophilic *Thioalkalivibrio denitrificans* at 0.8–19.6 g $S_2O_3^{2-}$-S L^{-1} and 0.2–0.58 g NO_2 L^{-1}, respectively. The experiments were 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 $S_2O_3^{2-}$-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 $S_2O_3^{2-}$-S L^{-1} at initial [$S_2O_3^{2-}$-S] of 14 g L^{-1}. S^0 accumulated at [$S_2O_3^{2-}$-S] ≥ 1.5 g L^{-1} (10% yield at initial 9.5 g $S_2O_3^{2-}$-S L^{-1}) and the highest S^0 yield estimated with the model was 61% with initial [$S_2O_3^{2-}$-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 NO_2^- 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 S^0 accumulated under anoxic conditions. Thus, future developments of biotechnical applications for the recovery of S^0 from haloalkaline streams from the process industry should focus on aerobic treatment.

Highlights

- Haloalkaline $S_2O_3^{2-}$ biotransformations kinetics by *Thioalkalivibrio denitrificans*
- Aerobic thiosulphate-S bioconversion up to 0.024 h^{-1} with K_s = 0.42 g $S_2O_3^{2-}$-S L^{-1}
- 10% S^0 yield with initial 9.5 g $S_2O_3^{2-}$-S L^{-1} in aerobic condition
- Anoxic NO_2 removal up to 0.01 h^{-1} with K_s = 0.84 g NO_2^- L^{-1}

ARTICLE HISTORY
Received 26 April 2021
Accepted 20 September 2021

KEYWORDS
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^-, $S_2O_3^{2-}$) 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

CONTACT Réka Hajdu-Rahkama reka.hajdu-rahkama@tuni.fi Faculty of Engineering and Natural Sciences, Bio- and Circular Economy Research Group, Tampere University, P.O. Box 541, FI-33014 Tampere University, Finland

Supplemental data for this article can be accessed https://doi.org/10.1080/09593330.2021.1985620
This article has been corrected with minor changes. These changes do not impact the academic content of the article.

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 steams. 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.

Haloalkalophilic 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^-, $S_2O_3^{2-}$, S_6^{2-}, S_8, SO_3^{2-}, $S_3O_6^{2-}$, $S_4O_6^{2-}$ and $S_5O_6^{2-}$) as a source of energy than for example those in the genus *Thiomicrospira* (HS^-, $S_2O_3^{2-}$, S_6^{2-}, S_8, $S_4O_6^{2-}$ and $S_5O_6^{2-}$) [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 $S_2O_3^{2-}$ 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 ± 1°C and 150 rpm under aerobic condition [24]. The growth medium included: 20 g L^{-1} Na_2CO_3, 10 g L^{-1} $NaHCO_3$, 5 g L^{-1} NaCl, 1 g L^{-1} K_2HPO_4, 0.5 g L^{-1} KNO_3, 0.05 g L^{-1} $MgCl_2$ and 2% (*v/v*) trace element solution (see preparation from [24]). As energy source, 4.5 g L^{-1} sterile filtered (0.2 µm 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 µm 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 the kinetic experiments (Section 2.2.1). Altogether 12 identical cultures were prepared with 9.5 g L^{-1} concentration of $S_2O_3^{2-}$-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 g NO_2^- 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 $S_2O_3^{2-}$ in the serum bottles was 3.9 ± 0.2 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 ($S_2O_3^{2-}$), 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} NO_2^- 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 $S_2O_3^{2-}$-S and NO_2^- utilisation by *T. denitrificans* were described by Monod equation (Equation (1)) [29–31]:

$$q = -\frac{q_m\,[S_2O_3\text{-}S]}{K_s + [S_2O_3\text{-}S]} \quad \text{and} \quad q = -\frac{q_m\,[NO_2]}{K_s + [NO_2]} \tag{1}$$

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 ($d[S_2O_3^{2-}\text{-}S]/dt$) 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'

[a] q_m is the maximum SUR; K_s is the half saturation constant.
[b] K_i is the inhibitory substrate concentration (g L^{-1}).
[c] f_1 is the fraction of $[S_2O_3^{2-}\text{-}S]$ to $[SO_4^{2-}\text{-}S]$; f_2 is the fraction of $[S_2O_3^{2-}\text{-}S]$ to $[S^0]$.
[d] μ 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 (g-S/ (g-VSS h)$^{-1}$ or simply h^{-1}), q_m is the specific maximum substrate ($S_2O_3^{2-}$-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:

$$\frac{d[S_2O_3\text{-}S]}{dt \cdot X} = q = -\frac{q_m \cdot [S_2O_3\text{-}S]}{K_s + [S_2O_3\text{-}S]} \quad (2)$$

and

$$\frac{d[S_2O_3\text{-}S]}{dt} = q \cdot X = -\frac{q_m \cdot [S_2O_3\text{-}S]}{K_s + [S_2O_3\text{-}S]} \cdot X \quad (3)$$

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 $S_2O_3^{2-}$-S utilisation, Haldane model has been also applied to see if there is inhibition by the substrate (Equation (4)) with the aerobic batch cultures:

$$q = -\frac{q_m [S_2O_3\text{-}S]}{K_s + [S_2O_3\text{-}S] + \frac{[S_2O_3\text{-}S]^2}{K_i}} \quad (4)$$

where K_i is the inhibitory concentration of [$S_2O_3^{2-}$-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*:

$$S_2O_3^{2-} + \frac{1}{2}O_2 \rightarrow S^0 + SO_4^{2-} \qquad \Delta G^0 = -231.6 \text{ kJ. (mol S-substrate)}^{-1} \quad (5)$$

$$S_2O_3^{2-} + 2O_2 + H_2O \rightarrow 2SO_4^{2-} + 2H^+ \qquad \Delta G^0 = -738.7 \text{ kJ. (mol S-substrate)}^{-1} \quad (6)$$

$$S^0 + \frac{3}{2}O_2 + H_2O \rightarrow SO_4^{2-} + 2H^+ \qquad \Delta G^0 = -507.4 \text{ kJ. (mol S-substrate)}^{-1} \quad (7)$$

$$S^0 + O_2 + H_2O \rightarrow SO_3^{2-} + 2H^+ \qquad \Delta G^0 = -249.4 \text{ kJ. (mol S-substrate)}^{-1} \quad (8)$$

$$SO_3^{2-} + \frac{1}{2}O_2 \rightarrow SO_4^{2-} \qquad \Delta G^0 = -258.0 \text{ kJ (mol S-substrate)}^{-1} \quad (9)$$

According to Equations (3) and (4), conversion of thiosulphate 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]:

$$3S_2O_3^{2-} + 8NO_2^- + 2H^+ \rightarrow 6SO_4^{2-} + 4N_2 + H_2O \qquad \Delta G^0 = -5515.4 \text{ kJ (mol S-substrate)}^{-1} \quad (10)$$

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:

$$2NO_{2(g)} \rightarrow 2NO_{(g)} + O_{2(g)} \quad (11)$$

$$2NO_{(g)} + N_{2(g)} \rightarrow 2N_2O_{(g)} \quad (12)$$

$$2NO_{(g)} \rightarrow N_{2(g)} + O_{2(g)} \quad (13)$$

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]:

$$\mu = \frac{\mu_m\ [S_2O_3\text{-}S]}{K_s + \ [S_2O_3\text{-}S]} \tag{14}$$

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:

$$\mu_m = q_m \cdot Y \tag{15}$$

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

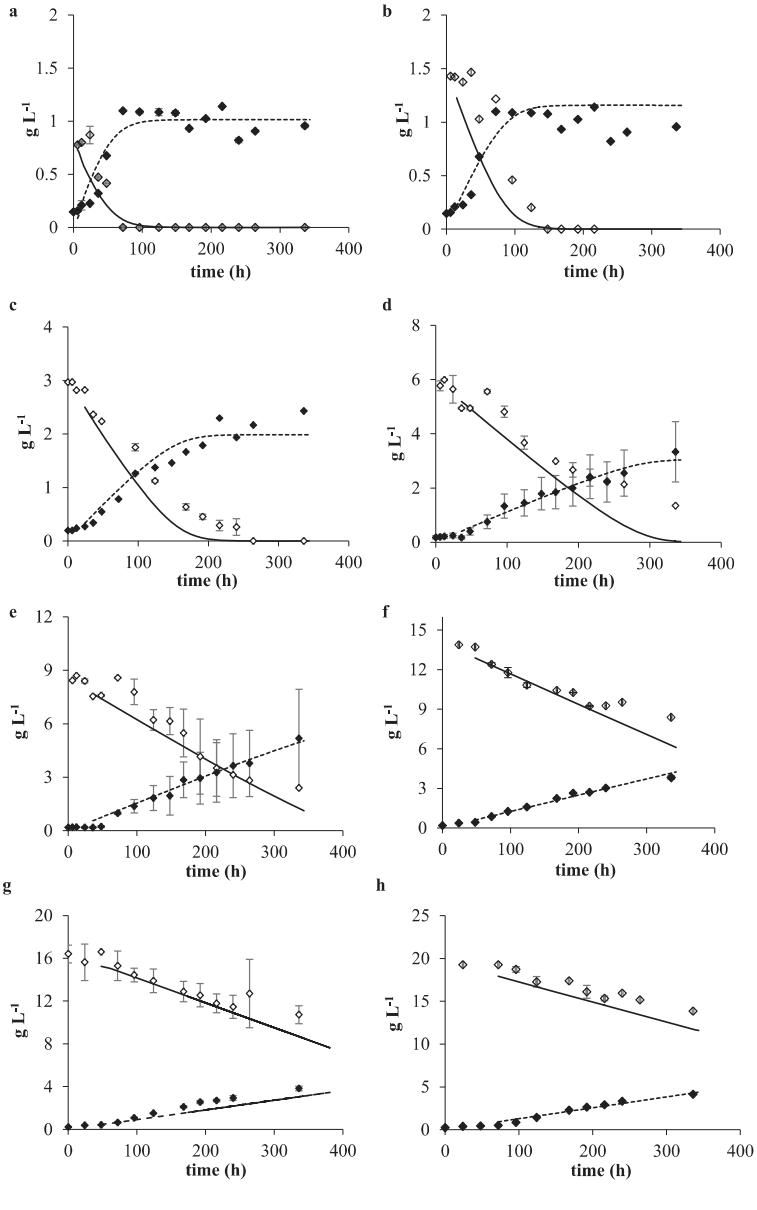
3.1.1. Kinetics of thiosulphate biotransformation

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°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 (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



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

$$SPR_1 = \left([S_2O_3\text{-}S]_0 - \frac{d[S_2O_3\text{-}S]}{dt}\right) \cdot f_1$$

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).

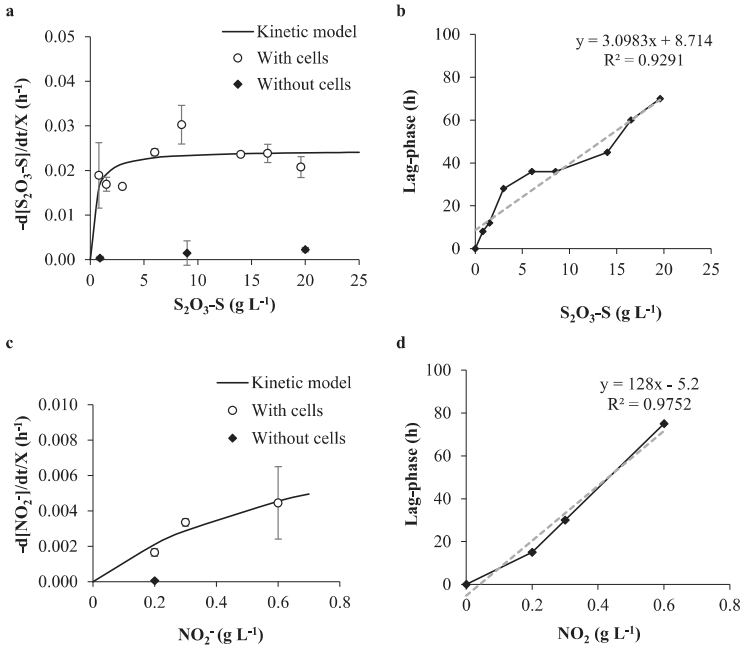

Figure 2. (a) Monod kinetics for aerobic thiosulphate utilisation (q_m = 0.024 h^{-1}; K_s = 0.42 g $S_2O_3^{2-}$-S L^{-1} for *T. denitrificans* and (b) lag phases of $S_2O_3^{2-}$-S removal at different initial concentrations. (c) Monod kinetics for anoxic nitrite removal (q_m=0.01 h^{-1}; K_s = 0.84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

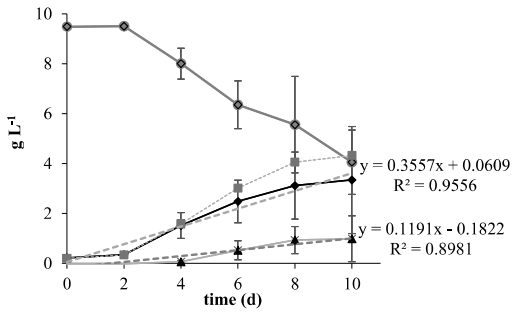

Figure 3. Thiosulphate biotransformation to elemental sulphur and sulphate by *T. denitrificans* under aerobic conditions. The symbols are (◇): $S_2O_3^{2-}$-S, (▲) S^0, (◆): SO_4^{2-}-S and (■) S^0+ SO_4^{2-}-S.

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 g NO_2^- L^{-1} (Figure 2(c)). The measured sulphate formation, especially at initial 0.2 and 0.3 g NO_2^- 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 g NO_2^- 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 g NO_2^- 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 g NO_2^- L^{-1} was 56%. Figure 6 shows that the calculated $S_2O_3^{2-}$-S to SO_4^{2-} conversion was linear.

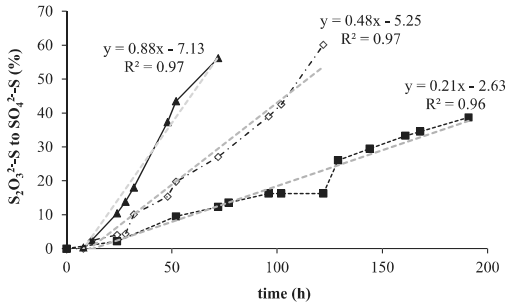

Figure 4. Sulphate formation yield as calculated based on the stoichiometry of thiosulphate biotransformation with nitrite as electron acceptor with *T. denitrificans* (Equation (10)). (▲) with 0.2 g L^{-1}, (◊): with 0.3 g L^{-1} and (■) 0.58 g L^{-1} initial NO_2^- concentrations.

3.3. Estimation of thiosulphate biotransformation under aerobic conditions

The fractions of thiosulphate biotransformed into SO_4^{2-}-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 $S_2O_3^{2-}$-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 g $S_2O_3^{2-}$-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 g S_2O_3-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 g $S_2O_3^{2-}$-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 g $S_2O_3^{2-}$-S L^{-1} of the SUR model. The maximum yield (Y_m) was 0.22 g cells/ g $S_2O_3^{2-}$-S.

3.4.2. Growth under anoxic conditions

The overall biomass growth was 10.2 and 10.7 mg cell h^{-1} at 0.2–0.3 g NO_2^- L^{-1}, respectively. The increase of

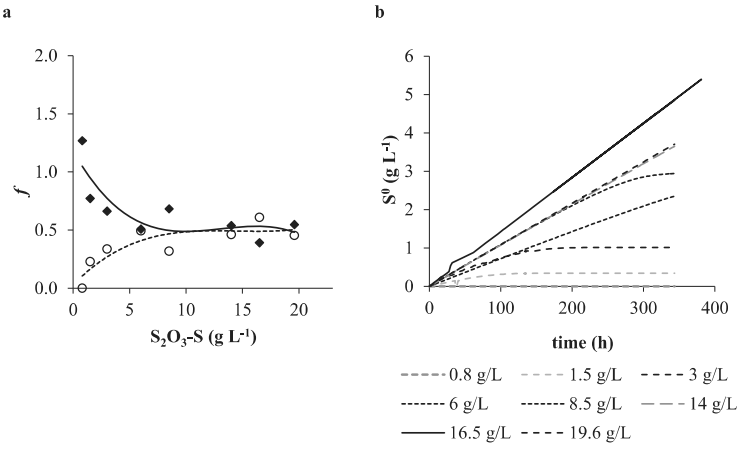

Figure 5. (a) Fractions of biotransformation of [$S_2O_3^{2-}$-S] to [SO_4^{2-}-S] (f_1, black rhombus) and [$S_2O_3^{2-}$-S] to [S^0] (f_2, black empty spheres) with *T. denitricans*. The solid line (—) shows the f_1 and the dashed (– – –) the f_2 based on the kinetic model. (b) modelling of elemental sulphur formation by different initial thiosulphate concentrations (these concentrations are shown below the figure as legends).

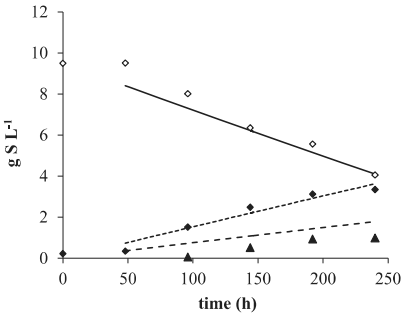

Figure 6. Validation of the SPR modelling with experimental data with initial 9.5 g $S_2O_3^{2-}$-S L^{-1} concentration in batch incubation with *T. denitrificans*. The (◇): $S_2O_3^{2-}$-S; (◆): SO_4^{2-}-S and (▲) S^0 data from batch assays. The solid line (—): $S_2O_3^{2-}$-S biotransformation kinetics model, dashed line (– – –): SO_4^{2-}-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 g NO_2^- L^{-1} was approximately 0.73 g cell L^{-1} (5 times) and 1.31 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 g $S_2O_3^{2-}$-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 g NO_2^- L^{-1} and 2.56 g $S_2O_3^{2-}$-S L^{-1}.

3.5. Limiting factors of aerobic biotransformations

Figure 7(b) shows that the rate of thiosulphate biotransformation did not increase after 0.03 h^{-1} (initial 8.6 g S_2O_3-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 g $S_2O_3^{2-}$ -S L^{-1}) than *T. denitrificans* (0.42 g $S_2O_3^{2-}$ -S L^{-1}), higher q_m (+0.059 h^{-1}) and higher yield (+0.09 g cell/g $S_2O_3^{2-}$S) of sulphur formation.

Table 3 compares the results obtained with initial 2.5 g $S_2O_3^{2-}$ -S L^{-1} in aerobic and anoxic conditions (containing 0.2–0.56 g NO_2^- L^{-1}). With 2.5 g $S_2O_3^{2-}$ -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 g $S_2O_3^{2-}$ -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

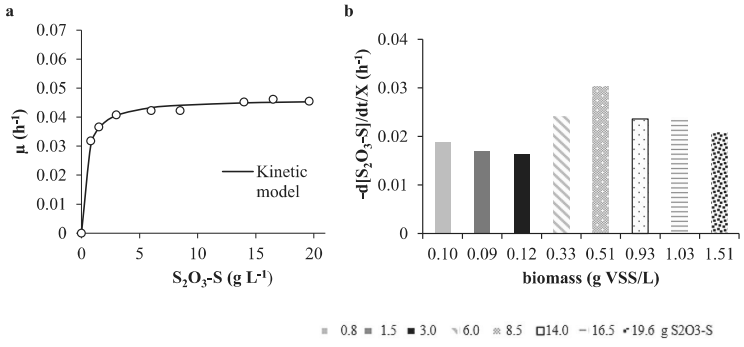

Figure 7. (a) Aerobic growth kinetics of *T. denitrificans*, $\mu_m = 0.046$ h^{-1}; $K_s = 0.42$ g $S_2O_3^{2-}$-S L^{-1}, Yield (Y_m) = 0.22 g cells (g $S_2O_3^{2-}$-S)$^{-1}$; and (b) specific thiosulphate-S utilisation rates and biomass concentrations with mean biomass concentrations.

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

Experimental conditions							Kinetic parameters				Reference
Microorganism	Experimental system	Temp. (°C)	pH	rpm	Salinity (g L^{-1} Na^+)	Initial $S_2O_3^{2-}$-S (g L^{-1})	q_m (h^{-1})	K_s (g L^{-1})	μ_m (h^{-1})	Y (g cell/g $S_2O_3^{2-}$)	
T. denitrificans	batch assays	30	10	150	24	14	0.024	0.42	0.046	0.22*	This study
T. denitrificans	batch assays	30	10	NR	0.6	2.56	N.D.	N.D.	0.028	0.038 g protein/ g $S_2O_3^{2-}$ (4.2 mg protein/ mmol $S_2O_3^{2-}$)	[17]
T. versutus	batch assays	30	10	150	26	17.2	0.083	1.74	0.048	0.31*	[24]
T. versutus	batch assays	30	10	150	N.R.	2.56	0.049**	N.D.	0.082	N.D.	[18]
T. versutus	batch assays	37	10	150	N.R.	2.56	0.064**	N.D.	0.095	N.D.	[18]
T. denitrificans	chemostat (lab-scale fermentor)	30	10	N.R.	N.R.	2.56	N.D.	N.D.	0.038	0.039 g protein/ g $S_2O_3^{2-}$ (4.4 mg protein/ mmol $S_2O_3^{2-}$)	[17]
T. versutus	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 $S_2O_3^{2-}$- 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 S^0 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.

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 $S_2O_3^{2-}$-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 $S_2O_3^{2-}$-S	0.04	0.02

[a]These results were calculated considering the initial 2.5 g $S_2O_3^{2-}$ -S L^{-1} concentration of the anoxic cultures.
[b]With 0.2–0.56 g [NO_2^-] L^{-1}.
[c]Based on theoretical calculation.

