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

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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

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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 mill's 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

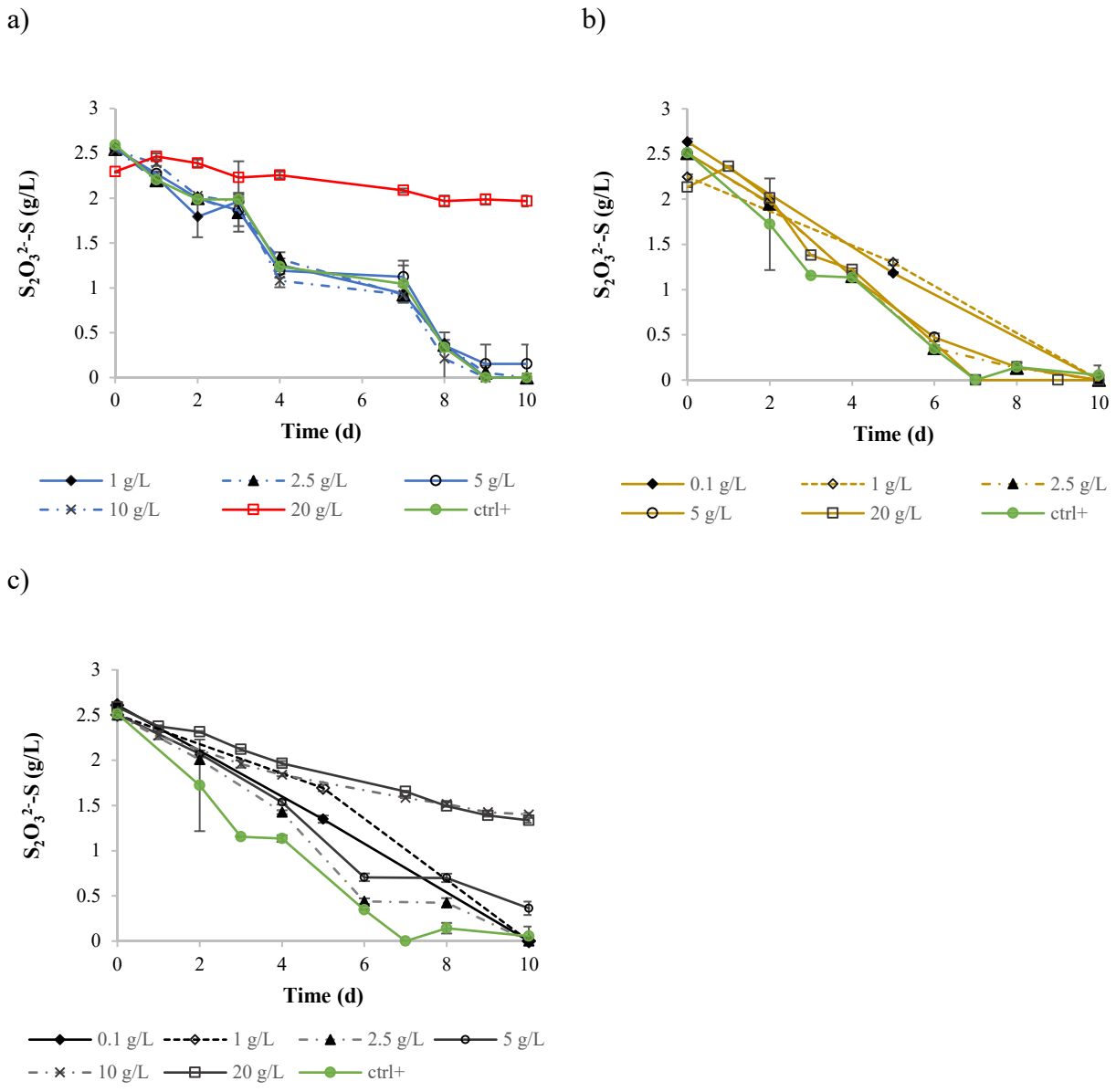

Fig. 1. The effects of (a) methanol, (b) acetate and (c) D-(+) xylose on thiosulphate biotransformation by *T. versutus*. The standard deviations are calculated from duplicate cultures.

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.

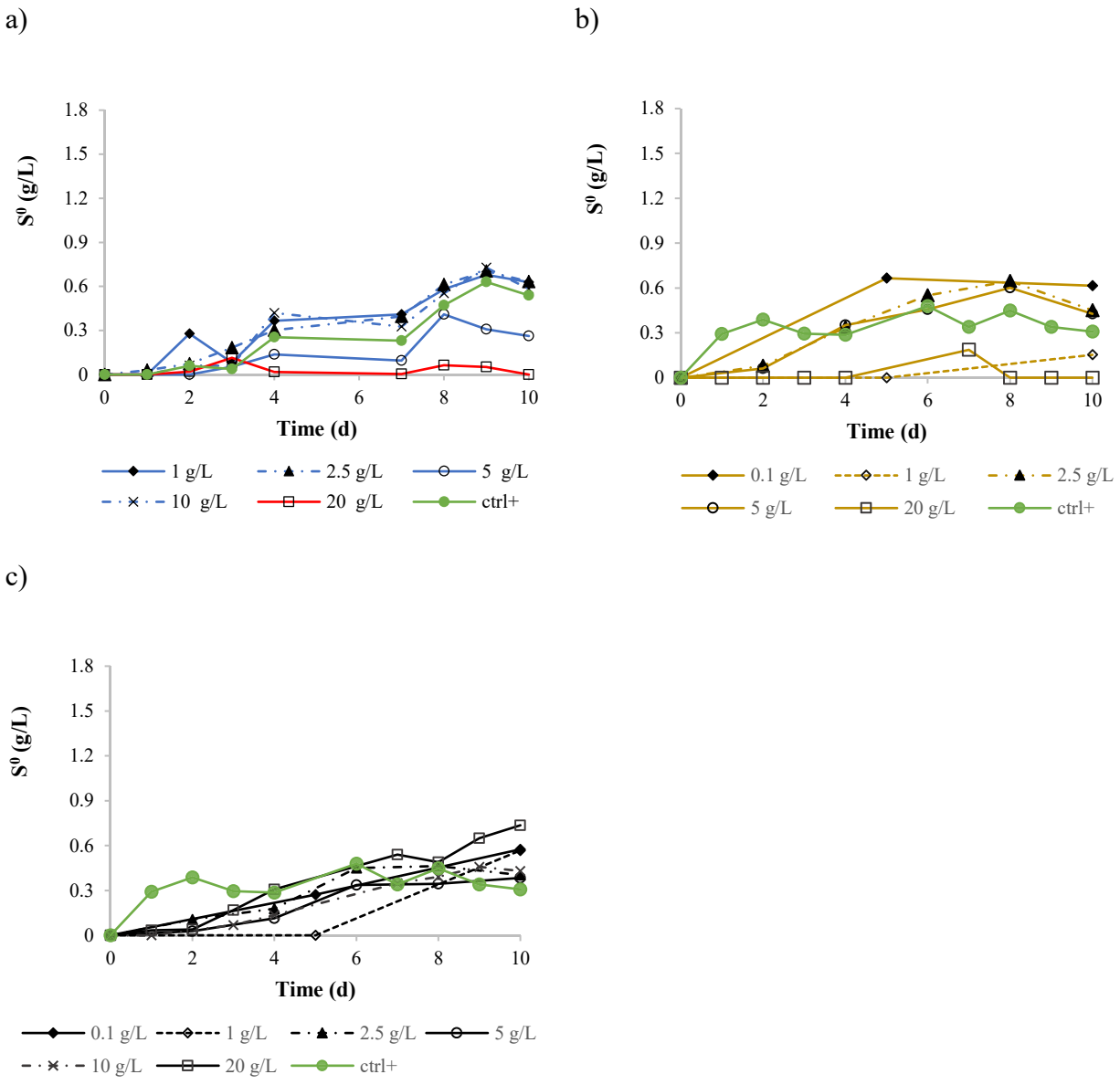

Fig. 2. Estimated biological S^0 production in the presence of methanol (a), acetate (b) and D(+)-xylose by *T. versutus*. The concentrations are calculated values and, thus without standard deviations.

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).