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 [$S_2O_3^{2-}$-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 $S_2O_3^{2-}$-S L^{-1}.
2. Elemental sulphur accumulated at ≥1.5 g $S_2O_3^{2-}$-S L^{-1}. The highest obtained elemental sulphur yield was 10% with an initial 9.5 g $S_2O_3^{2-}$-S L^{-1} and the estimated 61% with initial of 16.5 g $S_2O_3^{2-}$-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 NO_2^- L^{-1}.
4. The maximum aerobic and anoxic specific growth rates were 0.046 and 0.022 h^{-1}, respectively, which

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.

Acknowledgement

This work was supported by the Doctoral School of Tampere University, Finland.

Disclosure statement

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

Funding

This work was supported by Tampere University.

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.

ORCID

Réka Hajdu-Rahkama http://orcid.org/0000-0002-0500-4807
Bestamin Özkaya http://orcid.org/0000-0003-2358-3248
Aino-Maija Lakaniemi http://orcid.org/0000-0001-7189-8741
Jaakko A. Puhakka http://orcid.org/0000-0001-9423-6556

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Supplementary Material

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

Réka Hajdu-Rahkama[a]*, Bestamin Özkaya[a,b], Aino-Maija Lakaniemi[a], Jaakko A. Puhakka[a]

[a] Tampere University, Faculty of Engineering and Natural Sciences, P.O. Box 541, FI-33104 Tampere University, Finland

[b] Yildiz Technical University, Department of Environmental Engineering, Davutpasa, Istanbul, Turkey

Supplementary information: 14 pages, 8 figures, 2 tables,

* Corresponding author. Email address: reka.hajdu-rahkama@tuni.fi

S1: Different steps to obtain the different kinetic parameters and constants:

1.) First, the specific substrate utilization rates (q_1-q_8) was obtained by plotting the consumption of thiosulphate-S at each different initial concentration (Fig. S1 below) and the linear parts of the curves selected.

2.) Then, the linear regression analysis for these parts of the curves was performed in Excel. This resulted in 8 equations (y=m*x+b). From each equation, the slope (m) equals the "q" value (g/L/h). This way, we obtained $\boldsymbol{q_1}$-$\boldsymbol{q_8}$ with initial thiosulphate-S concentrations ranging from 0.8 to 19.6 g/L.

3.) For each thiosulphate consumption curve there was its own biomass growth curve. Thus, the concentration of biomass differed in the incubations with the different initial thiosulphate concentrations (see also Table 1). As illustrated in the Fig. S1, the different biomass concentrations were considered at constant consumption rates (q) for all substrate concentrations. In the selected linear curves, the lag-phases and increasing growth rates were neglected, thus the growth rates were considered constant.

4.) Based on this approach, the concentration of biomass has been taken into account in obtaining the q values. Therefore, the q values cannot be divided with biomass concentration and their units can be shown as h^{-1}.

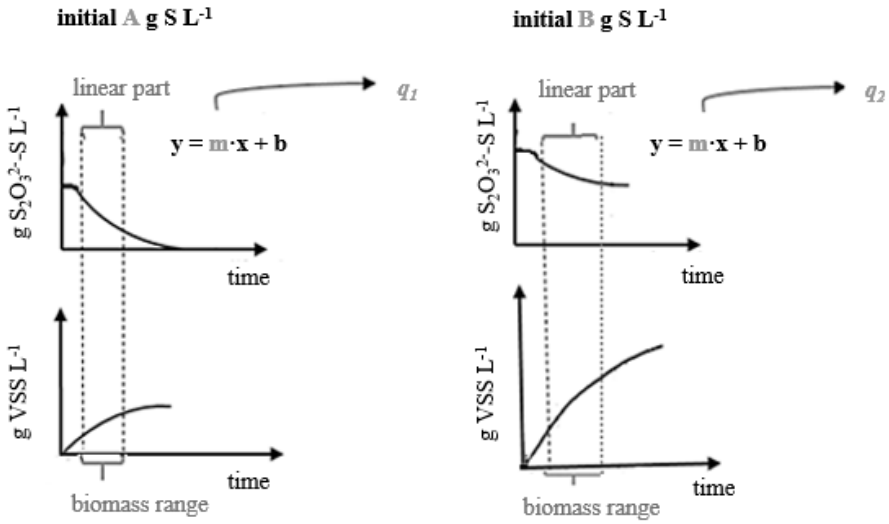

Fig. S1: Thiosulphate consumption and biomass growth at two different initial thiosulphate concentrations. The slope (m) gained by the linear regression equals the specific substrate utilization rate (q). The biomass concentration was considered at constant consumption rates. The figure is used here as an explanation of the approach rather than showing actual data.

Table S1: Different q and biomass concentrations (X) at each initial thiosulphate-S concentration

initial S2O3-S (g L^{-1})	**q (specific thiosulphate-S utilization rate, g L^{-1} h^{-1})**	**X (biomass, g L^{-1})**
0.8	*q1*	X_1
1.5	*q2*	X_2
3	*q3*	X_3
6	*q4*	X_4
8.5	*q5*	X_5
14	*q6*	X_6
16.5	*q7*	X_7
19.6	*q8*	X_8

5.) Then the q_m and K_s values were determined according to 95% confidence interval (to obtain high correlation) (Fig. S2). This step was done by using non-linear regression in Excel-Solver.

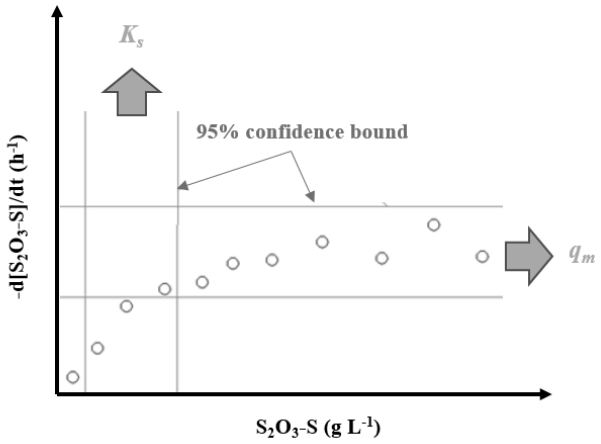

Fig. S2: Using 95% confidence interval to obtain q_m and K_s. The figure is used here as an explanation of the approach rather than showing actual data. The values within 95% confidence interval are used to estimate the kinetic constants.

6.) By using this approach, a model with kinetic parameters was obtained that fitted well the experimental data and it can be confidently used to estimate elemental sulphur and sulphate production from thiosulphate by *Thioalkalivibrio denitrificans*.

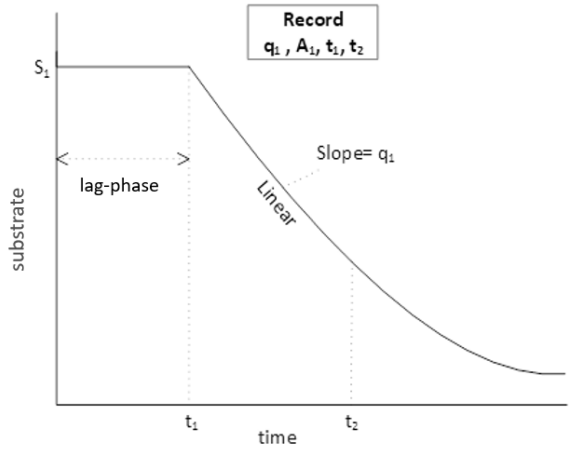

Fig. S3: Substrate consumption curve and time. The period of lag-phase (lag-time) and substrate utilization rate (q) are marked. The lag-phase lasts until the substrate consumption starts.

Day 0				
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	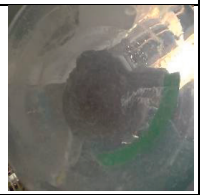	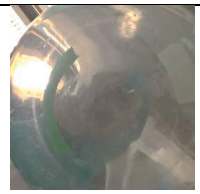		
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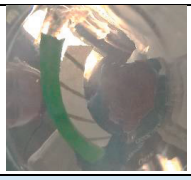				
Day 1				
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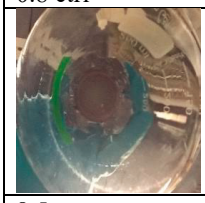
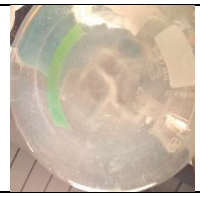
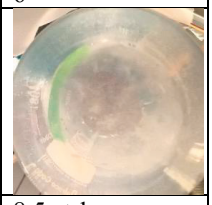
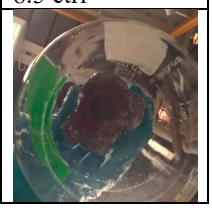
				
8.5	14	16.5	19.5	8.5 ctrl
				

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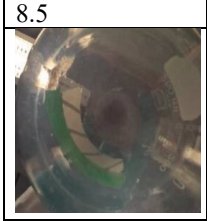				
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	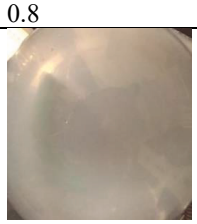			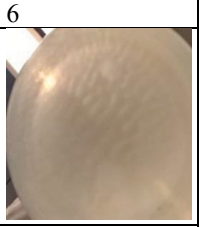
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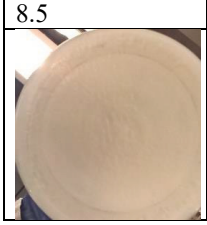	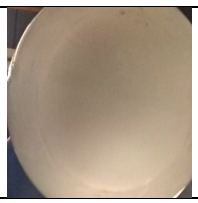	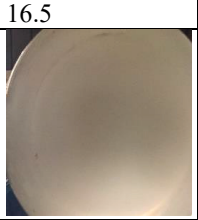	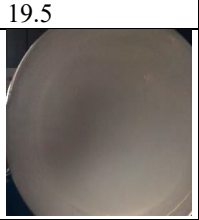	

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Day 7				
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	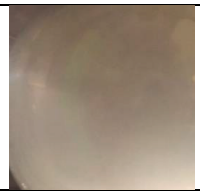			
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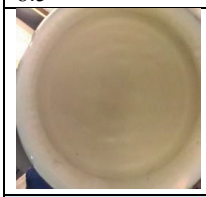				
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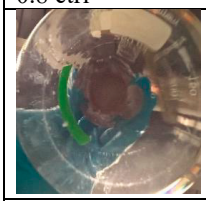
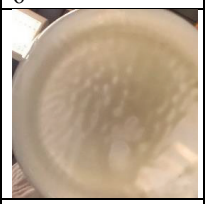
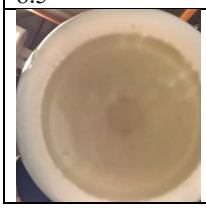
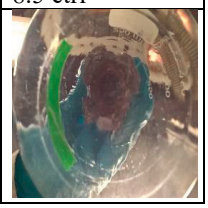
				
8.5	14	16.5	19.5	8.5 ctrl
				

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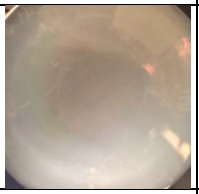
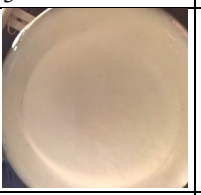
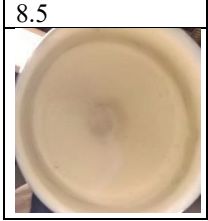
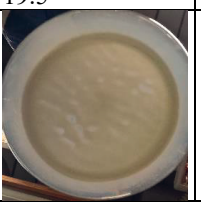
Day 14				
0.8 ctrl	0.8	1.5	3	6
				
8.5	14	16.5	19.5	8.5 ctrl
				

Fig. S4: Visual elemental sulphur formation in the aerobic cultures of *T. denitrificans*. The concentration of $S_2O_3^{2-}$-S (g L^{-1}) are indicated above the photos. The first and last photos of each day are negative controls with 8.5 and 19.6 g [$S_2O_3^{2-}$-S] L^{-1}. The numbers above the figures indicate the concentrations in g [$S_2O_3^{2-}$-S] L^{-1}.

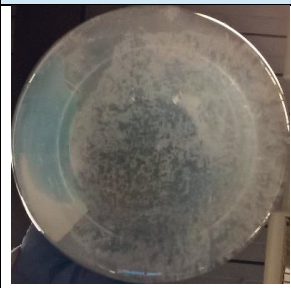
Day 0	Day 2	Day 4
		
Day 6	**Day 8**	**Day 10**
		

Fig. S5: Visual elemental sulphur formation in the aerobic cultures of *T. denitrificans* with initial 9.0 g [$S_2O_3^{2-}$—S] L^{-1}. The photos were taken on the same day of sulphur production measurements.

Table S2 16S rRNA gene copy numbers by different thiosulphate concentrations [22]

initial g $[S_2O_3\text{-}S]$ L^{-1}	copy number/ mL	change (copy number/mL)	change (%)	change (cells/mL)
inoculum for 0.8-8.5	108770.389			
0.8	1007629.66	898859.3	8.263823	154976
1.5	976556.223	867785.8	7.978144	149618
3	1251325.08	1142555	10.50428	196992
6	3193928.17	3085158	28.36395	531924
8.5	4863473.16	4754703	43.71321	819776
inoculum for 14-19.6	60119.681			
14	8651454.89	8591335	142.9039	1481265
16.5	9653203.49	9593084	159.5664	1653980
19.6	14102070.2	14041951	233.5666	2421026

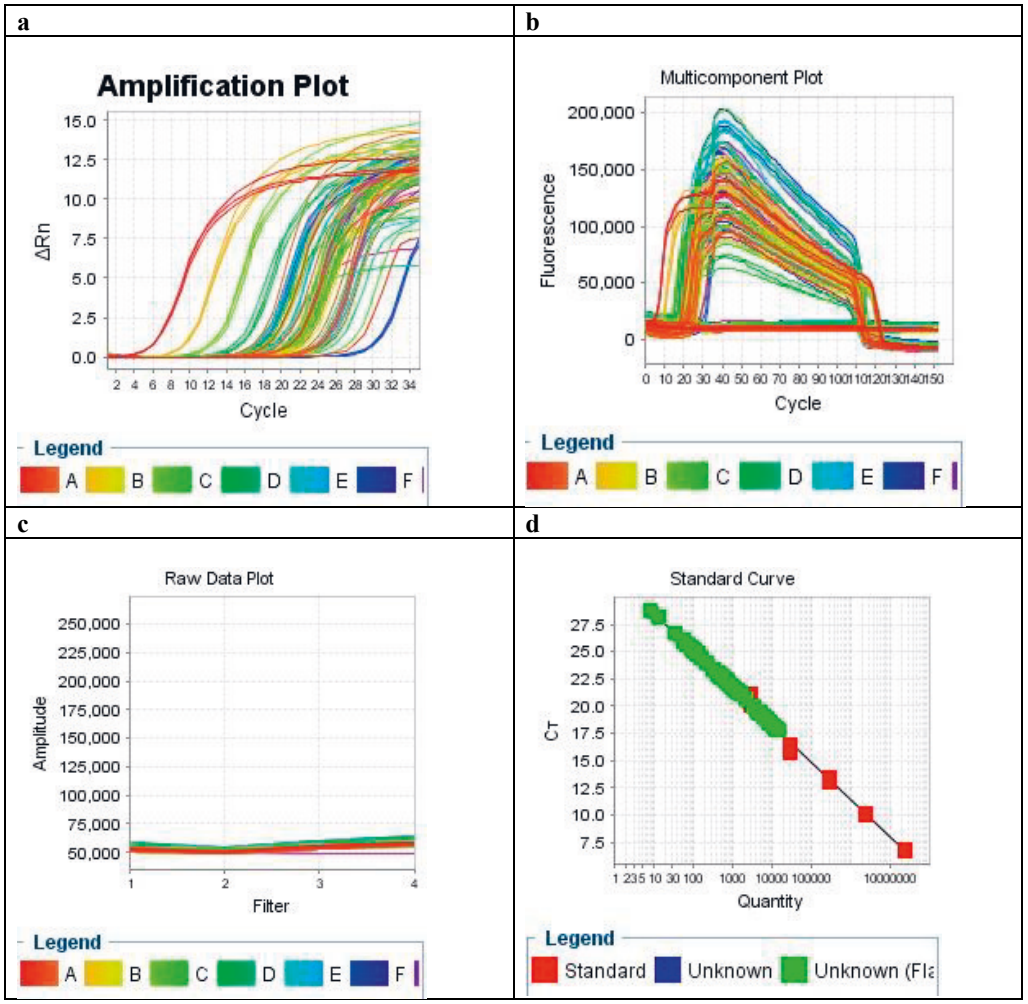
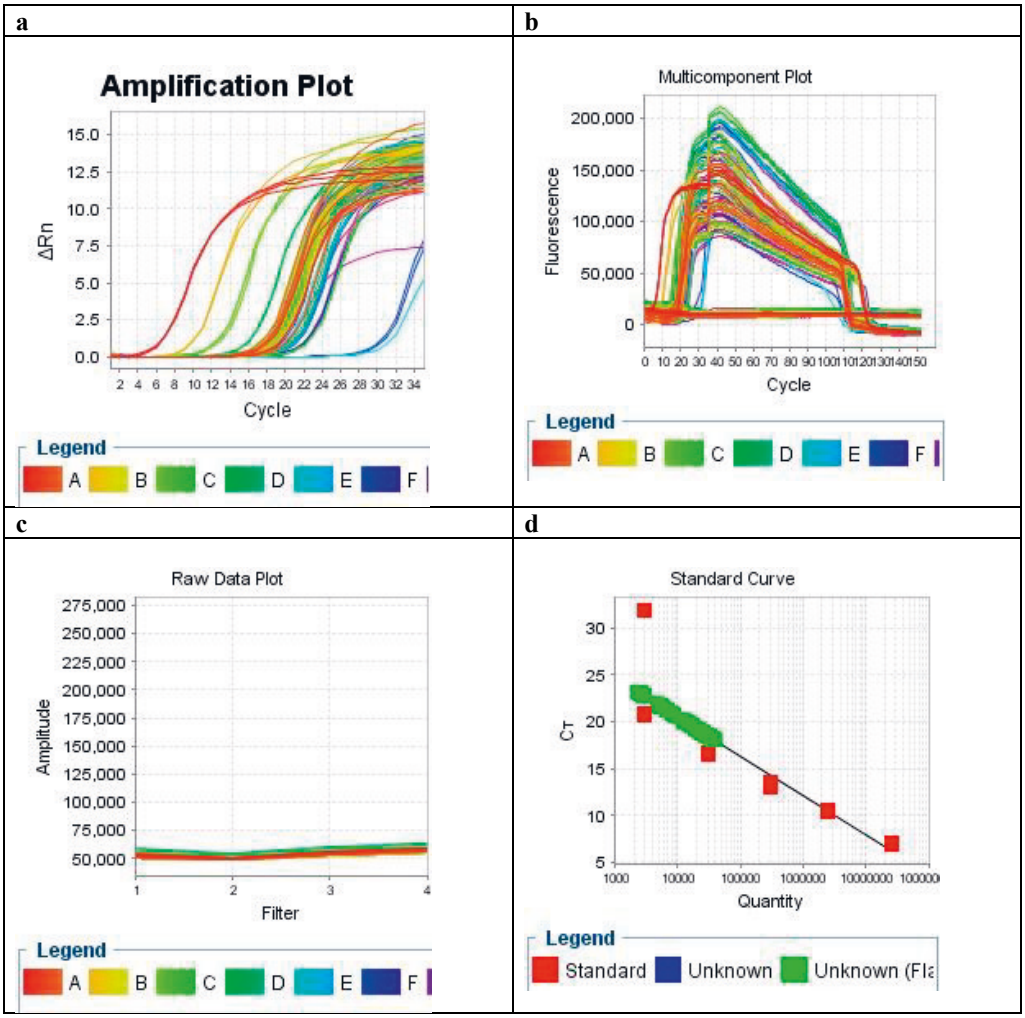


Fig. S6: Results of qPCR with initial 0.8-8.5 g $[S_2O_3^{2-}\text{-}S]$ L^{-1} in aerobic condition. In the figure (a) is amplification plot, (b) multicomponent plot, (c) raw data plot and (d) standard curve showing results as well.



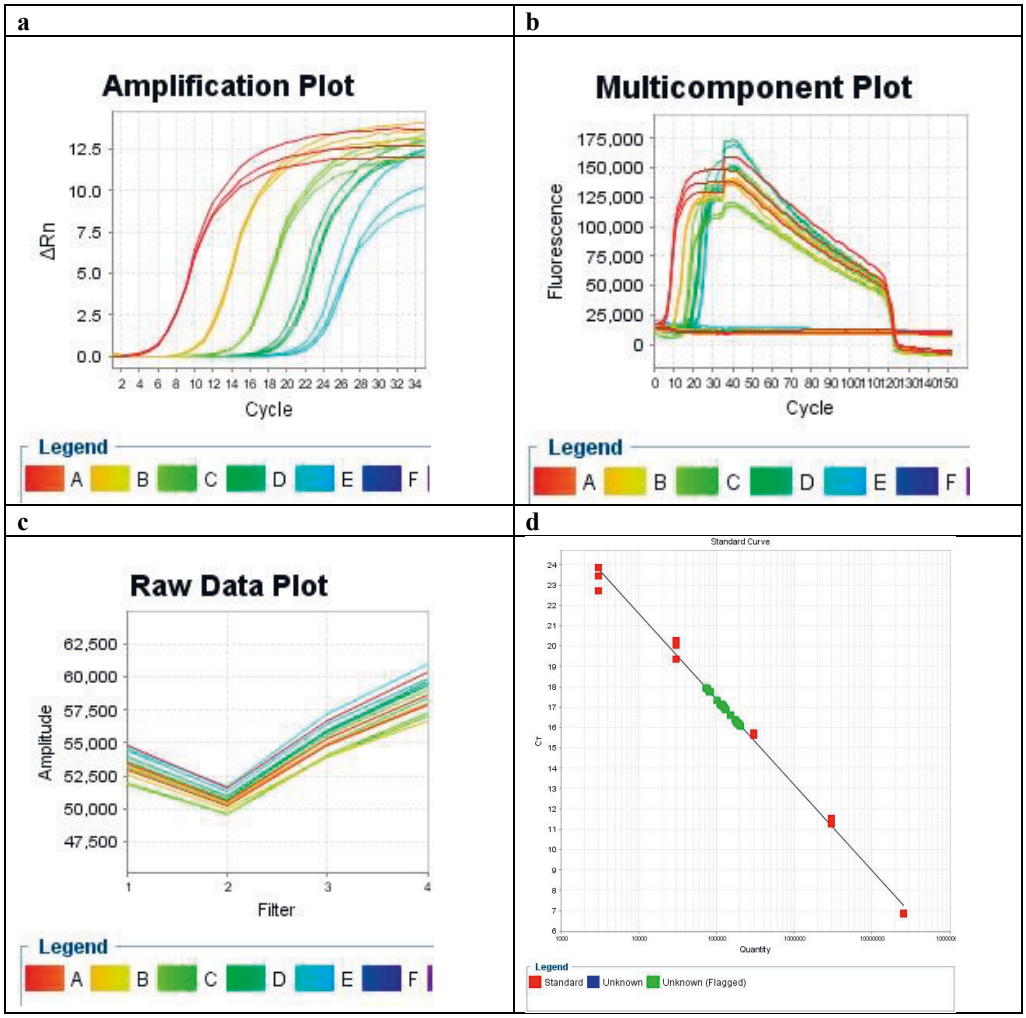
101

102 Fig. S7: Results of qPCR with initial 14-19.6 g $[S_2O_3^{2-}\text{-}S]$ L^{-1} in aerobic condition. In the figure

103 (a) is amplification plot, (b) multicomponent plot, (c) raw data plot and (d) standard curve

104 showing results as well.