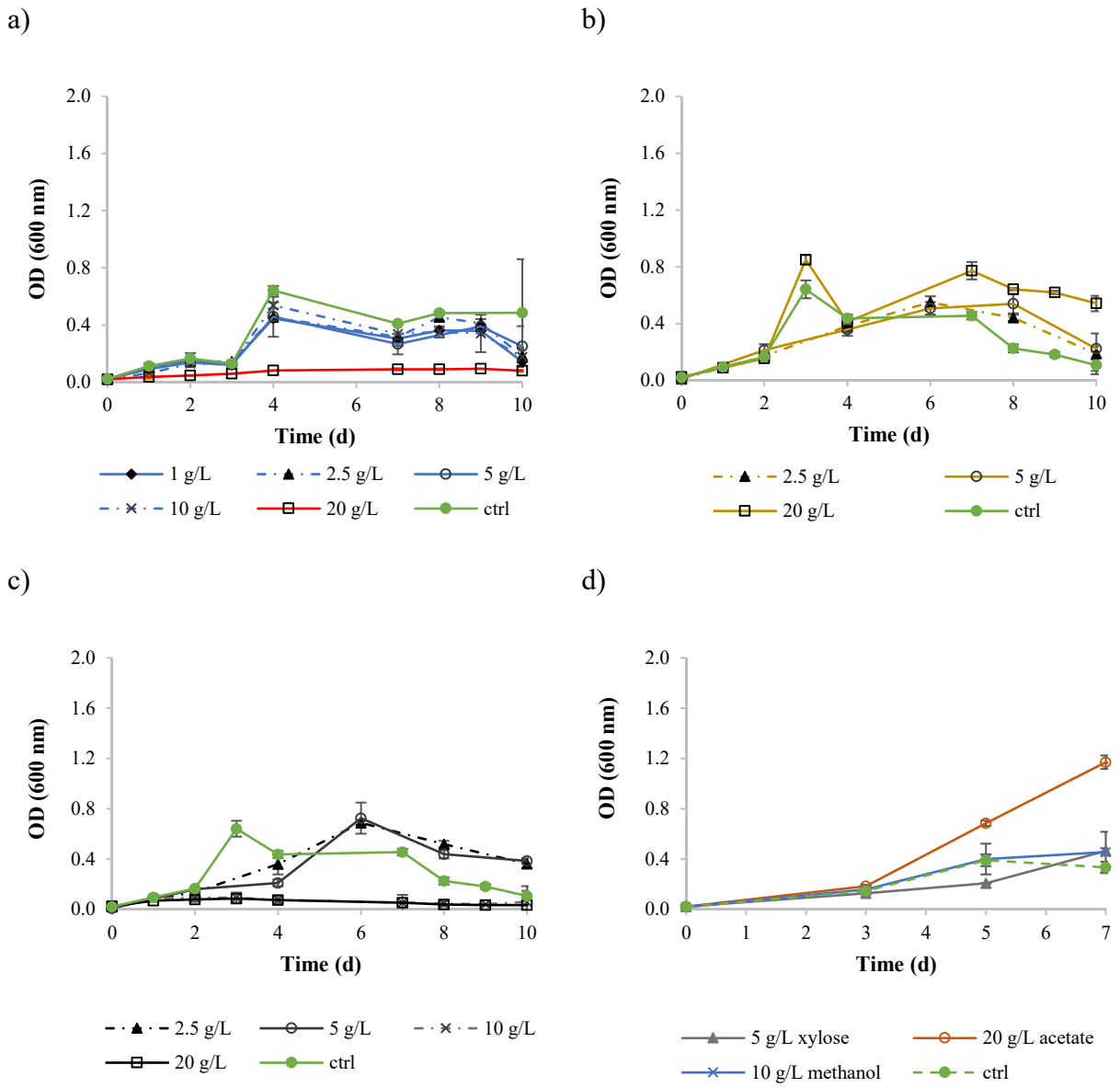

Fig. 3. Changes in optical density (OD_{600}) during thiosulphate incubation of *T. versutus* in the presence of methanol (a), acetate (b) and D-(+) xylose (c). OD_{600} of additional incubations with sterile methanol, acetate and xylose (d) to eliminate contamination and investigate cell growth. The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