105



106

107 Fig. S8: Results of qPCR with initial 0.2-0.58 g [NO_2^-] L^{-1} and 9 g [$S_2O_3^{2-}$-S] L^{-1} in aerobic

108 condition. In the figure (a) is amplification plot, (b) multicomponent plot, (c) raw data plot and

109 (d) standard curve showing results as well.

110

PUBLICATION III

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

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Biochemical Engineering Journal, 172, 108062
DOI: 10.1016/j.bej.2021.108062

Biochemical Engineering Journal 172 (2021) 108062

Contents lists available at ScienceDirect

Biochemical Engineering Journal

journal homepage: www.elsevier.com/locate/bej

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

Alessio D'Aquino *,[1], Réka Hajdu-Rahkama, Jaakko A. Puhakka

Tampere University, Faculty of Engineering and Natural Sciences, P.O. Box 541, FI-33104 Tampere University, Finland

ARTICLE INFO

Keywords:
Alkaline
Biofilm reactor
Biological sulphur recovery
Biotransformation
Saline solution
Sulphur oxidizing bacteria

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 thiosulphate 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

* Corresponding author.
E-mail address: alessiodaquino.env@gmail.com (A. D'Aquino).
[1] Visiting M.Sc. thesis student from University of Trento, Department of Civil, Environmental and Mechanical Engineering, Trento, Italy, at the time of the experiments.

https://doi.org/10.1016/j.bej.2021.108062
Received 7 January 2021; Received in revised form 23 April 2021; Accepted 10 May 2021
Available online 13 May 2021

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 THIOPAQ™ 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 FBRR 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 precultivated 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 Na_2CO_3, 10 g/l $NaHCO_3$, 5 g/l NaCl, 1 g/l K_2HPO_4), 0.5 g/l KNO_3, 0.048 g/l $MgCl_2$, 2% (*v/v*) trace element solution (TES) and $Na_2S_2O_3$. 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 KNO_3, $MgCl_2$, and $Na_2S_2O_3$ 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 approximatively 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) (Filtrasorb 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 % O_2, 78 % N_2 and 1% CO_2), 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):

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

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,

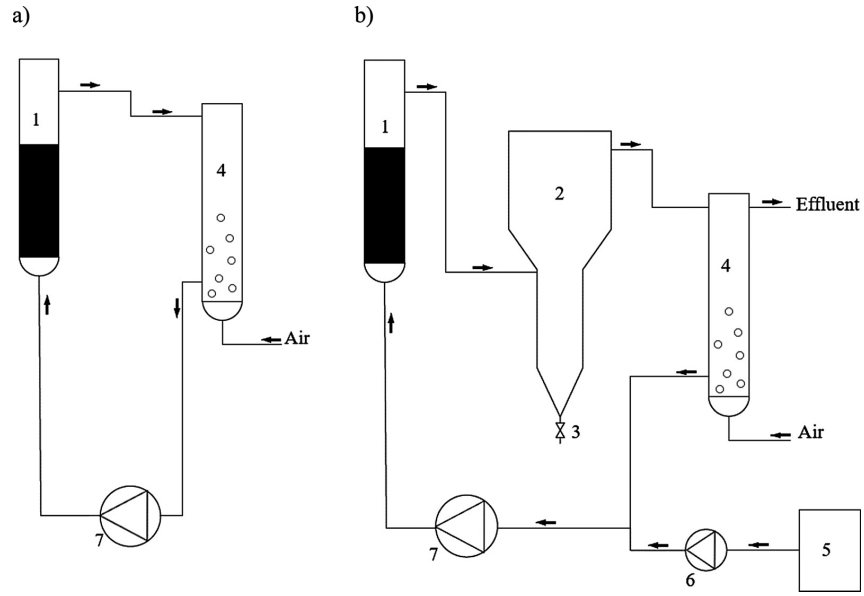

Fig. 1. Schematic diagram of the fluidized bed bioreactor system: (1a) during the semi-batch operation, (1b) during the continuous operation. Units: (1) fluidized bed bioreactor (FBBR), (2) settling tank, (3) S^0 outlet, (4) recirculation unit (RU), (5) feed tank, (6) feed pump, (7) recirculation pump. 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):

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

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 form 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 ± 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 ± 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 ± 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 μm. 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 ± 1% and 8 ml, respectively), while ferric chloride was the second most efficient (76 ± 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

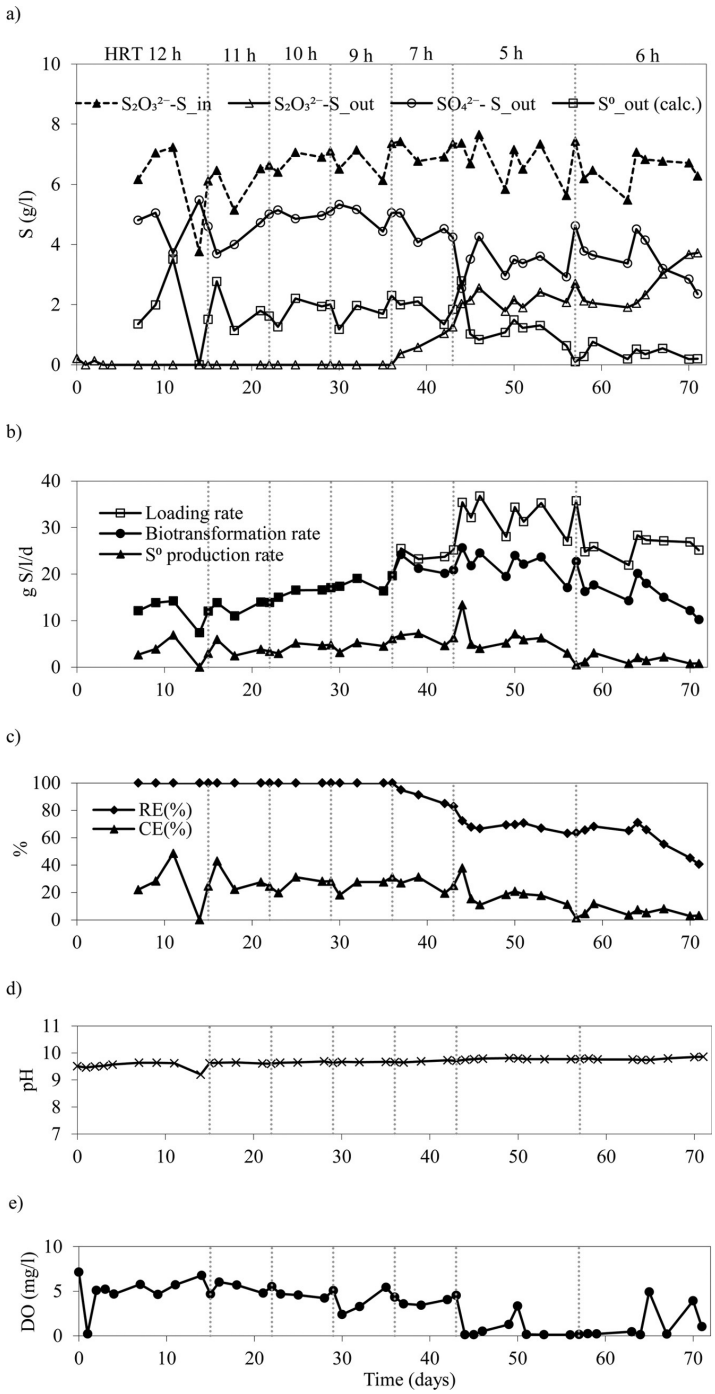

Fig. 2. Fluidized bed bioreactor performances during the continuous operation. Time course profiles of sulphur compounds concentration (a); loading rate, biotransformation rate, elemental sulphur production rate (b); removal efficiency (RE), conversion efficiency (CE) (c); pH and dissolved oxygen (DO) (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
$\overline{S^0}$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 ± 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 ± 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. Annachhatre 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 Annachhatre 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

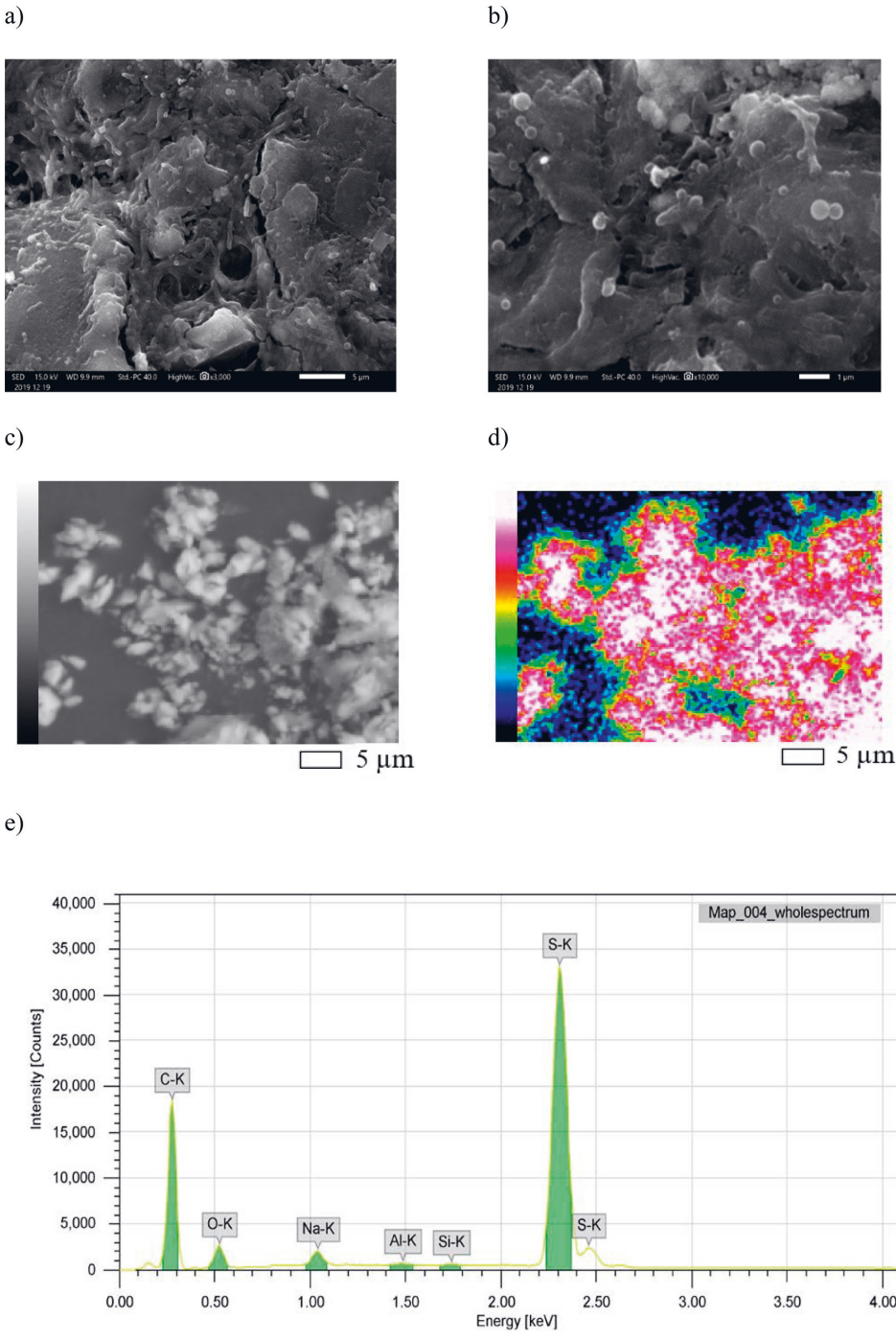

Fig. 3. Scanning electron microscope (SEM) images of the activated carbon (a-b) and sulphur from the settler (c-e) taken on day 69 of continuous bioreactor operation (HRT 6 h). a-b) biofilm formed on the surface of the activated carbon in the fluidized bed bioreactor; c) sulphur and ᴅ-e) SEM and energy-dispersive 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.

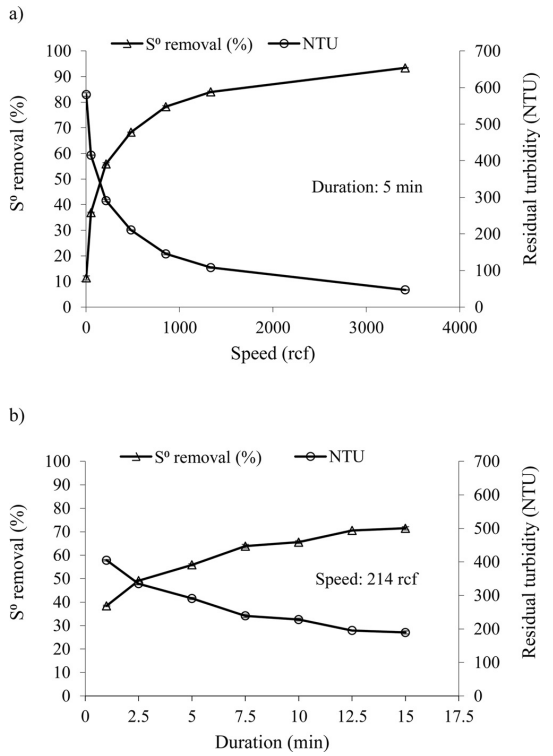

Fig. 4. Centrifugation tests results. Speed test (a) and duration test (b). Initial turbidity and TS in the effluent batch were 640 NTU and 58.1 g/l, respectively.

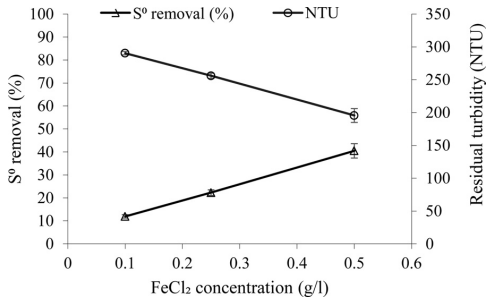

Fig. 5. Coagulation of sulphurous effluent from fluidized bed bioreactor after 30 min settling and as a function of $FeCl_2$ concentration. Initial turbidity and TS in the effluent batch were 320 NTU and 47.5 g/l, respectively.

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 FBBR 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 FBBR system was likely due to the vertical up-flow in the settling tank. Besides, the consistent turbulence in the FBBR 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 FBBR 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 share stress caused by the up-flow in the FBBR. 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 Annachhatre [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 FBBR 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 FBBR 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 THIOPAQ™ 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 FBBR 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 ± 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.

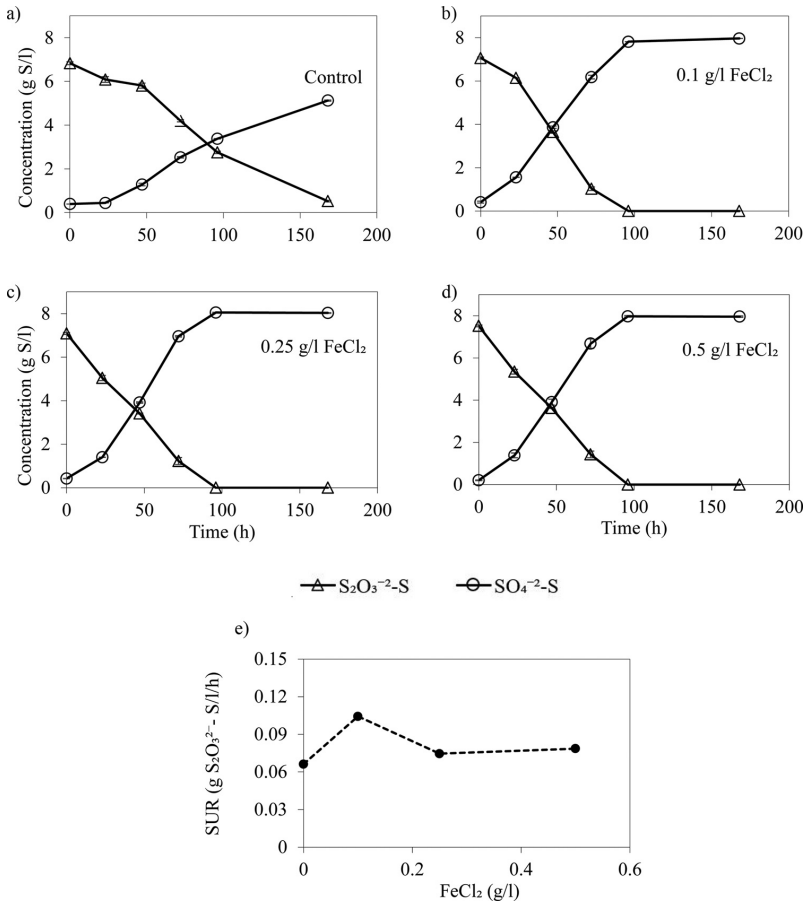

Fig. 6. Effect of $FeCl_2$ on thiosulphate biotransformation by T. versutus. SUR represents the substrate utilisation rate.

Table 2
Comparison of laboratory-scale studies using different SOBs and $S_2O_3^{2-}$ 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	$S_2O_3^{2-}$	~6	30 ± 2	~10	*T. versutus*	9–7	~18–24	~18–22	99.9–88	27 ± 2	This study
Batch assays	$S_2O_3^{2-}$	17.6	30	10	*T. versutus*	/	/	2	99.9	45	[17]
Upflow bioreactor with suspended biomass	$S_2O_3^{2-}$	6.8	30	5–5.5	*Thiobacilli spp.*	N.R.	3.4	N.R.	≥90	60	[25]
Supernatant-recycling settler bioreactor with PVC packing	$S_2O_3^{2-}$	6.4	30	5–5.5	*Thiobacilli spp.*	N.R.	< 8	N.R.	>90	77	[14]
Biotrickling filter system	$S_2O_3^{2-}$	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	*T. versutus*	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	*Thiobacillus denitrificans*	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	*Thiobacillus*-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 S^0 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.

Acknowledgments

The authors would like to thank Prof. Bestamin Özkaya and Dr. Emre Oğuz Köroğlu from Yildiz Technical University, Turkey, for planning the fluidized bed bioreactor system. The advice of Dr. Aino-Maija Lakaniemi during the laboratory experimentation is greatly appreciated. We also thank Kati Valtonen (Staff Scientist) from Tampere University for performing the scanning electron microscopy of the biofilm samples. This study was funded by the Doctoral School of Tampere University. The exchange period of Mr. D'Aquino was partially funded by the Thesis Research Abroad Mobility Programme offered by University of Trento, Italy. Finally, Mr. D'Aquino would like to thank Prof. Paola Foladori (University of Trento) for having supported the M.Sc. thesis study realization abroad.

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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Supplementary Material

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

Alessio D'Aquino[ab*], Réka Hajdu-Rahkama[a] and Jaakko A. Puhakka[a]

[a] Tampere University, Faculty of Engineering and Natural Sciences, P.O. Box 541, FI-33104 Tampere University, Finland

[b] Visiting M.Sc. thesis student from University of Trento-Department of Civil, Environmental and Mechanical Engineering, Trento, Italy, at the time of the experiments.

Supplementary information: 8 pages, 8 Figures, 2 Tables, References

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*Corresponding author. Email address: alessiodaquino.env@gmail.com

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Table S1. Thiosulphate biotransformation reactions and related Gibbs-free energy changes.

Reaction	Gibbs free energy change ΔG^0 [kJ/(mol S-substrate)]	Symbol used in the text
$S_2O_3^{2-}+2O_2+H_2O \rightarrow 2SO_4^{2-}+2H^+$	-738.7	(a)
$S_2O_3^{2-}+\frac{1}{2}O_2 \rightarrow S^0+SO_4^{2-}$	-231.6	(b)
$S^0+\frac{3}{2}O_2+H_2O \rightarrow SO_4^{2-}+2H^+$	-507.4	(c)
$S^0+O_2+H_2O \rightarrow SO_3^{2-}+2H^+$	-249.4	(d)
$SO_3^{2-}+\frac{1}{2}O_2 \rightarrow SO_4^{2-}$	-258.0	(e)

Figure S1. Elemental sulphur collected from the effluent of fluidized bed bioreactor (FBBR) system into an Erlenmeyer flask during operation at HRT 5 h. The figure was taken from the bottom of the Erlenmeyer flask.

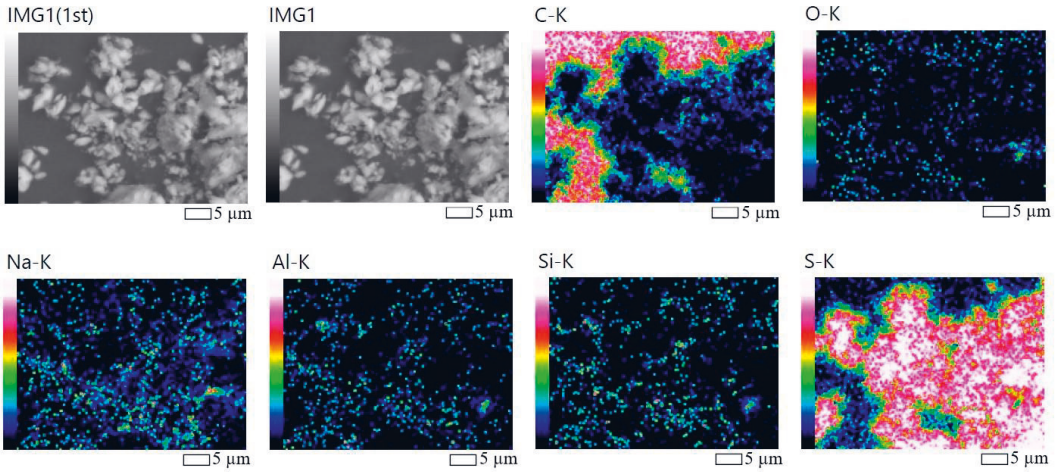
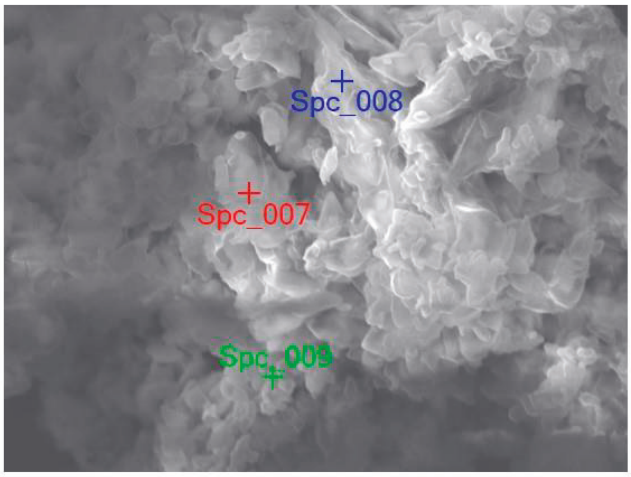


Figure S2. Scanning electron microscopy (SEM) and energy-dispersive X-ray spectrometry (EDS) mapping results of the solid sample collected from the settling tank of the fluidized bed bioreactor system treating a thiosulphate solution. The sample was dried at 105°C, prior to the preparation for SEM analysis. The first two figures (IMG1(1st) and IMG1) show the SEM images of the sulphur sample which are followed by the images showing the abundances of the different elements (C, O, Na, Al, Si, S) in the sample. The different elements are marked above the image where the "-K" means K(alpha)-radiation of the element. The colour bar on the left-hand side indicates the concentration of the certain element. When going from down (black) to up (white), the concentration increases. Carbon was the second most abundant element that mainly originated from the carbon coating of the SEM sample and possibly due to the AC carryover from the FBBR to the settler.

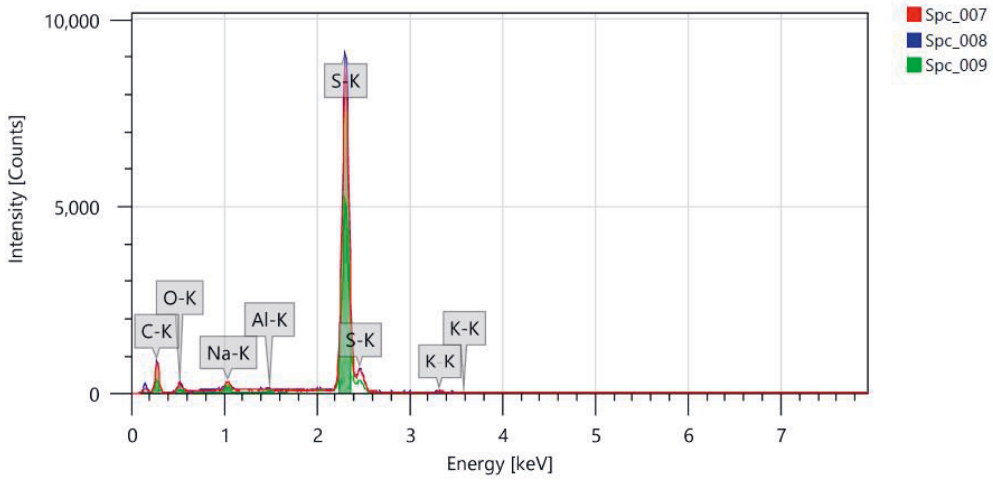
a)



Signal SED
Landing Voltage 15.0 kV
WD 10.4 mm
Magnification x3,000
Vacuum Mode HighVacuum

5 µm

b)



Name	C	O	Na	Al	S	K	Total
Spc_007	51.89	4.55	0.88	0.12	42.55		100.00
Spc_008	51.90	4.27	0.73		43.11		100.00
Spc_009	46.15	2.23	1.17		49.78	0.67	100.00
Average	49.98	3.68	0.93	0.12	45.15	0.67	
StandardDeviation	2.71	1.03	0.18		3.29		

Mass%

Figure S3. Point analysis of the scanning electron micrograph (SEM) about the elemental sulphur collected from the settling tank from the fluidized bed bioreactor. The SEM was equipped with an energy-dispersive X-ray spectrometer (EDS). The figure a) is SEM image with 3 points selected for elemental distribution analysis, b) quantitative analysis and mass percentage of elements in the three points analysed. After carbon, sulphur was the most abundant element. For elemental analysis, standardless quantitative analysis by Burgess [1] was used.

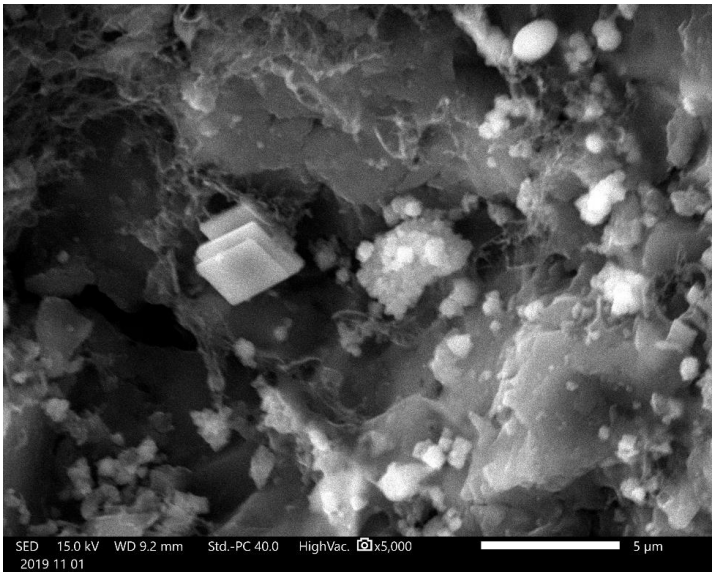


Figure S4. Scanning electron micrograph (SEM) from the activated carbon carrier material from the fluidized bed bioreactor operated at 12 h HRT. The sample was dried at 105 °C and carbon coated. The bright spherical particles/globules are elemental sulphur. No bacterial cells were detected due to the sample preparation protocol.

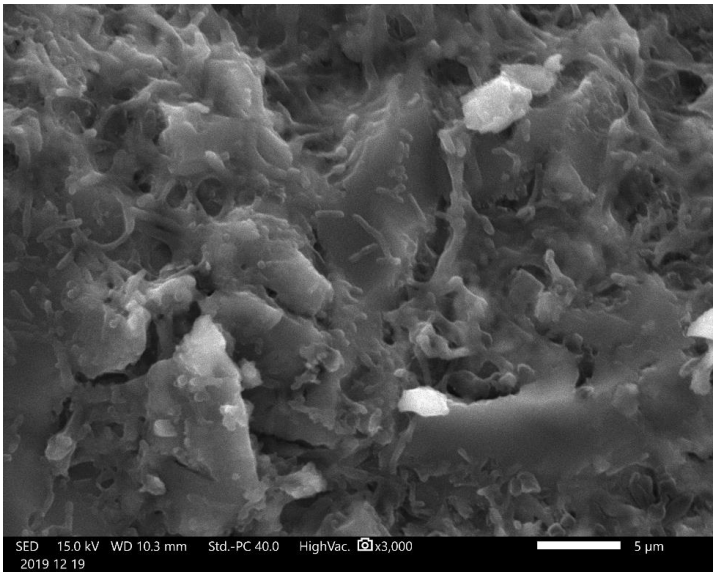
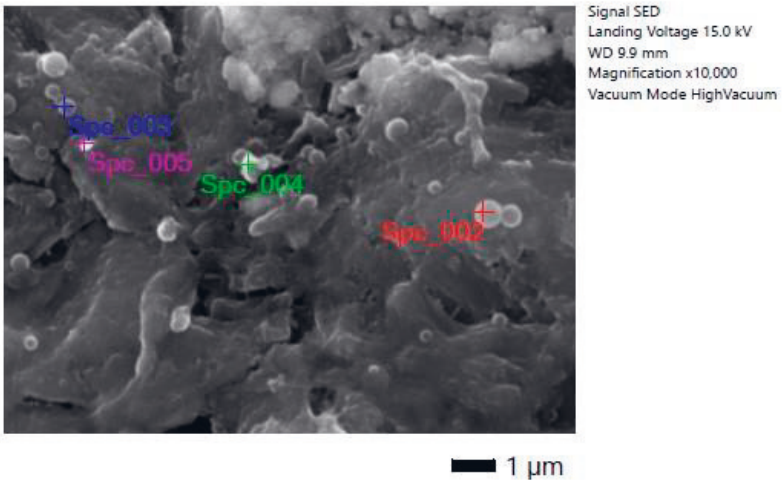
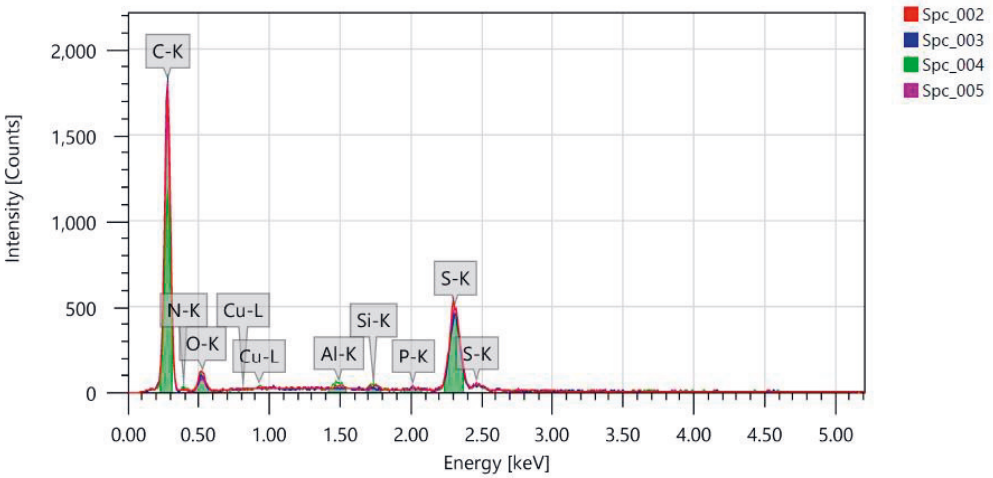


Figure S5. Scanning electron micrograph (SEM) of elemental sulphur particles (whitish) on the activated carbon carrier material covered with vibrio shaped cells (*T. versutus*). The sample was taken from fluidized bed bioreactor on day 69 (HRT 6 h). The sample was primary fixed with glutaraldehyde in phosphate buffered saline (PBS) containing Alcian Blue. Then it was washed with PBS and dehydrated in ethanol series prior to critical-point dehydratation in ethanol-acetone series. Finally, the sample was carbon coated.

a)



b)



Name	C	N	O	Al	Si	P	S	Cu	Total
Spc_002	81.94		8.99	0.17	0.24	0.20	8.46		100.00
Spc_003	84.58		7.34				8.08		100.00
Spc_004	72.97	6.00	10.28	0.59	0.52		9.18	0.45	100.00
Spc_005	84.54		6.37				9.09		100.00
Average	81.01	6.00	8.24	0.38	0.38	0.20	8.70	0.45	
StandardDeviation	4.76		1.50	0.21	0.14		0.45		

Mass%

Figure S6. Scanning electron microscopy (SEM)- energy-dispersive X-ray spectrometer (EDS) mapping point analysis of activated carbon carrier material taken on day 69 (HRT 12 h) from the settling tank of the fluidized bed bioreactor system. a) Scanning electron micrograph with four points selected for elemental analysis, b) quantitative analysis and mass percentage of elements in the four points selected. Sulphur was the second most abundant element after carbon. For elemental analysis, standardless quantitative analysis by Burgess [1] was used.

Table S2. The effect of different coagulants (0.5 g/l) on gravity settling (30 minutes) of elemental sulphur from the fluidized bed bioreactor effluent as indicated by % turbidity removal and settled volume.

Coagulation -Test 1			
Coagulant	**Turbidity removal (%)**	**Settled Volume (ml)**	**pH**
$Al_2(SO_4)_3$	72±1	6.5	9.8
$Fe_2(SO_4)_3$	69	3.5±1	9.8
$FeCl_2$	76±1	8	9.9
$FeCl_3$	76±1	7	9.9

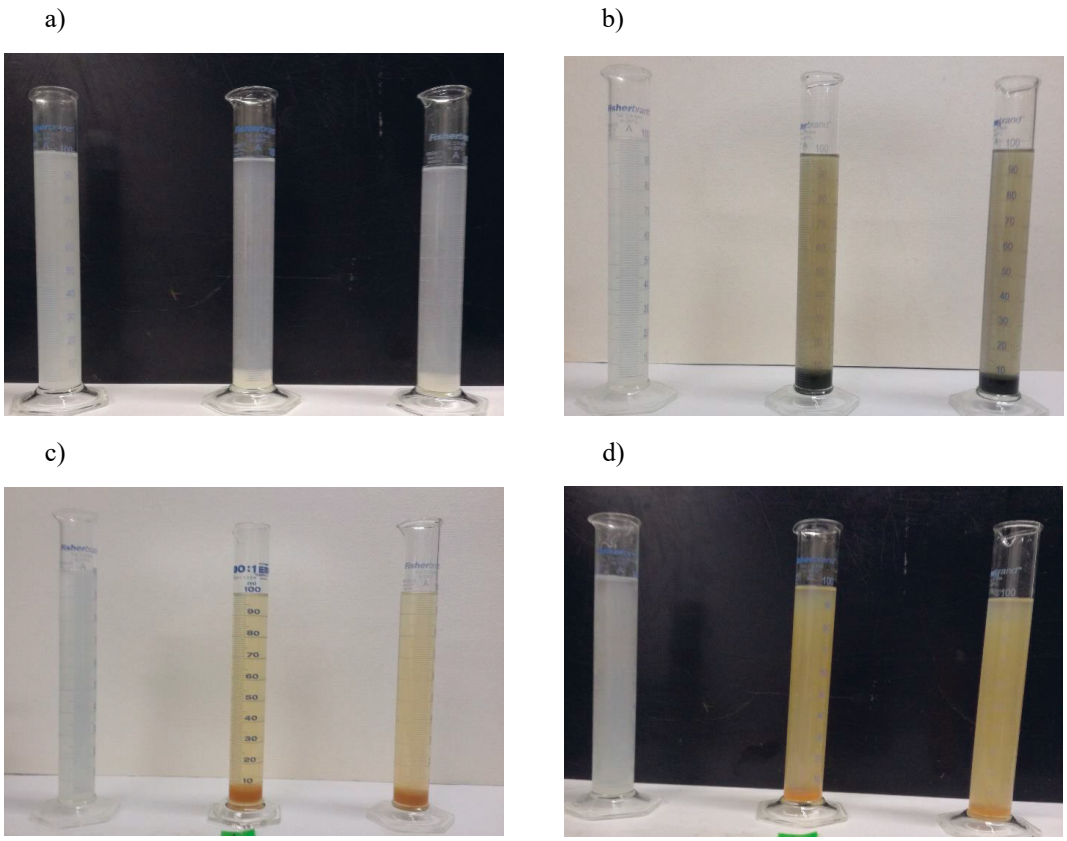


Figure S7. The effect of different coagulants (0.5 g/l) on gravity settling (30 minutes) of elemental sulphur from the fluidized bed bioreactor effluent. The coagulants used were: a) $Al_2(SO_4)_3$ b) $FeCl_2$ c) $FeCl_3$ d) $Fe_2(SO_4)_3$. In each figure, the cylinder on the left contains effluent only, whereas the cylinders in the middle and on the right contain effluent supplemented with the coagulants.

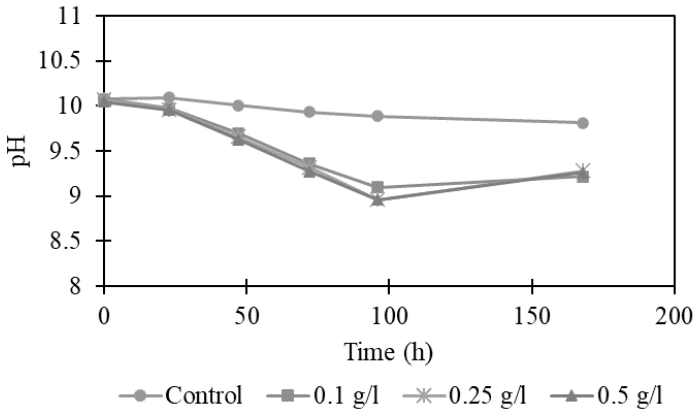


Figure S8. pH changes during thiosulphate biotransformation by *T. versutus* in the presence of different concentrations of $FeCl_2$. The bioassays were conducted in shake flasks and incubated in an orbital shaker at 150 rpm and 30 °C.

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PUBLICATION IV

High tolerance of chemolithoautotrophic sulphur oxidizing bacteria towards pulp and paper mill wastewaters and their organic constituents supporting sulphur recovery in alkaline conditions

Chemical Engineering Journal, 450, 137972
DOI: 10.1016/j.cej.2022.137972

Chemical Engineering Journal 450 (2022) 137972

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

High tolerance of chemolithoautotrophic sulphur oxidizing bacteria towards pulp and paper mill wastewaters and their organic constituents supporting sulphur recovery in alkaline conditions

Réka Hajdu-Rahkama *, Jaakko A. Puhakka

Tampere University, Faculty of Engineering and Natural Sciences, Bio- and Circular Economy Research Group, P.O. Box 541, FI-33014 Tampere University, Finland

ARTICLE INFO

Keywords:
Biotransformation
Haloalkaline
Organic toxicity
Resource recovery
Thioalkalivibrio versutus
Thiosulfate

ABSTRACT

This study reports the tolerance of chemolithoautotrophic biotransformation of sulphurous compounds towards pulp and paper (P&P) mill wastewaters (primary filtrate of bleaching (PFB) and composite wastewater (WW)) and their constituents under haloalkaline conditions. The effects of organic compounds (methanol, acetate, D (+)-xylose, phenol and benzene) that may be present in P&P wastewaters, and yeast extract, a complex organic compound on thiosulphate biotransformation by *Thioalkalivibrio versutus* were investigated. All experiments were carried out in batch bioassays at pH 10 and 13–23 g Na^+/L. Phenol and benzene reduced thiosulphate biotransformation by 88 and 94% at 0.25 and 1 g/L, respectively in 10 days. 20 g/L methanol, 20 g/L yeast extract and 10 g/L xylose reduced the biotransformation by 90, 88 and 56%, respectively. No inhibition of biotransformation occurred with acetate at concentrations up to 20 g/L. The growth was also enhanced by 1 to 10 g/L yeast extract likely serving as additional nutrients. At pH (~10), the studied organic acids remain mostly unprotonated and, thus control their access through the cell membrane. Therefore, the inaccessibility of these compounds to the cytosol is a likely mechanism for having non-inhibitory effects. The 87% (*v/v*) WW did not affect thiosulphate biotransformation efficiency while 87% (*v/v*) PFB reduced it by 36% by day 10. The resistance of *T. versutus* to common organics present in P&P wastewaters indicates its potential use for sulphur recovery from P&P mill wastewaters at haloalkaline conditions and thus, supports the circular economy approach.

1. Introduction

Pulp and paper manufacturing, petroleum refining, mining, agriculture, tanning, and food processing represent major anthropogenic sources of sulphur releases [1–3]. In petroleum refining and mining, sulphur is present in crude oil or minerals, respectively [4,5], while it is a process chemical in other industries [6,7]. Sulphur gas emissions are effectively controlled by modern technologies such as scrubbers [8]. Many of these processes transfer sulphurous compounds including H_2S to the liquid phase i.e., to process and wastewaters. Reduced sulphurous compounds are toxic, corrosive and increase operational costs [7,9,10]. The recovery of sulphurous chemicals from industrial water solutions supports circular economy, in addition to environmental sustainability.

Technologies such as Claus-process and amine-treatment recovering sulphur from wastewaters and process streams are energy-intensive, generate chemical side-streams and are often maintenance-costly due to corrosion [11]. Biological sulphur recovery as elemental sulphur at ambient temperature and atmospheric pressure is gaining increasing attention [12]. Bioprocessing for sulphur recovery involves two steps, i.e., reduction of sulphur oxyanions to hydrogen sulphide followed by oxidation of H_2S to elemental sulphur [13]. Another less studied alternative for elemental sulphur production is using chemolithoautotrophic sulphur oxidizing bacteria (SOB) [14–17]. These bacteria disproportionate partially oxidized sulphur oxyanions into hydrogen sulphide and sulphate followed by oxidation of hydrogen sulphide to elemental sulphur [10]. During the biotransformation of thiosulphate, balanced internal oxidation–reduction reactions take place in SOB. The electrons released in the oxidation of one S-atom in thiosulphate to sulphate are accepted by the reduction of the other thiosulphate S-atom to sulphide. This reaction is then followed by oxidation of the sulphide to elemental sulphur [17–19]. Biogenic elemental sulphur is easy to separate, hydrophilic, non-corrosive, and can be used in wide-range applications [15,20–24].