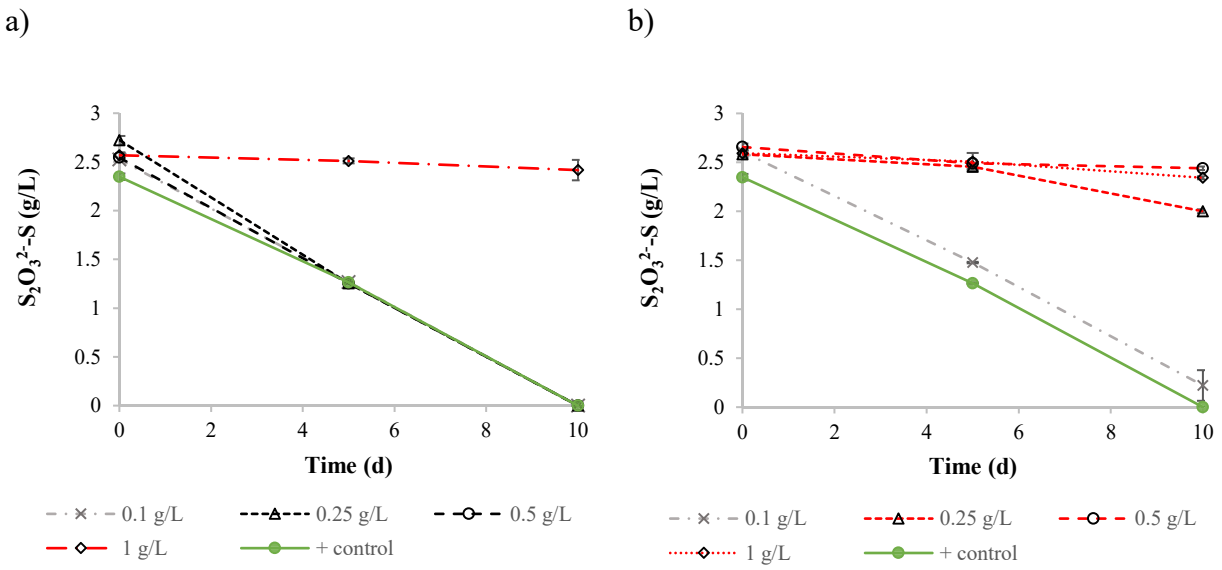

Fig. 4. Thiosulphate biotransformation in the presence of (a) benzene and (b) phenol. The standard deviations are from duplicate cultures.

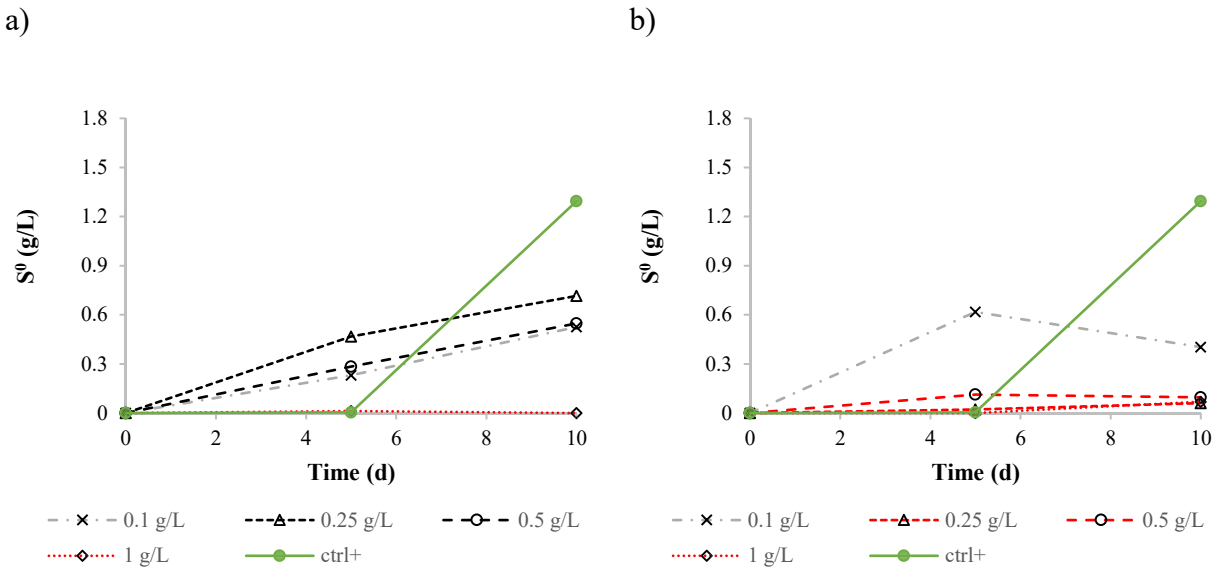

Fig. 5. Calculated biological S^0 production from thiosulphate in the presence of benzene (a) and phenol (b). The concentrations are calculated values, thus without standard deviations.