Chemolithoautotrophic SOB gain energy from bioconversion of

* Corresponding author.
E-mail address: reka.hajdu-rahkama@tuni.fi (R. Hajdu-Rahkama).

https://doi.org/10.1016/j.cej.2022.137972
Received 24 March 2022; Received in revised form 29 June 2022; Accepted 5 July 2022
Available online 11 July 2022

reduced sulphurous compounds and produce elemental sulphur as a metabolic intermediate [25]. Acidophilic chemolithoautotrophs are widely applied in biomining for metal recovery [23]. On contrary, many of the streams of P&P and petrochemical industries are alkaline and saline (Na^+) [26,27], and therefore, their treatment would require haloalkaliphilic SOB. Bacteria belonging to the genus *Thioalkalivibrio* are characterized by extreme tolerance to high pH and high Na^+ concentrations [28], thus, potent organisms for engineering applications in such environments.

Organic raw-material processing-based industries produce process and wastewaters containing multiple dissolved organic compounds in addition to sulphurous and inorganic process chemicals. P&P mill wastewaters contain wood-based organics and their chemical transformation products. The chemical oxygen demand (COD) and the composition of these solutions depend on the characteristics of the raw material and the pulping process. For example, pulp bleaching wastewaters contain about 0.3–4.3 g COD/L. Evaporator condensates, accounting for 40% of a pulp mills effluent, represent another organic-rich stream with 0.6–6.5 g COD/L [29]. Methanol, acetic acid and furfural are major organics in evaporator condensates and bleaching liquors [30,31]. The bleaching effluents also contain low concentrations of organohalogens [32] with varying degrees of aerobic or anaerobic biodegradabilities [33–35]. In the petrochemical industry, on the other hand, organic compounds in the sulphide-rich sulphidic spent caustics include phenol, benzene and toluene in addition to the sulphurous organics methanethiol, ethanethiol and disulphides [36–38].

Acidophilic chemolithoautotrophic bacteria such as those belonging to the genus *Acidithiobacillus* that use various inorganic sulphurous compounds as electron donors are very sensitive to organic compounds [39–42] whereas the effects of organic compounds on haloalkaliphilic chemolithoautotrophic sulphur oxidizing bacteria (SOB) have, to the best of our knowledge, not been comprehensively documented [14,43,44]. For biological sulphur recovery using chemolithoautotrophic SOB from organic-rich industrial processes and wastewaters such as P&P and petroleum production, this is a critical factor to be delineated.

Earlier studies [15,16,45] have demonstrated the sulphur recovery potential from thiosulphate by haloalkaliphilic *Thioalkalivibrio versutus*. Therefore, this study aimed to investigate the potential of recovering sulphur by chemolithoautotrophic *T. versutus* from solutions containing elevated concentrations of organic compounds. The effects of several organic compounds and two P&P mill wastewaters including bleaching process filtrate and composite wastewater on thiosulphate biotransformation and growth by *T. versutus* were studied. Phenol and benzene, methanol, acetate and D(+)-xylose were selected as typical constituents in pulping wastewaters. Moreover, the effects of yeast extract as a complex mixture of organics were also determined.

2. Materials and methods

2.1. Model microorganism and growth medium

Thioalkalivibrio versutus (DSM 13738), obtained from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ), was used in this study. According to the recommendation of DSMZ [46], the culture was maintained in 925 medium for the alkaliphilic sulphur respiring strain. The medium consisted of base medium (20 g/L Na_2CO_3, 10 g/L $NaHCO_3$, 5 g/L NaCl, 1 g/L K_2HPO_4), 2% (*v/v*) trace element solution (TES) and separately added nutrient solutions (0.5 g/L KNO_3, 0.05 g /L $MgCl_2$). Initially, the stock culture was supplied with 4.5 g/L $S_2O_3^{2-}$ (prepared from $Na_2S_2O_3{\cdot}5H_2O$). A detailed description of the medium and stock preparations was as reported by Hajdu-Rahkama et al. [16]. The stock culture was routinely grown at 150 rpm and 30 ± 1 °C in an arbitrary shaker.

Table 1
Analysed constituents of primary filtrate of bleaching (PFB) and mixed wastewater from main sever (WW) samples.

Constituent	PFB (g/L)	WW (g/L)
CHO_2^-	0.004	0.001
Cl^-	0.007	0.004
NO_3^-	0.001	0.001
NO_2^-	N.D.	N.D.
PO_4^-	N.D.	N.D.
SO_4^{2-}	0.28	0.22
$S_2O_3^{2-}$	N.D.	N.D.
Na^+	1.93	0.55
NH_4^+	0.09	0.09
Mg^+	0.09	N.D.
K^+	0.01	0.03
Ca^+	0.11	0.62
acetic acid	0.08	0.07
methanol	0.17	0.14
propionate	0.01	0.01
isobutyrate	0.01	0.01
dissolved organic carbon (DOC)	1.39	0.51

N.D.: not detected.

Table 2
Experimental designs of bioassays with organic compounds and wastewaters.

Organic compound/ wasters	Selected concentrations	Total Na^+ (g/L)	pK_a	Controls
Methanol	1, 2.5, 5, 10 and 20 g/L	13	15.5	Positive[a] controls
Acetate	0.1, 1, 2.5, 5, 10 and 20 g/L	13–23	4.9	Positive controls
D(+)-xylose	0.1, 1, 2.5, 5, 10 and 20 g/L	13	12.1	Positive controls
Benzene	0.1, 0.25, 0.5 and 1 g/L	13	43	Positive controls
Phenol	0.1, 0.25, 0.5 and 1 g/L	13	10	Positive controls
Yeast extract	1, 2.5, 5, 10 and 20 g/L	13	4.9	Positive controls
PFB	87% *(v/v)*	3		Positive and negative[b] controls
WW	87% *(v/v)*	2		Positive and negative controls
Acetate[c]	20 g/L	23	4.9	Positive controls
Methanol[d]	10 g/L	13	15.5	Positive controls
D(+)-xylose[c]	5 g/L	13	12.1	Positive controls

[a] *T. versutus* positive controls without organics or wastewaters.
[b] Controls without added inoculum of *T. versutus*.
[c] Investigation of cell growth.
[d] Elimination of contamination and investigation of cell growth.

2.2. Organic compounds

The studied organic compounds contained typical P&P mill wastewater constituents. These wood-and process-based compounds were methanol (CH_3OH), acetate ($C_2H_3O_2^-$) and D-(+)-xylose ($C_5H_{10}O_5$). Phenol (C_6H_5OH) and benzene (C_6H_6) can occur in both P&P mill wastewaters and streams of petrochemical industry. Further, yeast extract ($C_{19}H_{14}O_2$), a complex organic compound, was also studied.

Concentrated analytical grade methanol (99.8%, Fischer Chemicals, Trinidad and Tobago), phenol (99%, Acros Chemicals, India) and benzene (99%, Sigma Aldrich, Germany) were used. From acetate (sodium acetate, Merk, Germany), D-(+)-xylose (99%, Sigma Aldrich, China) and yeast extract (Lab M Limited, United Kingdom), 200 g/L stock solutions were prepared. The stock solutions of yeast extract, acetate and xylose were 0.2 μm sterile filtered (polyethersulfone membrane syringe filter, VWR International, North America) whilst the other organic stock

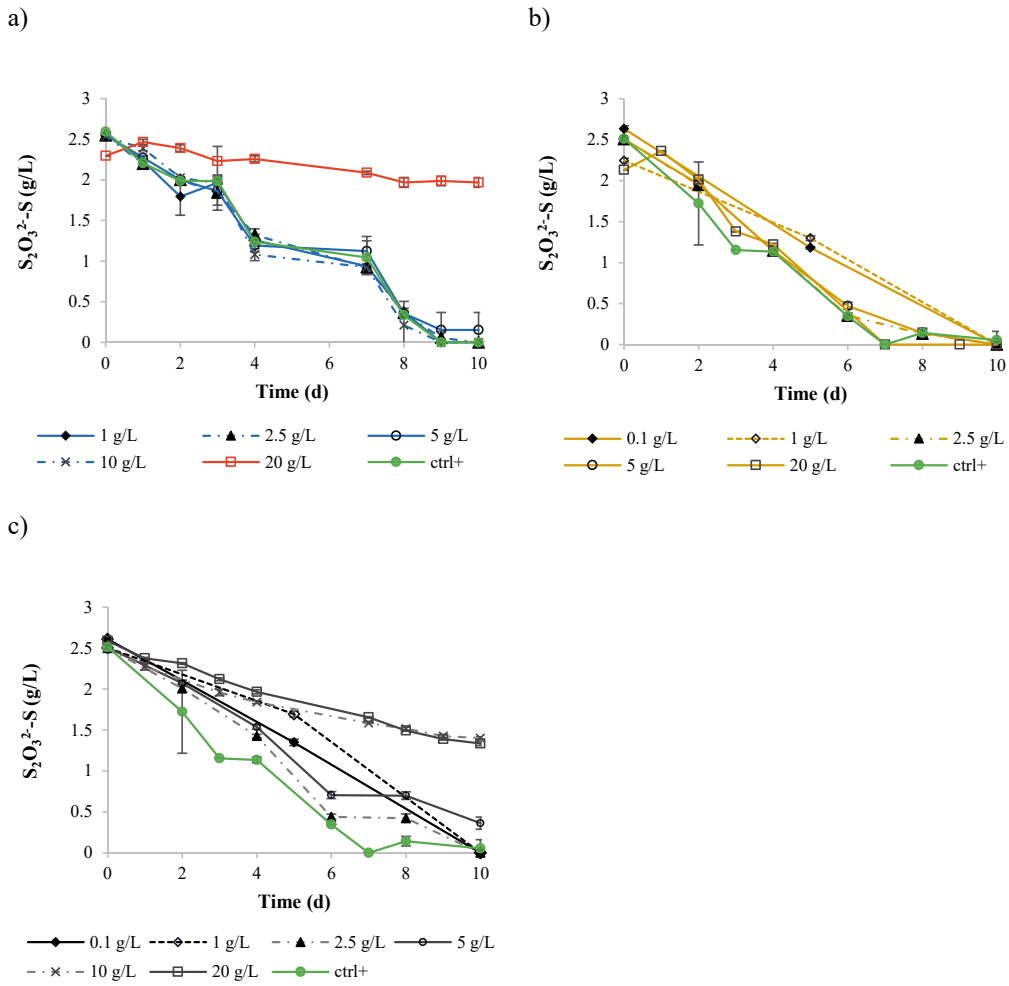

Fig. 1. The effects of (a) methanol, (b) acetate and (c) D-(+) xylose on thiosulphate biotransformation by *T. versutus*. The standard deviations are calculated from duplicate cultures.

solutions were not sterilized. The possibility of contamination from methanol was ruled out in a complementary experiment with 0.2 μm sterile filtered analytical grade methanol.

2.3. Pulp and paper wastewaters

Two P&P wastewater streams, primary filtrate of bleaching (PFB) and mixed wastewater from the main sewer (WW), of a Finnish mill were used as real wastewaters with high organic load. The pH of the PFB and WW were 9.2 and 7.4, respectively. Some of the constituents present in the wastewaters were as shown in Table 1.

2.4. Thiosulphate biotransformation bioassays

All experiments (Table 2) were implemented as batch bioassays in 160 mL duplicate serum bottles (64 mL working volume). The bottles were kept in an arbitrary shaker at 150 rpm and 30 ± 1 °C. The initial pH of the media was pH 10 ± 0.2. The pH of wastewater samples was adjusted with NaOH.

The headspace of the bottles with organic compounds or wastewaters was regularly flushed with technical sterile filtered (0.2 μm polyethersulfone membrane syringe filter, VWR International, North America) air for 10 min. The air-purging of incubations took place every second day. The media were supplemented with the same concentration of TES, KNO_3, $MgCl_2$ and S_2O_3 (approx. 2.5 g/L $S_2O_3^{2-}$-S) as the stock culture. To ensure the same working volume of all media, the volumes of organic additions were subtracted from the volume of the mineral base. The media with wastewaters did not contain mineral base. In the beginning, a 10% (*v*/*v*) inoculum of *T. versutus* culture incubated for 7 days on thiosulphate was added. Positive controls without organic compounds or wastewaters were used. During the incubations with wastewaters, negative controls with 10% (*v*/*v*) autoclaved MilliQ-water instead of inoculum were also prepared. The average initial optical densities (OD_{600}) in incubations with organic compounds (acetate, xylose, methanol and yeast extract) and the positive controls were 0.03 ± 0.01.

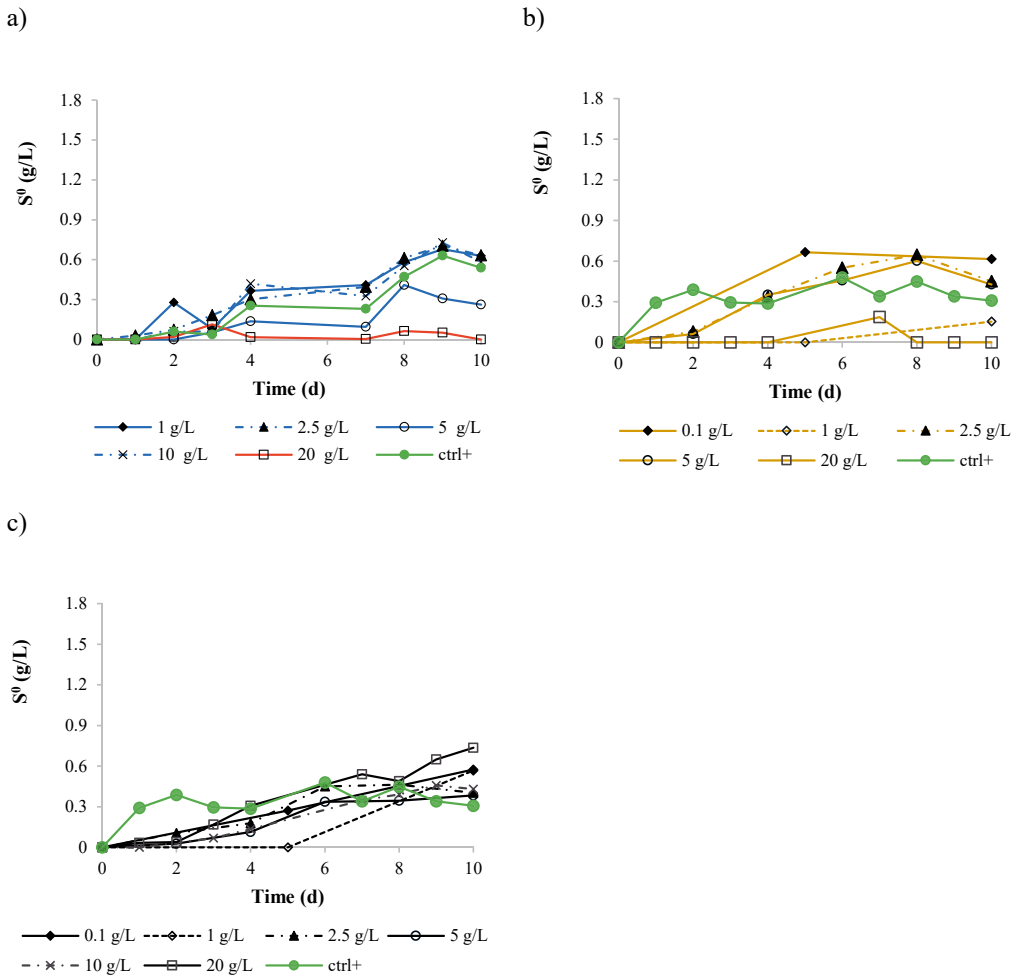

Fig. 2. Estimated biological S^0 production in the presence of methanol (a), acetate (b) and D(+)-xylose by *T. versutus*. The concentrations are calculated values and, thus without standard deviations.

2.5. Analysis

Different anions of original wastewaters (Table 2) and $S_2O_3^{2-}$ and SO_4^{2-} concentrations of other samples were analysed by ion chromatography (Integrion, Thermo Scientific) equipped with Dionex IonPac AS22 anion exchange column (Thermo Scientific), Dionex GM-4 (2 mm) guard column and an autosampler (Dionex AS-DV). The cations (Table 2) present in PFB and WW were analysed by using Dionex DX-120 ion chromatograph (Thermo Fischer Scientific, USA), equipped with IonPac CS12A (4 × 250 mm) cation exchange column, Dionex IonPac CG12A (4 × 50 mm) guard column and an autosampler (Dionex AS40). The dissolved organic carbon (DOC) was measured with TOC-VCPH/CPN analyser (Shimadzu, Japan) by using the method of non-purgeable organic carbon (NPOC) according to SFS-EN 1484 standard (Finnish Standards Association, 1997). Optical density (OD) was measured at 600 nm by using UV-1900i UV–Vis spectrophotometer (Shimadzu Corporation, Japan). The total cell count of additional incubations with 20 g/L acetate, 10 g/L methanol, 5 g/L xylose and positive controls were calculated from 4́,6-diamino-2-phenylindole (DAPI) stained samples under epifluorescence microscopy [47].

The volatile fatty acids (VFAs) and methanol were measured by using Shimadzu GC-2010 Plus chromatograph equipped with a Zebran ZB-WAX Plus column and a 218 flame ionization (FID) detector [48]. The initial and end-point pH of the bioassays was measured with pH 3110 m (WTW, Germany) and Slim Trode electrode (Hamilton®).

The elemental sulphur formation was confirmed by high-performance liquid chromatography (HPLC, Shimadzu, Japan) equipped with Luna 5u C18 (2) reverse-phase column (250x4.6 mm), security guard and UV-detector at 260 nm. The flow of the mobile phase (100% methanol) was 1 mL/min, injection volume 20 μL and the column temperature 40 °C.

Before the ion chromatography and DOC analysis, the samples were filtered with 0.45 μm sterile filter (CHROMAFIL® Xtra polyester membrane filter, Macherey-Nagel, Germany) and stored at −20 °C. The samples used with the GC-FID and HPLC were 0.20 μm sterile filtered (CHROMAFIL® Xtra PET-20/25, Macherey-Nagel, Germany).

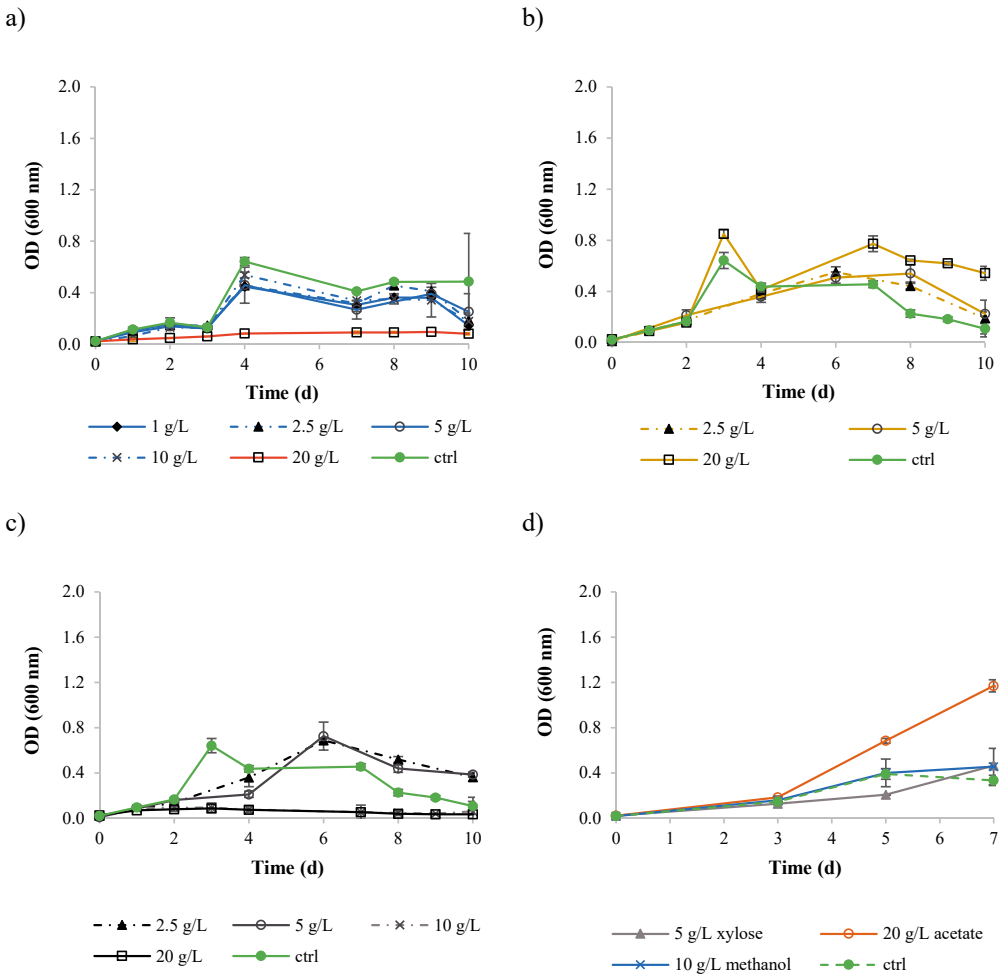

Fig. 3. Changes in optical density (OD_{600}) during thiosulphate incubation of *T. versutus* in the presence of methanol (a), acetate (b) and D-(+) xylose (c). OD_{600} of additional incubations with sterile methanol, acetate and xylose (d) to eliminate contamination and investigate cell growth. The standard deviations were calculated from duplicate cultures.