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

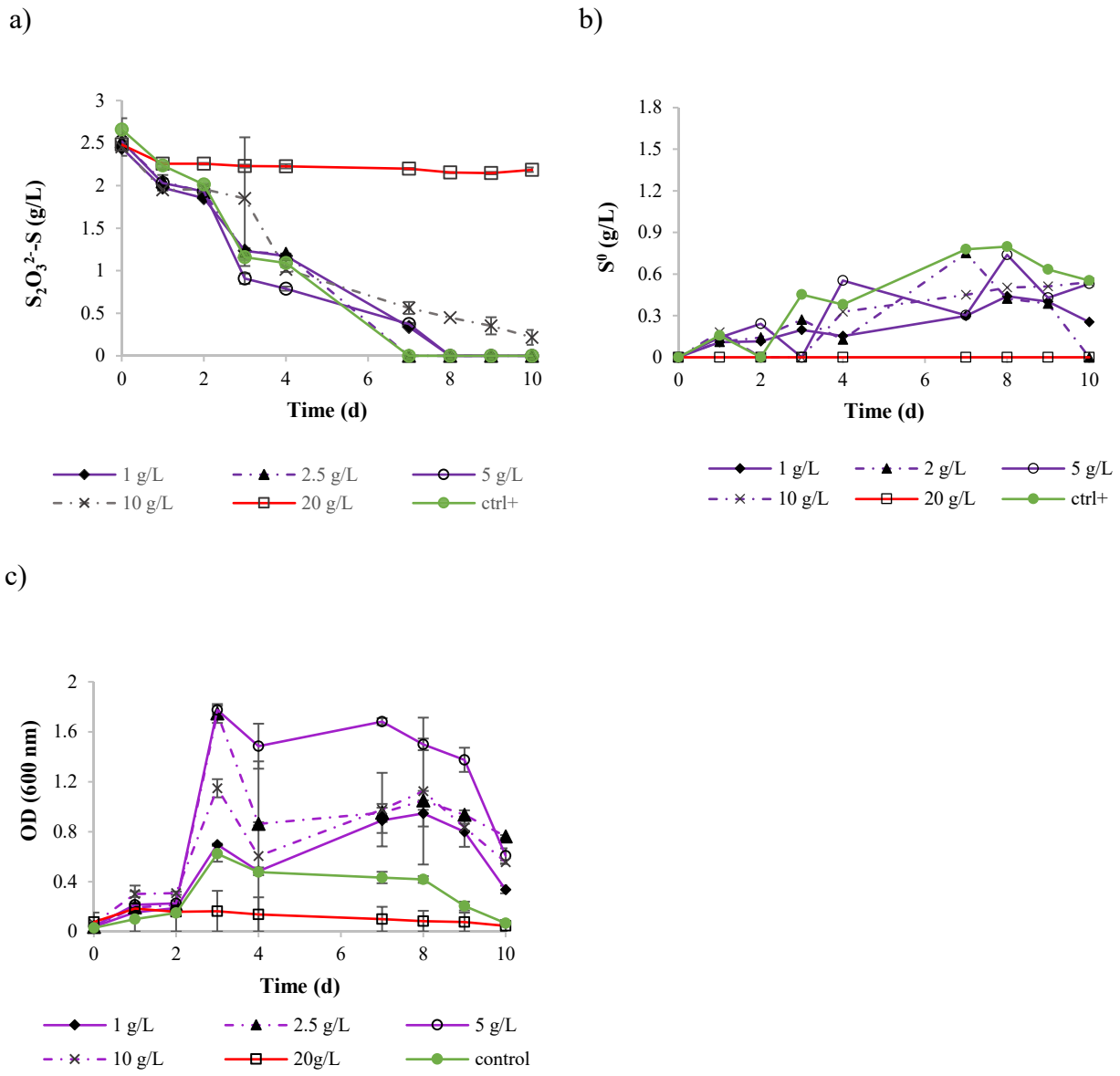

Fig. 6. Thiosulphate biotransformation by *T. versutus* (a), estimated S^0 formation (b) and changes of optical density in the presence of different concentrations (1–20 g/L) of yeast extract. The standard deviations of biotransformation and optical densities are calculated from duplicates. The S^0 concentrations 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