3. Results

3.1. Biotransformation of thiosulphate and growth of T. versutus in the presence of methanol, acetate and D(+)-xylose

The effects of methanol, acetate and xylose on thiosulphate biotransformation by *T. versutus* were studied, and the results were as shown in Figs. 1 and 2. Also, the effect of these organics on the development of optical density, as a result of both biomass formation and S^0 production, was monitored (Fig. 3).

With 1 to 10 g/L methanol, thiosulphate was removed similarly in inoculated and control bottles (Fig. 1a), while at 20 g/L it was inhibited. The biotransformation rates at 10 g/L methanol and below were between 0.25 and 0.28 g/L/d. The main bioconversion product was sulphate, while elemental sulphur formation was visual in all inoculated bottles (Fig. S1). Based on mass balance estimation, the highest elemental sulphur production (Fig. 2a) by *T. versutus* with 1, 2.5 and 10 g/L methanol were 25%, 28% and 29%, respectively. These concentrations were slightly higher than in the positive controls (24%). The elemental sulphur formation at 5 and 20 g/L methanol was 16% and 5%, respectively.

Acetate at concentrations up to 20 g/L did not affect the thiosulphate biotransformation (Fig. 1b). However, the estimated elemental sulphur formation was reduced at 20 g/L acetate (Fig. 2b). At 5 g/L acetate and below, the thiosulphate biotransformation rates and elemental sulphur formation yields were similar as in the positive controls. The highest thiosulphate biotransformation rates with 2.5, 5, and 20 g/L acetate and positive controls were, 0.36, 0.34, 0.4 and 0.38 g/L/d, respectively. The highest calculated sulphur yields (%) 2.5 and 5 g/L acetate were 19 and 15, respectively, which were similar to the positive controls (19%). With 20 g/L acetate, the main product of thiosulphate biotransformation was sulphate, with elemental sulphur yield of 9%.

With 0.1–20 g/L D-(+) xylose, the biotransformation of thiosulphate was reduced (Fig. 1c). In 10 days, all thiosulphate was removed at xylose concentrations of 0.1–2.5 g/L, while it was around 7 days in the positive control. At 5, 10 and 20 g/L D-(+) xylose the biotransformation

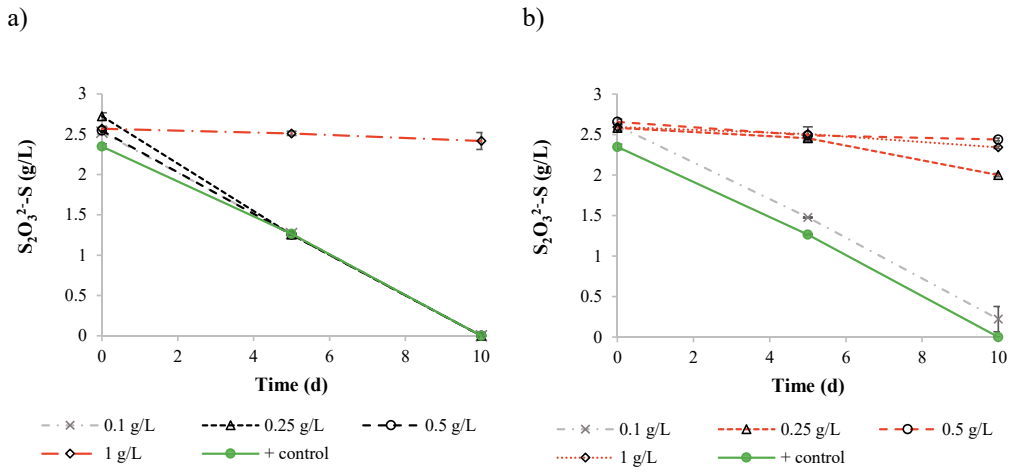

Fig. 4. Thiosulphate biotransformation in the presence of (a) benzene and (b) phenol. The standard deviations are from duplicate cultures.

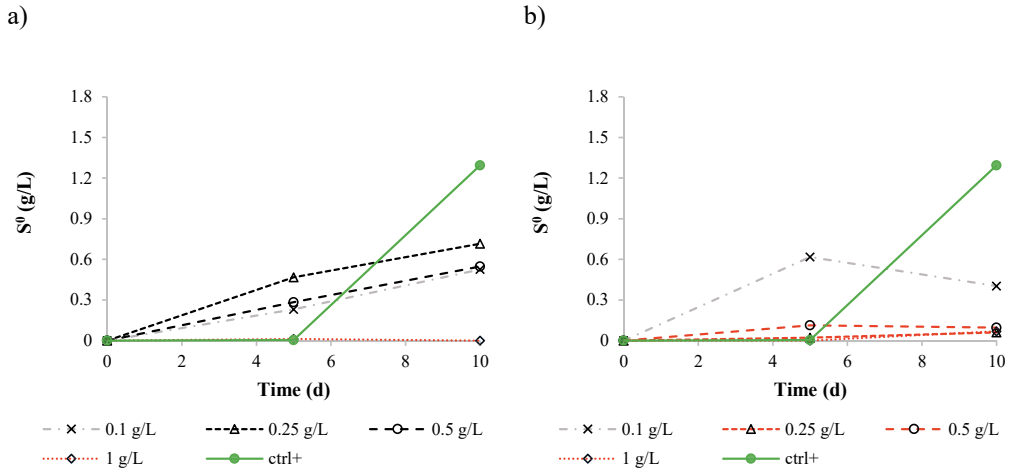

Fig. 5. Calculated biological S^0 production from thiosulphate in the presence of benzene (a) and phenol (b). The concentrations are calculated values, thus without standard deviations.

efficiency was reduced to 86%, 44% and 48%, respectively, in 10 days. The rate of biotransformation decreased by the increase of xylose concentration as follows: The rates were 0.34, 0.3, 0.11 and 0.13 g/L/d with 2.5, 5, 10 and 20 g/L xylose, respectively. The highest calculated elemental sulphur yield (%) (Fig. 2c) at 10 g/L xylose and below was approximately 20%, while it was 28% at 20 g/L xylose. Once thiosulphate was biotransformed (Fig. 1), elemental sulphur concentrations started to decrease (Fig. 2) indicating oxidation of elemental sulphur by *T. versutus*. The S^0 formation by *T. versutus* was confirmed with HPLC analysis of 10 g/L methanol, 20 g/L acetate and positive control samples of additional incubations (Fig. S12). The thiosulphate biotransformation proceeded similarly with sterile filtered 10 g/L methanol as with non-filtered.

The ODs with 1–10 g/L methanol, 2.5–5 g/L acetate and 2.5–5 g/L xylose and *T. versutus* developed similarly as in the positive controls, while at 20 g/L methanol and 10–20 g/L xylose, the ODs did not change during the incubation (Fig. 3a-b). From the three test compounds, only acetate at 20 g/L increased the OD. The total cell counts at the end of additional incubations with 10 g/L methanol, 20 g/L acetate and 5 g/L xylose (Table S1 and Fig. S13) were similar. The pH in the *T. versutus* cultures with methanol (1–20 g/L), acetate (10–20 g/L) and xylose (10–20 g/L) was 9.9 to 9.7, being the same as in the positive controls. The DOC concentrations did not change during incubations (Figs. S4-S6).

3.2. Biotransformation of thiosulphate and growth of T. versutus in the presence of benzene and phenol

The effects of benzene and phenol on thiosulphate biotransformation by *T. versutus* were studied and the results were as shown in Figs. 4 and 5.

Thiosulphate removal was similar with 0.1–0.5 g/L benzene and 0.1 g/L phenol as in the positive controls. At 0.25 g/L phenol, the lag phase was elongated, and the biotransformation was partial. Benzene at 1 g/L

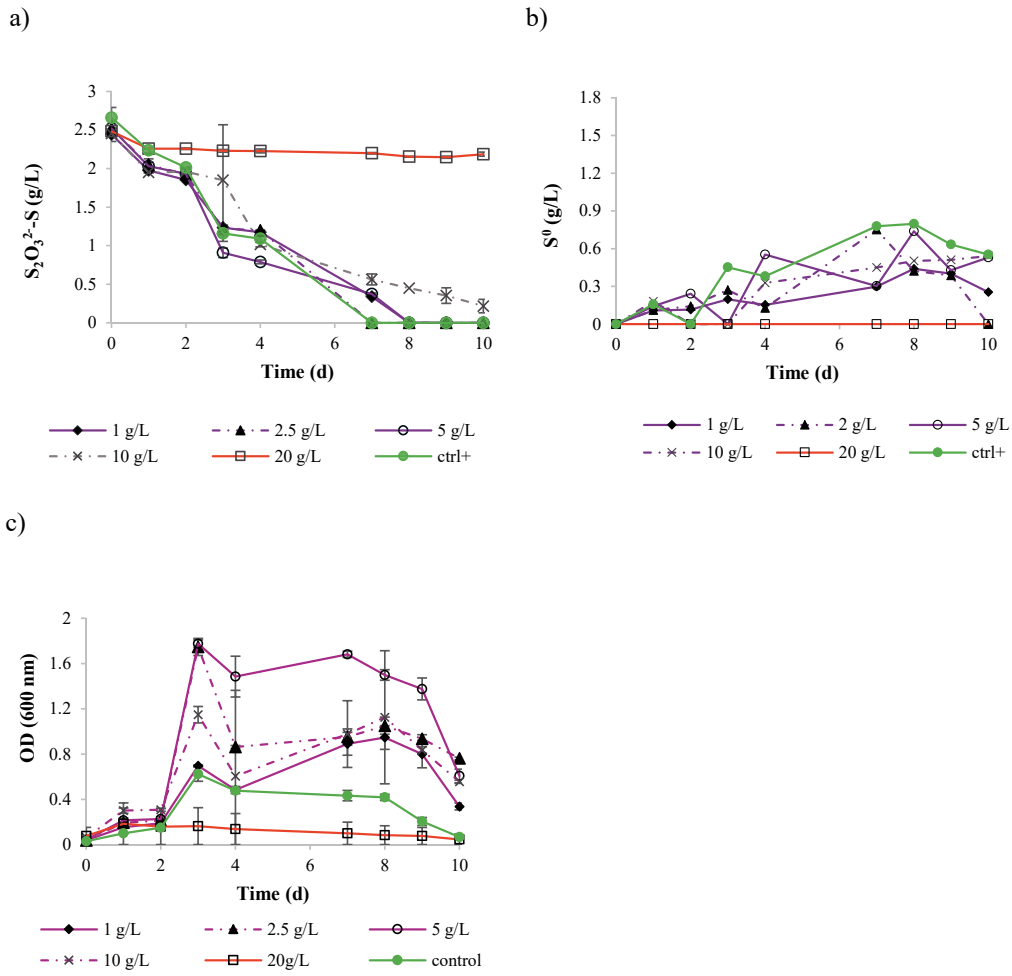

Fig. 6. Thiosulphate biotransformation by *T. versutus* (a), estimated S^0 formation (b) and changes of optical density in the presence of different concentrations (1–20 g/L) of yeast extract. The standard deviations of biotransformation and optical densities are calculated from duplicates. The S^0 concentrations are calculated values and thus, presented without the standard deviations.

and phenol at 0.5–1 g/L inhibited thiosulphate biotransformation. The main product of thiosulphate conversion was sulphate with both benzene and phenol. The share of the calculated elemental sulphur yields (%) (Fig. 5a) was 21, 26 and 22 with 0.1, 0.25 and 0.5 g/L benzene, respectively. The corresponding sulphur yields (%) were 24, 2 and 4 with 0.1, 0.25 and 0.5 g/L phenol (Fig. S1).

The inertness of phenol and benzene biodegradation under the experimental conditions was confirmed at 0.1 and 0.25 g/L concentrations.

3.3. Enhancement of thiosulphate biotransformation and growth of *T. versutus* by yeast extract

The impact of yeast extract concentration on thiosulphate biotransformation and growth of *T. versutus* were investigated and the results were as shown in Fig. 7.

Biotransformation of thiosulphate by *T. versutus* in the presence of 1–5 g/L yeast extract was similar to the positive controls (Fig. 6a). The biotransformation at 10 g/L yeast extract was similar to the positive controls with the exception that after day 7, the rate slowed down. The highest biotransformation rate was 0.57 g/L/d, with 5 g/L/d yeast extract, which was the same as in the positive controls. The rates of biotransformation were 0.28, 0.4, 0.26 g/L/d with 1, 2.5 and 10 g/L yeast extract, respectively. At 20 g/L yeast extract, no thiosulphate removal occurred in 10 days. The estimated elemental sulphur formation in the cultures with yeast extract remained below that of the positive controls (Fig. 6b). The growth of *T. versutus* measured as OD was enhanced by 1–10 g/L yeast extract (Fig. 6c and Fig. S1). Adding 1, 2.5, 5 and 10 g/L yeast extract increased the ODs by 1.5, 2.8, 2.8 and 1.8 times, respectively, compared to the positive controls. After day 3, the highest OD was apparent with 5 g/L yeast extract. At the end of the experiment, the ODs of the cultures with up to 10 g/L yeast extract remained above the positive controls. In 10 days, the OD at 20 g/L yeast extract remained close to the initial. The DOC concentrations of the cultures with 2.5–20 g/L yeast extract increased by day 10 (Fig. S7). In the positive controls and with 1 g/L yeast extract, the DOC decreased

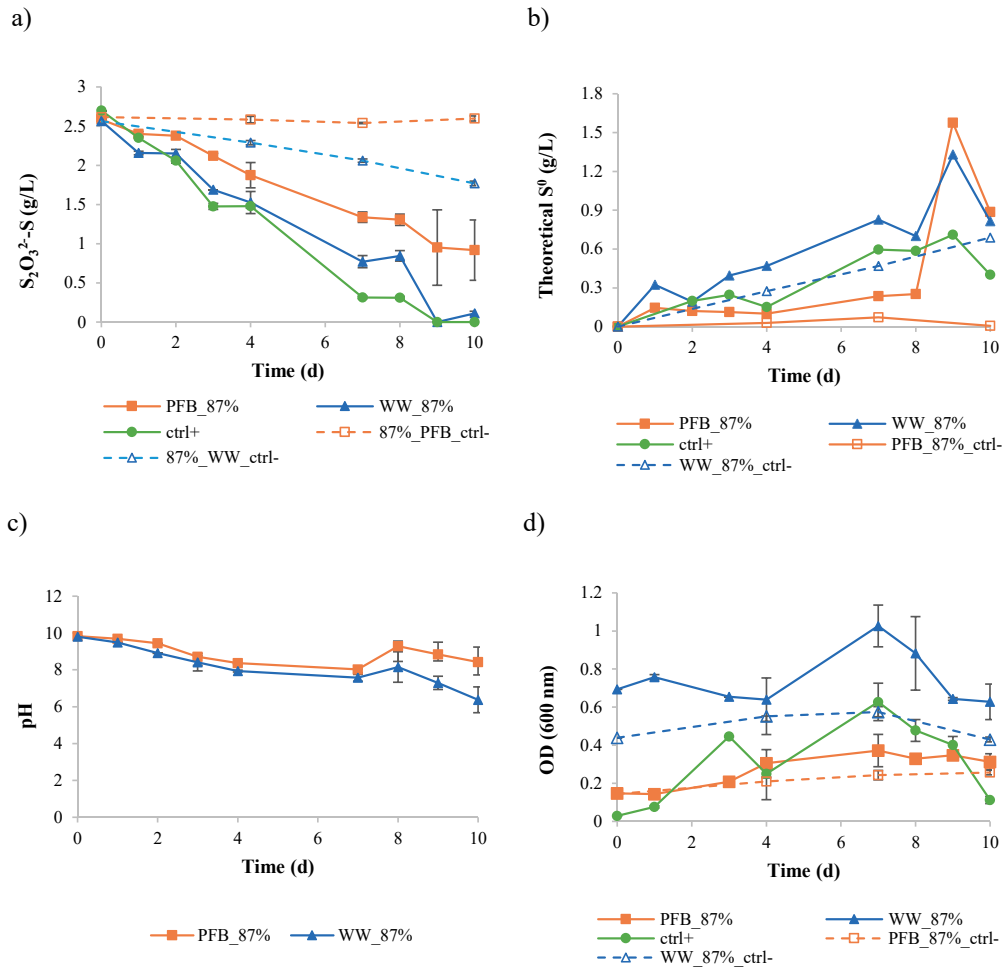

Fig. 7. Thiosulphate biotransformation (a) in 87% concentrated primary filtrate of bleaching (PFB_87%) and mixed wastewater from the main sewer (WW_87%). Positive (ctrl +) and non-inoculated (ctrl-) controls are also included. The theoretical S^0 production (b) in the wastewaters was calculated as missing sulphur values. The development of the pH (c) and optical density (d) during the incubation was also followed. The standard deviations of biotransformation and pH are calculated from duplicate cultures.

during the experiment.

3.4. Biotransformation of thiosulphate in the presence of P&P mill wastewaters

The effects of two selected P&P mill wastewaters (primary filtrate of bleaching, PFB and wastewater from main sewer, WW) on thiosulphate biotransformation were investigated. The results of the experimentation were as shown in Fig. 7 and Fig. S1.

The highest biotransformation rate (Fig. 7a) with PFB (87%) and WW (87%) were 0.20 and 0.26 g/L/d, respectively, which were lower than in the positive controls (0.33 g/L/d). All thiosulphate was removed in the WW in 9 days whilst some remained in the PFB even on day 10. Thiosulphate was partially removed in the negative controls of WW (0.08 g/L/d), but not in the negative controls of PFB. The calculated elemental sulphur formation was higher in the WW and lower in the PFB than in the positive controls (Fig. 7b). A separate set of batch assays with inoculated PFB and WW controls showed a slow pH decrease from 10 to 8.4 and 6.4, respectively (Fig. 7c). After the 3-day lag phase, the ODs of the cultures with PFB increased more than in the negative controls but remained below that of the positive controls (Fig. 7d). The initial ODs of the inoculated cultures with WW were higher than in corresponding negative controls. This gap increased after day 4, however, remaining below the positive controls.

4. Discussion

This study revealed the non-inhibition of haloalkaline chemolithoautotrophic SOB towards organic compounds and thus, the potential of using these bacteria for recovery of sulphur from industrial wastewaters and process streams.

Table 3
Toxicity of organic compounds on chemolithoautotrophs.

Compound	Inhibitory concentration (g/L)	Experimental design	Acidophilic microorg.	Haloalkaliphilic microorg.	Ref.
citric acid, galacturonic acid, glucose and cellobiose	9.6–25 (50–130 mM), 8.5–44.6 (29–230 mM), 12.6–50.4 (70–280 mM) and 2.6–51.3 (7.5–150 mM)[a]	shake flasks	*Acidothiobacillus ferrooxidans*		[56]
glucose	1	shake flasks	*At. ferrooxidans*		[57]
formic acid	0.077 (1.67 mM)	shake flasks	*At. thiooxidans* and *At. ferrooxidans*		[39]
acetic acid, propionic acid and butyric acid[b]	0.375, 0.308 and 0.275	shake flasks	*At. ferrooxidans*		[39]
oxaloacetate, acetate and 2-ketoglutarate	0.033 (0.25 mM), 0.12 (5 mM) and 0.73 (5 mM)	shake flasks	*At. caldus*		[58]
methanethiol	0.031 (0.65 mM)	thermostated glass chamber		*Thioalkalivibrio* dominated mixed culture	[59]
phenol, benzene, methanol, and yeast extract	0.25, 1, 20 and 20	serum bottles		*Thioalkalivibrio versutus*	This study

[a] depending on the strain.
[b] ~ 94% inhibition.

4.1. Responses of chemolithoautotrophs towards organic compounds

The toxicity of organic compounds toward chemolithoautotrophic bacteria has mainly been reported for acidophilic iron and sulphur oxidizers and for haloalkaliphiles, only towards organosulphur compounds (Table 3). In both alkaliphiles and acidophiles, the pH of the cytosol must be maintained in the neutral range [49–51] whilst their environmental pH is drastically different. This allows a comparison of the effects of organic acids with different acid-base dissociation constants (pK_a) on biotransformation in a wide pH range.

Fig. 8 summarizes the responses of thiosulphate biotransformation to the organic compounds of this study. Thiosulphate biotransformation by *T. versutus* (Figs. S2 and S3) was inhibited at 0.25 g/L phenol, 0.5 g/L benzene, 20 g/L methanol and 20 g/L yeast extract. Xylose at 10–20 g/L reduced the rate of biotransformation whilst acetate up to 20 g/L had no inhibitory impact. Yeast extract at 2.5 and 5 g/L enhanced the growth of *T. versutus* and stimulated thiosulphate biotransformation. The highest rate of biotransformation was with 2.5 g/L yeast extract. Yeast extract as a nitrogen source for *Sulfurimonas gotlandica* [52], *Acidithiobacillus ferrooxidans* [53] and some strains of *Thiomicrospira* (synonym: *Thioalkalimicrobium*) [44] growth has been reported in several studies and was a likely mechanism also in this study. Sorokin et al. [54] also reported growth stimulation of various haloalkaliphilic *Thioalkalivibio* strains by yeast extract and peptone. This stimulatory effect of yeast extract has been reported even with mixed cultures oxidizing multimetal sulphidic ore [55]. Acetate increased the turbidity (OD_{600}) which would suggest growth stimulation and therefore, mixotrophy. However, the DAPI cell counts did not show growth enhancement. Some of the strains of haloalkaliphilic *Thiomicrospira* were also able to assimilate a limited amount of acetate [44]. The turbidity of the incubations with 10 g/L sterile and non-sterile filtered methanol developed similarly, indicating no contamination from analytical grade methanol. *T. versutus* produced elemental sulphur in the presence of non-inhibitory concentrations of the studied organics. The sulphur particles were similar as reported by ÓAquino et al. (Figs. S10-11) [15].

4.2. Possible mechanisms of inhibition by organic compounds

The inhibition by organic compounds may depend on several factors, including pK_a and the protonation of the organic compound [56,60], which is determined by the pH of the surrounding environment [42]. Based on the pK_a, a given acid is weak or strong and the weaker the acid is, its dissociation in aqueous solutions decreases. With increasing $pK_a >$ pH the protonation of organic compounds increases [60,61]. The protonated organic acids diffuse through the cytoplasmic membrane, dissociating within the neutral cytoplasm and dissipating the transmembrane pH gradient by proton accumulation [62,63]. At high concentrations, weak organic acids and also anions may penetrate the cell membrane and thus, accumulate in the cytosol [50]. Once the protonated acids have entered the cells, they dissociate into protons and corresponding ions, which leads to an increase in intracellular acidity and accelerates the metabolic disorders of the cells [50,64,65]. Further, chemolithoautotrophic microorganisms (both alkaliphiles and acidophiles) lack enzymatic pumps for transporting organic compounds from outside to inside or from inside to outside the cell membrane as well as the enzymatic machinery for heterotrophic catabolism. For these reasons at highly alkaline conditions, diffusion of non-ionized (protonated) organic compounds through the cell membrane is the most likely mechanism to become transported to the cytosol.

Table 4 shows that the inhibition of thiosulphate biotransformation at pH 10 increased by the increase of pK_a values, thus the decrease of dissociation of the organic compounds, except for phenol. Based on the pK_a values, acetate and yeast extract were dissociated at the medium pH. Due to its negative charge, acetate probably did not diffuse through the cell membrane of *T. versutus* [61] and, therefore, did not affect the biotransformation efficiency. The inflow of the ions of yeast extract (pK_a 4.84) was also similarly limited. The high molecular weight of yeast extract and D(+)-xylose may also be associated with their limited inhibitory effect on biotransformation [42]. Although phenol is poorly diffusible at pH 10, it was inhibitory for *T. versutus*. As the pK_a value of phenol (~10) equals the pH of the medium (~10); approximately half of it was dissociated and half undissociated. Phenol is toxic to microorganisms [2] and, therefore, the share of undissociated form that entered the cytosol at pH 10 was enough to [66] cause inhibition. Inhibition of *At. ferrooxidans* due to the electronegativity of simple organic compounds was reported by Tuttle et al. [67]. In their study, negatively charged simple organic compounds inhibited iron and sulphur oxidation due to an abiological reaction with Fe^{2+}. The thiosulphate biotransformation rates decreased with PFB of this study as compared to positive controls, which might be due to the decrease of pH during incubation increasing diffusion of some of the wastewater constituents through the cytoplasmic membrane [60].

In summary, the inhibitory effect of organic compounds is not due to chemolithoautotrophy *per se*, it is more affected by the environmental pH, which largely defines the entrance to the cytosol. Diffusion of protonated organic compounds poses a challenge to maintaining internal homeostasis both due to decreasing pH and accumulating organic anions in alkaliphilic bacteria. Once the organic compounds are in the cytosol, the chemolithoautrotrops do not have enzymological means for degrading or pumping out these compounds. Therefore, the non-

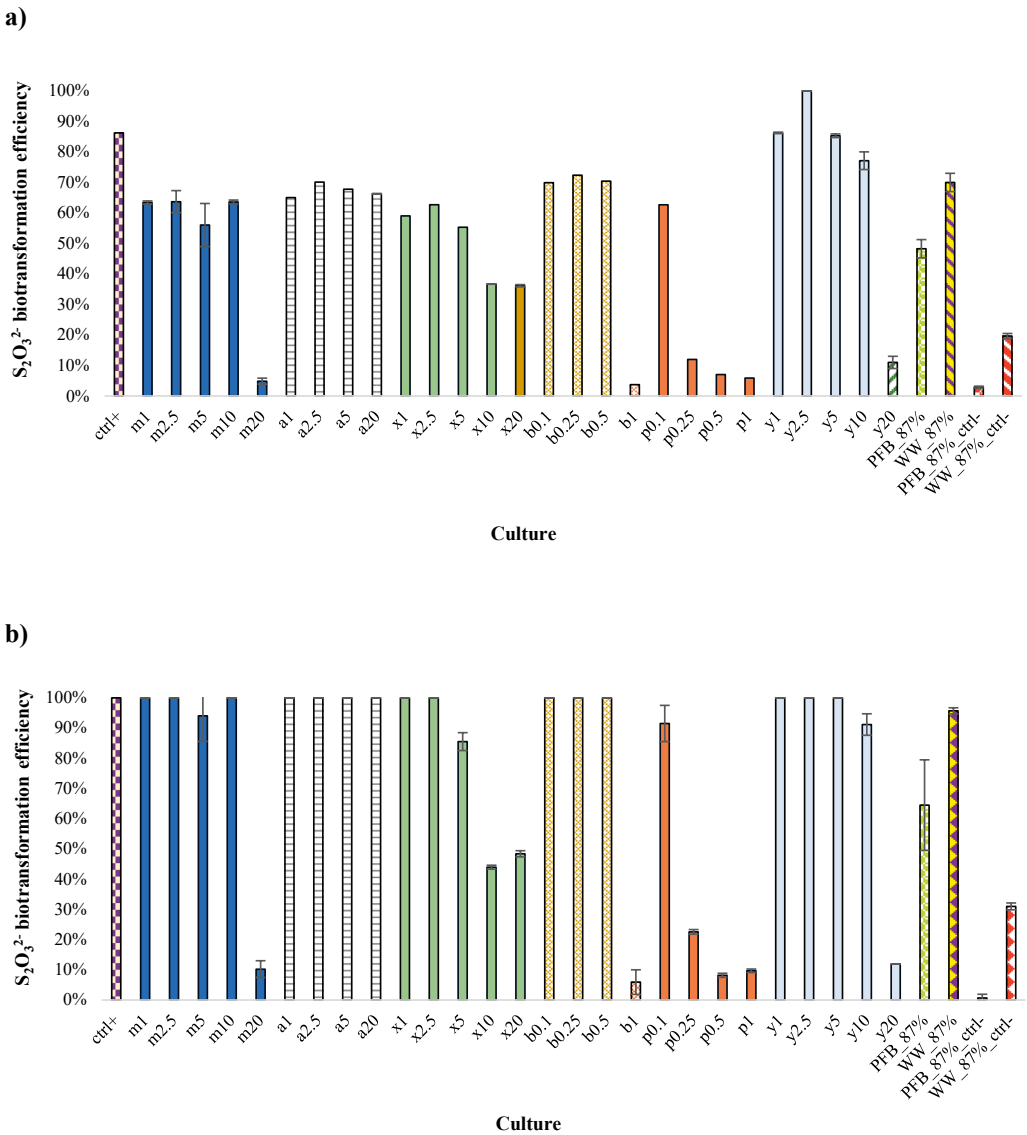

Fig. 8. Thiosulphate biotransformation efficiency by *T. versutus* in the presence of different organic compounds and wastewaters in 7 (a) and 10 (b) days. Some of the results were calculated, thus the standard deviations are missing. In the names, the letter indicates the compound, and the number is the concentration in g/L.

Table 4
pK_a values and molecular weight (MW) of tested organic compounds and their inhibition of thiosulphate biotransformation during this study. The darkness of the background colour in the compound row indicates increasing toxicity.

Compound	acetic acid	yeast extract	D-xylose	methanol	benzene	phenol
pK_a	4.76	4.84	12.14	15.5	43	9.99
MW (g/mol)	59	274*	150	32	78	94

* Molar weight was taken from: https://pubchem.ncbi.nlm.nih.gov/compound/Bakers-yeast-extract.

inhibition of *T. versutus* towards organic compounds is probably due to the high environmental pH (~10) that efficiently limits the access of these compounds through the cell membrane.

4.3. The applicability of biological sulphur recovery from organic solutions

Haloalkaliphilic *T. versutus* and *T. denitrificans* have been demonstrated to recover elemental sulphur from reduced and partially oxidized sulphur oxyanions at yields ranging from 25 to 86% [15–17,68–70] and thus, indicate the potential for developing sulphur recovery processes from industrial process and waste streams. These waste streams contain various organic compounds that may affect the activity of these haloalkaliphilic SOB.

The results of this study demonstrated the high resistance of *T. versutus* to organic compounds. Phenol and benzene were inhibitory at 0.25 and 0.5 g/L, respectively, and thus, not of concern in P&P mill wastewaters [29]. As the access of organic compounds to the cytosol depends on the pH of the medium, maintaining it high (~pH 10) is important. High pH at the same time decreases the competition for oxygen by chemoheterotrophs.

The two wastewaters (PFB and WW) did not inhibit thiosulphate biotransformation and therefore, would not require dilution. P&P wastewaters that contain sulphate would be also potent streams for biological sulphur recovery by haloalkaliphilic SOB. In this case, the first step would involve sulphate reduction [13] and followed by sulphide oxidation by haloalkaliphilic SOB. This kind of two-step process has been already used on industrial scale (SULFATEQ®, Paques) for example, with acid-mine wastewaters using non-haloakaline SOB.

5. Conclusions

This study demonstrated the non-inhibition of haloalkaliphilic chemolithoautotrophic *T. versutus* towards pulp and paper mill wastewaters and their constituents. From the studied compounds and wastewaters, only yeast extract (2.5–5 g/L) enhances thiosulphate biotransformation by *T. versutus*. Yeast extract (2.5–5 g/L) also stimulated microbial growth serving likely as a nutrient source. D(+)-xylose > 5 g/L decreases biotransformation efficiency while phenol, benzene, methanol and yeast extract inhibits growth and biotransformation at 0.25, 1, 20 and 20 g/L. Acetate (0.1–20 g/L) and composite pulp and paper mill wastewater have no effect, whilst primary filtrate of bleaching partially decreases the rate of thiosulphate biotransformation. High environmental pH (~10) probably limits the access of the studied compounds through the cell membrane and thus, decreases their inhibitory effects. Organic compounds present in P&P wastewaters, and the primary filtrate of bleaching and composite wastewater from sewer are non-inhibitory for *T. versutus* and, therefore, has potential for use in recovering elemental sulphur from these liquid streams. In summary, biological sulphur recovery from organic-rich sulphurous wastewaters and process streams by chemolithoautotrophic bacteria has great future potential.

Funding

This work was supported by the Doctoral School of Tampere University.

CRediT authorship contribution statement

Réka Hajdu-Rahkama: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Visualization, Writing - original draft. **Jaakko A. Puhakka:** Conceptualization, Methodology, Supervision, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The financial support of the Doctoral School of Tampere University is highly appreciated. We would like to thank the analytical help of Donya Kamravamanesh (Ph.D.) and Hui Yao (M.Sc.) from Tampere University. The constructive comments by the anonymous reviewers are also acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2022.137972.

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Supplementary Material

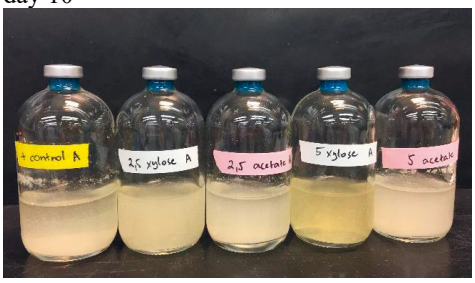
High tolerance of chemolithoautotrophic sulphur oxidizing bacteria towards pulp and paper mill wastewaters and their organic constituents supporting sulphur recovery in alkaline conditions

Réka Hajdu-Rahkama*[a] and Jaakko A. Puhakka[a]

[a] Tampere University, Faculty of Engineering and Natural Sciences, Bio- and Circular Economy Research Group, P.O. Box 541, FI-33014 Tampere University, Finland

*Corresponding author. Email address: reka.hajdu-rahkama@tuni.fi

13 figures. 1 table

0.1-1 g/L phenol	0.1-1 g/L benzene
day 0	day 0
	
day 10	day 10
	
2.5-5 g/L xylose and acetate	10-20 g/L xylose and acetate
day 0	day 0
	
day 10	day 10
	

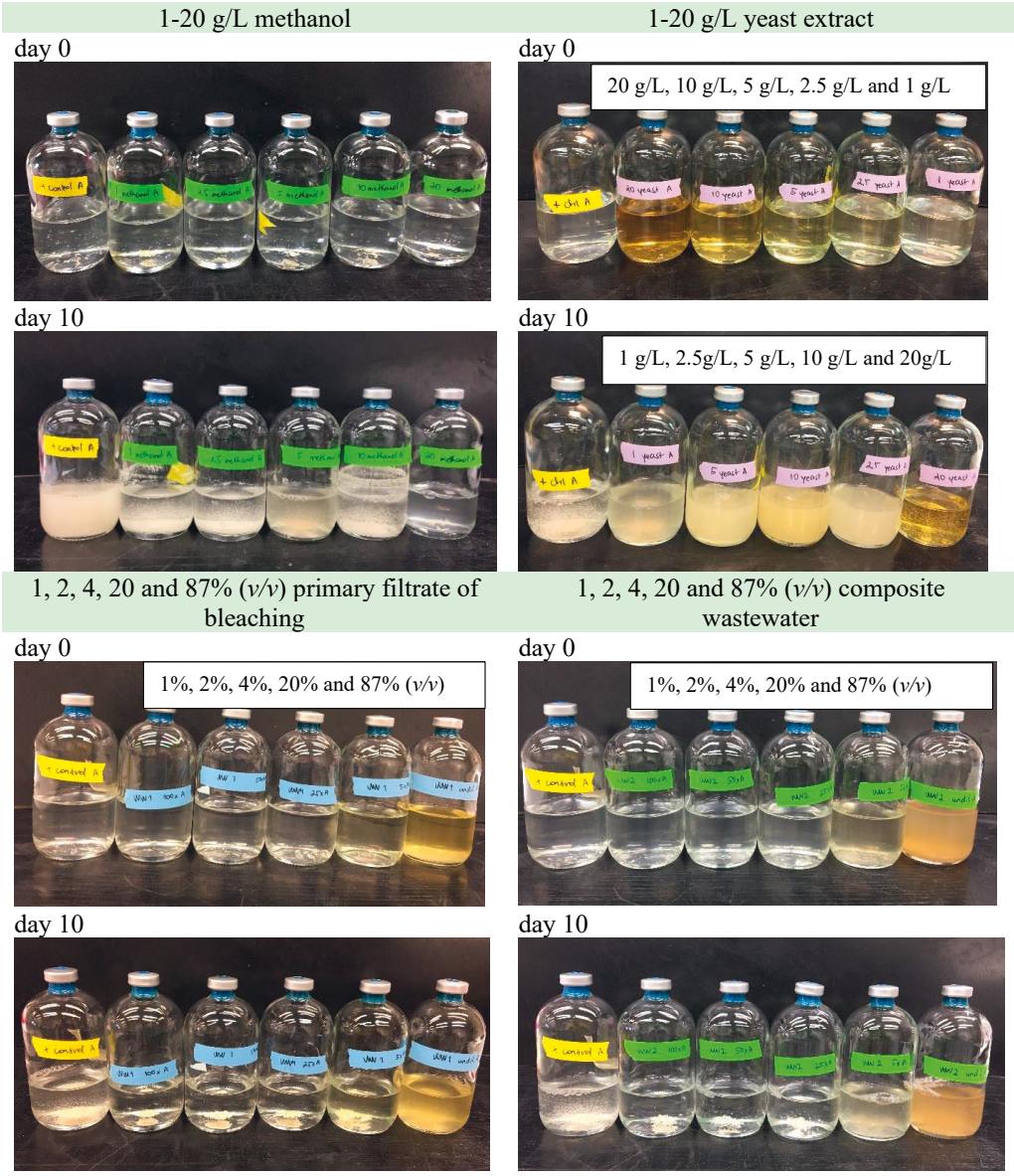


Fig. S1: Photos taken from the *Thioalkalivibrio versutus* incubations on thiosulphate with different organic compounds and P&P wastewaters on days 0 and 10.

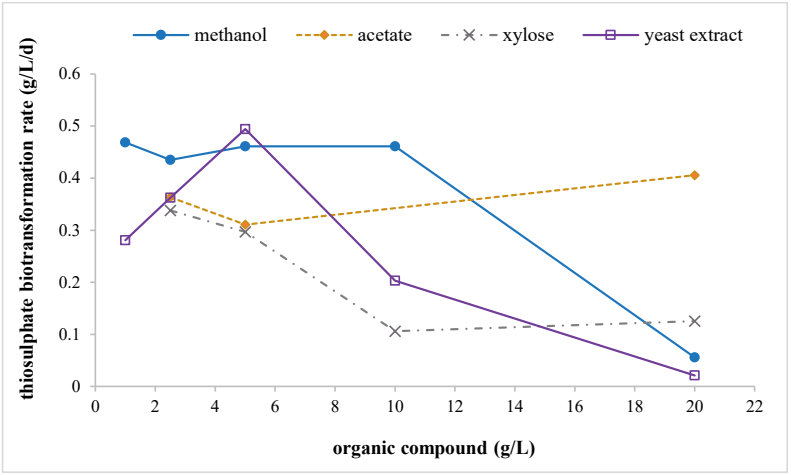


Fig. S2. Rate of thiosulphate biotransformation by *Thioalkalivibrio versutus* in the presence of methanol, acetate, xylose and yeast extract.

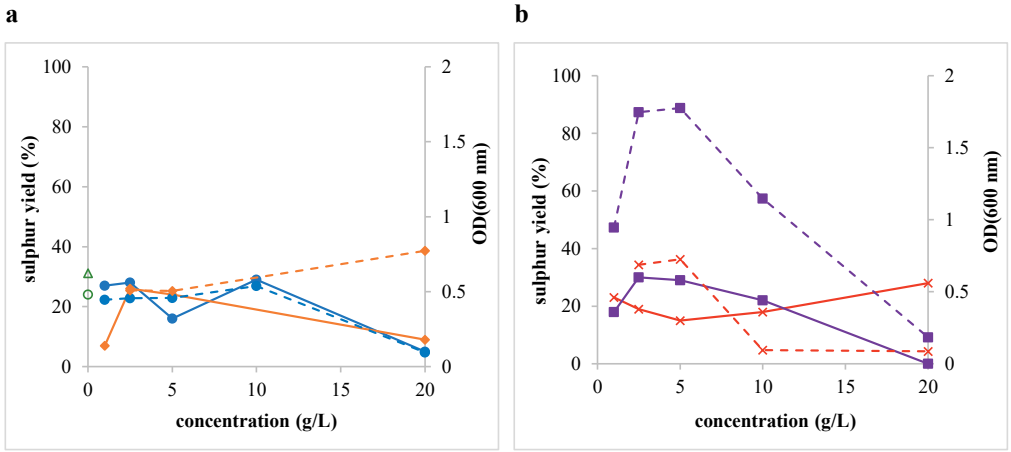


Fig. S3. Comparison of highest S^0 formation yields (continuous lines) and highest optical densities (OD_{600}) (dashed lines) by *Thioalkalivibrio versutus* grown on thiosulphate with different concentrations of (a) methanol (●) and acetate (◆); (b) xylose (×) and yeast extract (■). The S^0 yield (○) and OD_{600} (△) of inoculated controls are also shown.

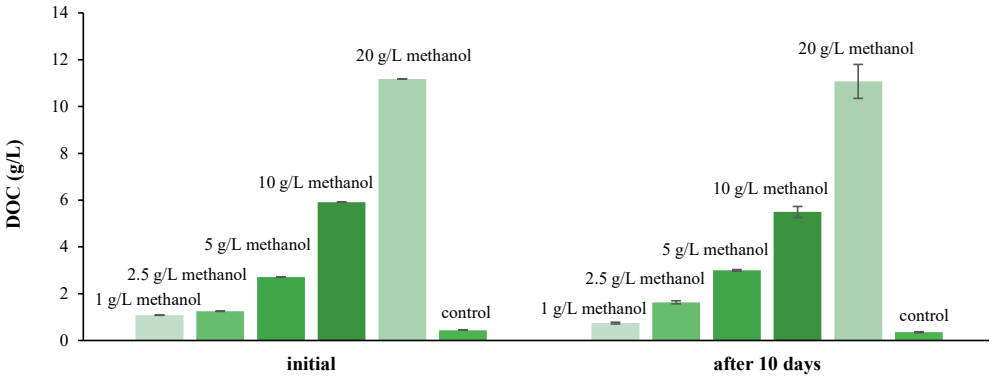


Fig. S4: Initial and final DOC concentrations during incubation of *T. versutus* with thiosulphate and methanol concentrations.

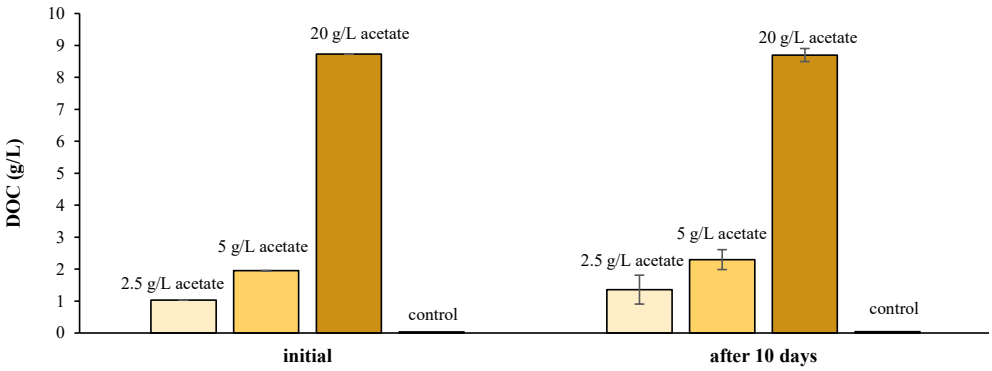


Fig. S5: Initial and final DOC concentrations during incubation of *T. versutus* with thiosulphate in the presence of different acetate concentrations.

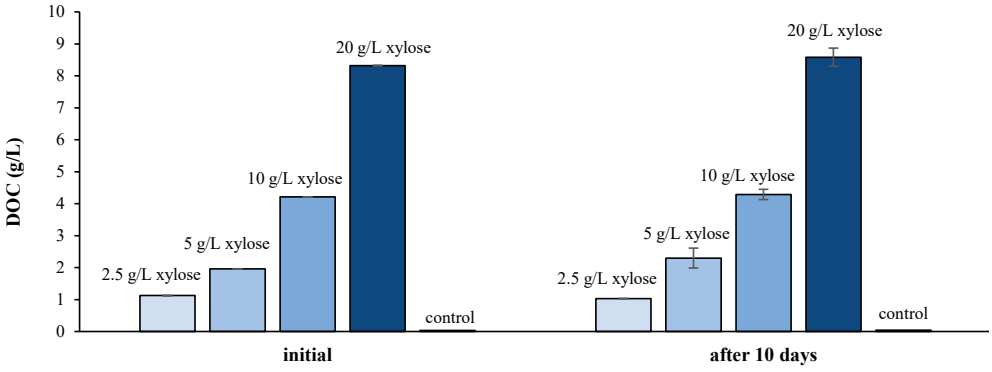


Fig. S6: Initial and final DOC concentrations during incubation of *T. versutus* with thiosulphate in the presence of different xylose concentrations.

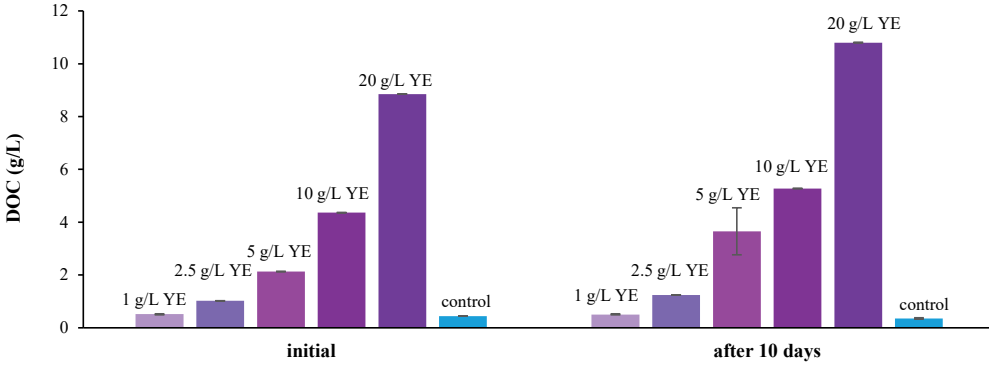


Fig. S7: Initial and final DOC concentrations during incubation of *T. versutus* with thiosulphate in the presence of different yeast extract (YE) concentrations

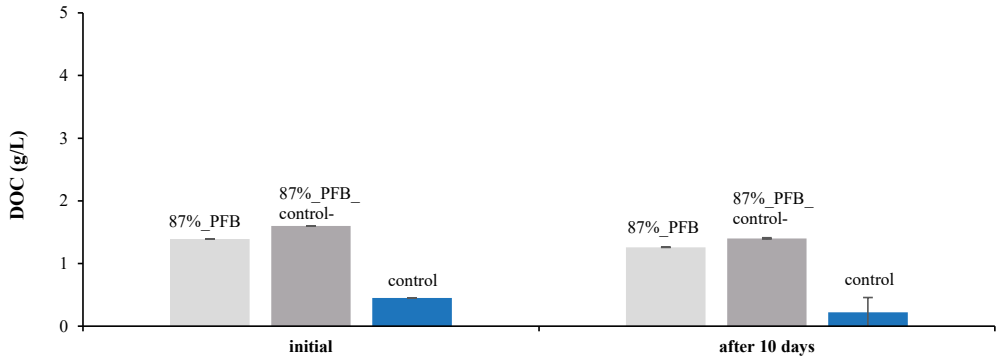


Fig. S8: Initial and final DOC concentrations during incubation of *T. versutus* with thiosulphate ain the presence primary filtrate of bleaching PFB.

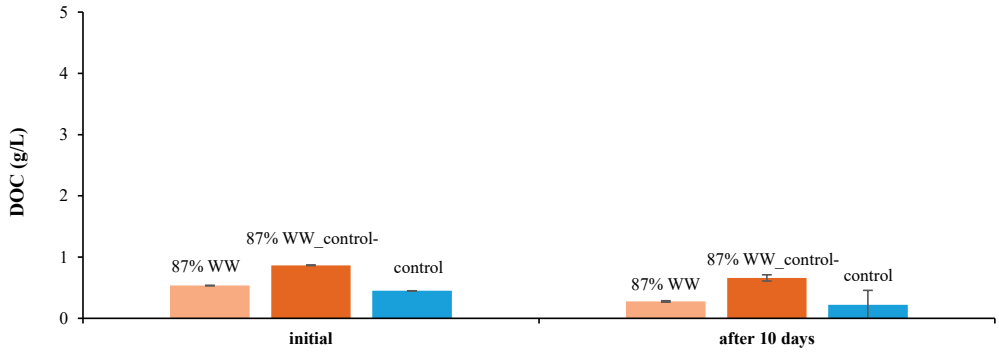


Fig. S9: Initial and final DOC concentrations during incubation of *T. versutus* with thiosulphate and composite wastewater (WW) concentrations.

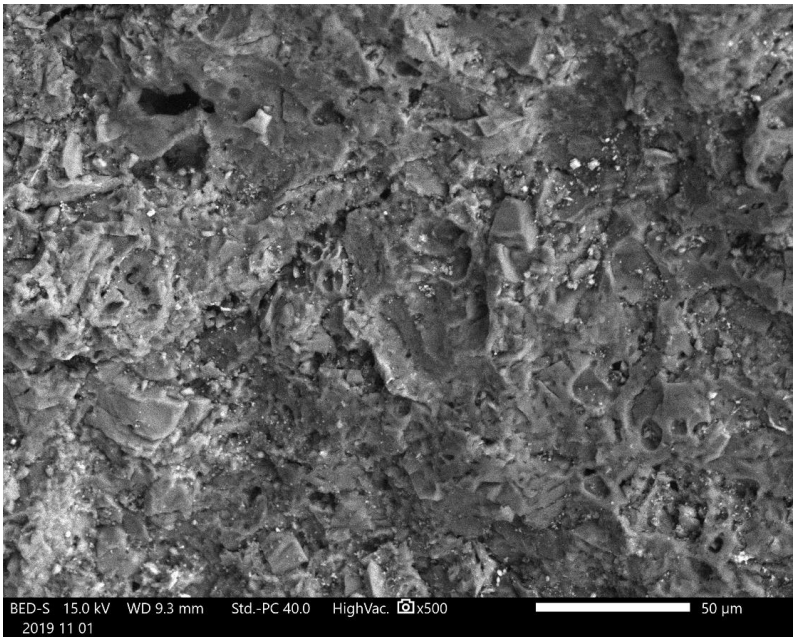


Fig. S10: Scanning electron microscope (SEM) image of the sulphur formation from thiosulphate by *Thioalkalivibrio versutus* on activated carbon carrier. Particle analysis and biomass visualization were performed with scanning electron microscopy (SEM, Jeol JSM-IT500, Japan) equipped with energy-dispersive X-ray spectrometer (EDS). The sample preparation was according to [1].

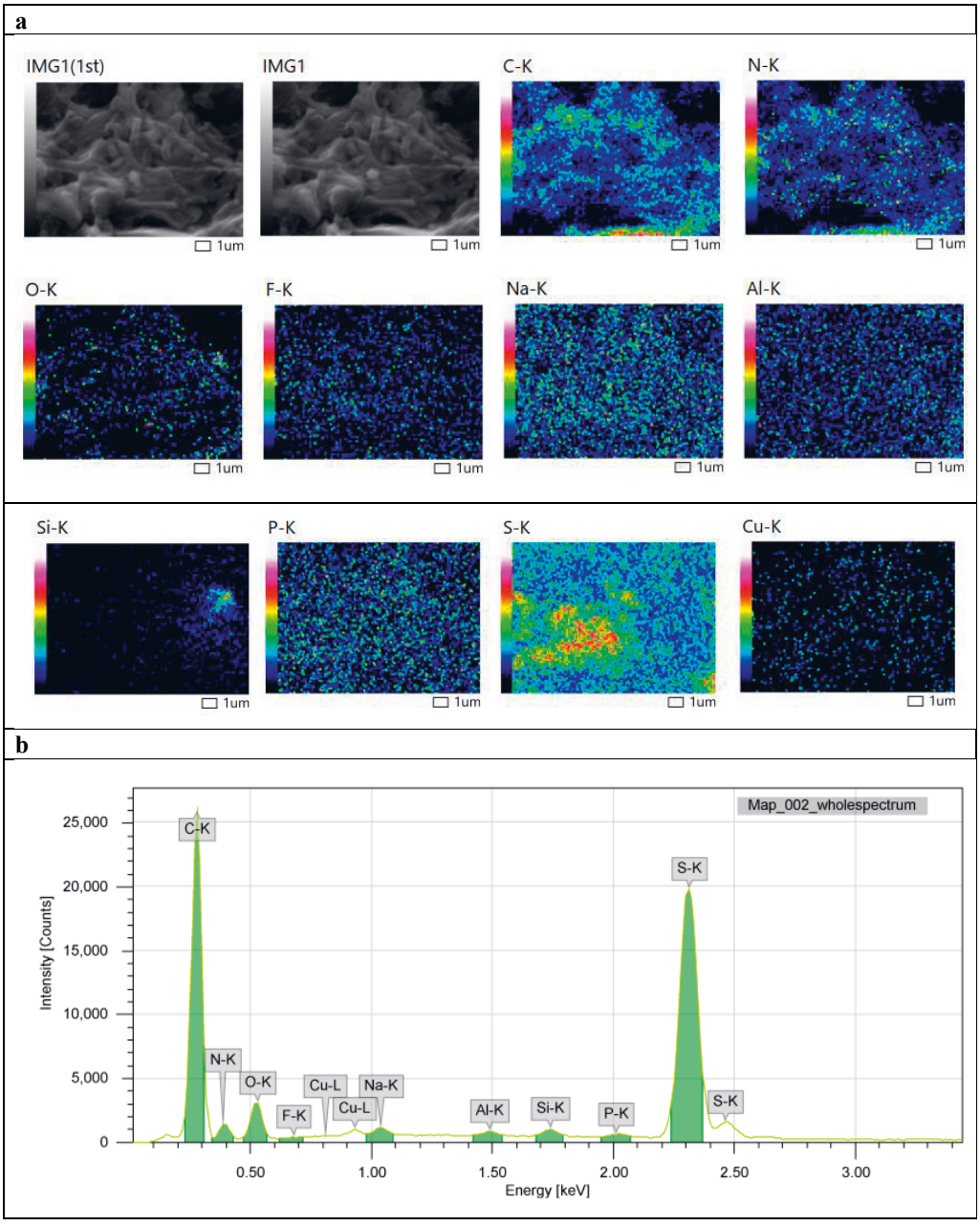
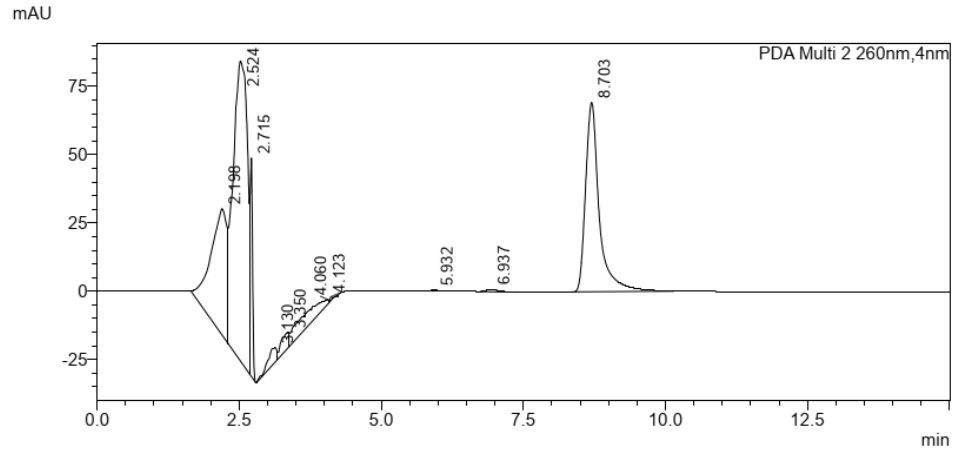


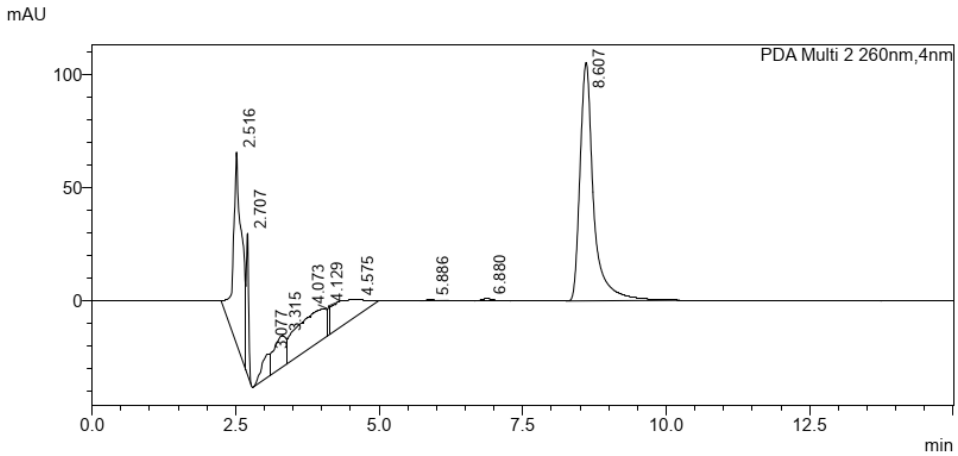
Fig. S11: Scanning electron microscope (SEM) images of the quantitative analysis of the elements present in *Thioalkalivibrio versutus* grown on thiosulphate fed FBR. The original

image examined is on the top left. a) concentrations of certain elements increase by changing colour from black to white (see colour bar on right side of the small images); b) elemental map. The first letters indicate the element and the “K” are K(alpha) radiation. The analytical device used was Jeol JSM-IT500 scanning electron microscope (Japan) equipped with energy-dispersive X-ray spectrometer (EDS). A detailed description of sample preparation prior to SEM can be found in D´Aquino et al. [1].

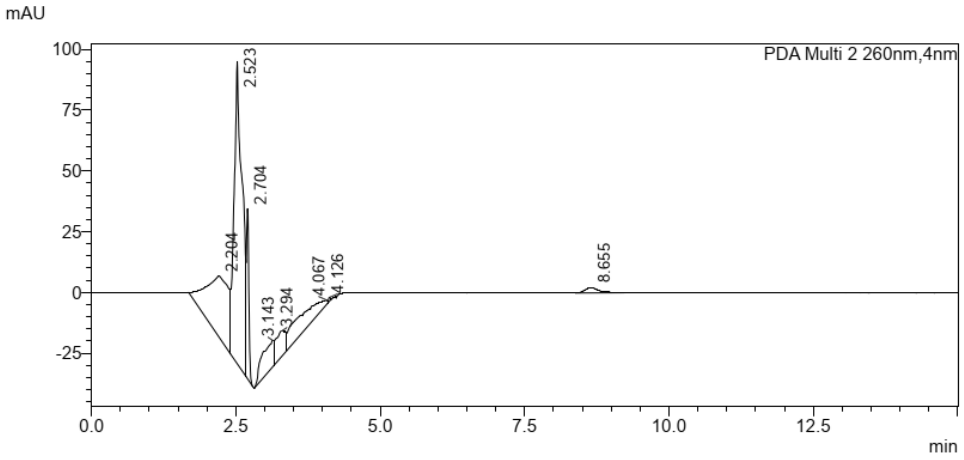
a



b



c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PDA Multi 2 260nm,4nm
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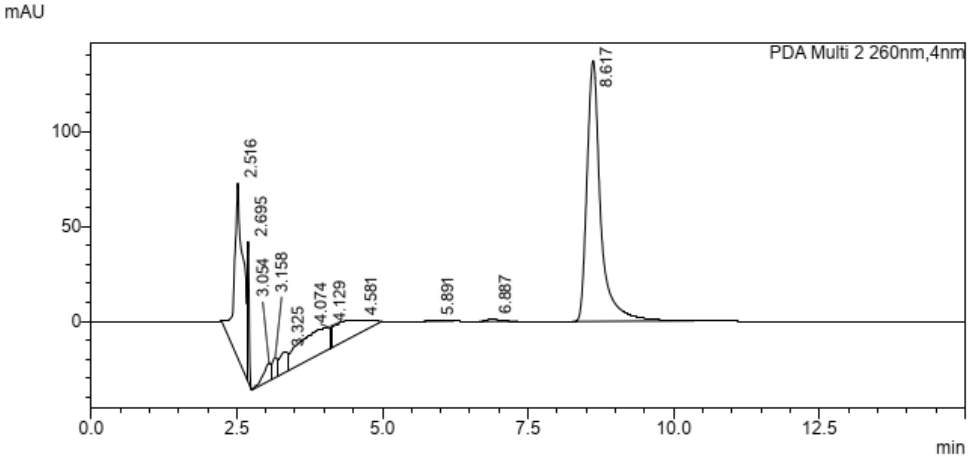
2.704
2.204
3.143
3.294
4.067
4.126
8.655
min

d


mAU
PDA Multi 2 260nm,4nm
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2.695
3.054
3.158
3.325
4.074
4.129
4.581
5.891
6.887
min

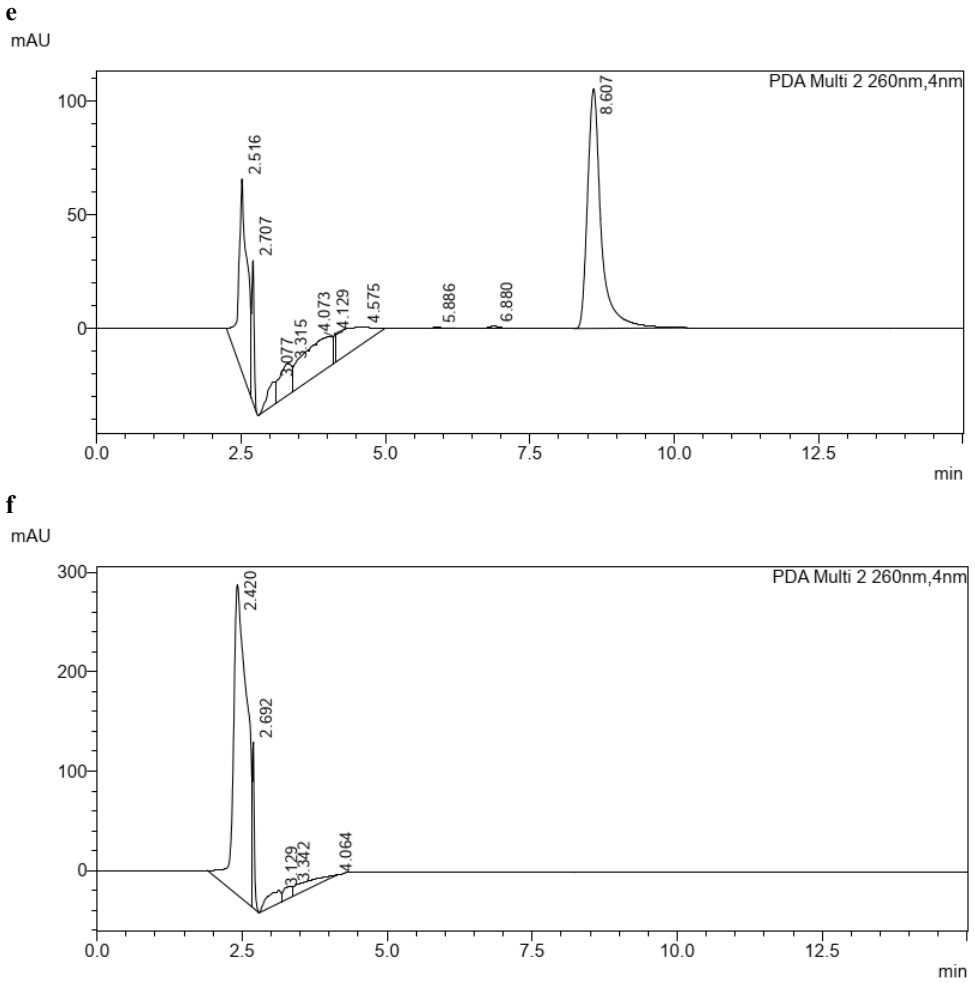

Fig. S12: High performance liquid chromatographs (HPLC) showing elemental sulphur in the samples with a) 20 g/L acetate, b) 10 g/L methanol, c) positive control, d) abiotic elemental sulphur, e) biosulphur from earlier experiments with *T. versutus* and f) Milli-Q water. 1 mL samples were taken from13 days old acetate, methanol and positive control assays.

Table S1: Total cell counts determined by doing DAPI (4′,6-diamidino-2-phenylindole) staining and counting with epifluorescence microscopy

Organic compound	Initial log (cell/mL)	day 7 log (cell/mL)
5 g/L xylose	4.56	9.91
20 g/L acetate	4.58	9.95
10 g/L methanol	4.56	9.94
+control	4.56	9.92

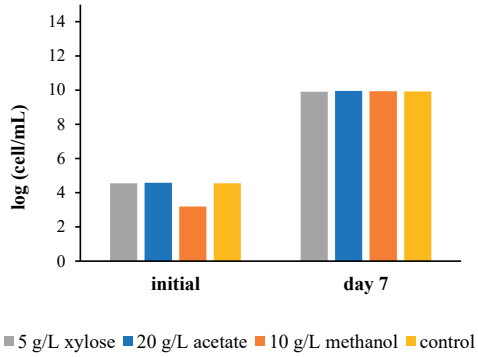

Fig. S13: Graphical visualization of total cell counts determined by doing DAPI (4′,6-diamidino-2-phenylindole) staining and count epifluorescence microscopy of initial and end-point (day 7) samples.

Reference:

[1] A. D'Aquino, R. Hajdu-Rahkama, J.A. Puhakka, Elemental sulphur production from thiosulphate under haloalkaline conditions in a *Thioalkalivibrio versutus* amended fluidized bed bioreactor, Biochem. Eng. J. 172 (2021) 108062. doi:10.1016/j.bej.2021.108062.