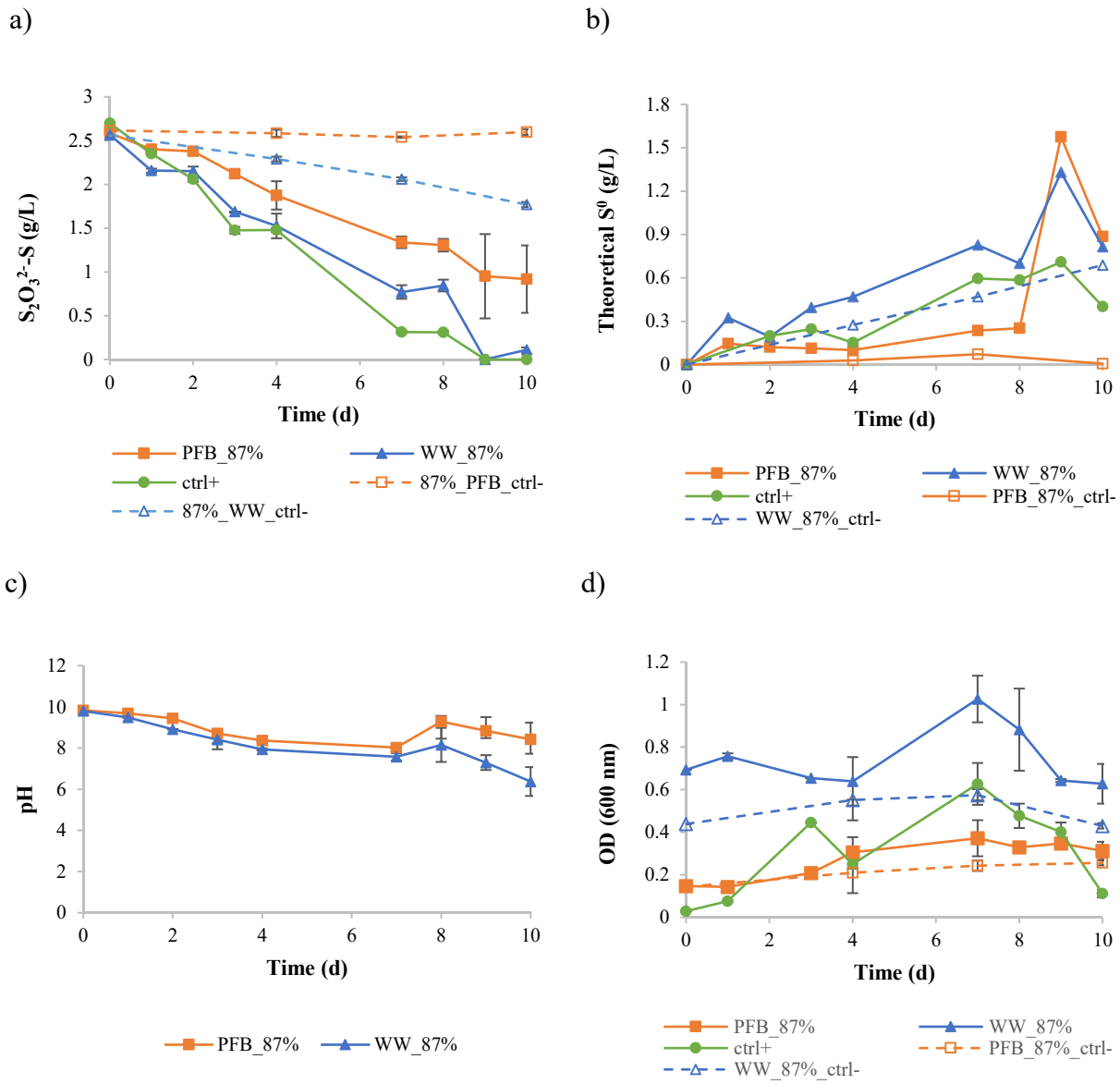

Fig. 7. Thiosulphate biotransformation (a) in 87% concentrated primary filtrate of bleaching (PFB_87%) and mixed wastewater from the main sewer (WW_87%). Positive (ctrl +) and non-inoculated (ctrl-) controls are also included. The theoretical S^0 production (b) in the wastewaters was calculated as missing 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 ĎAquino 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-

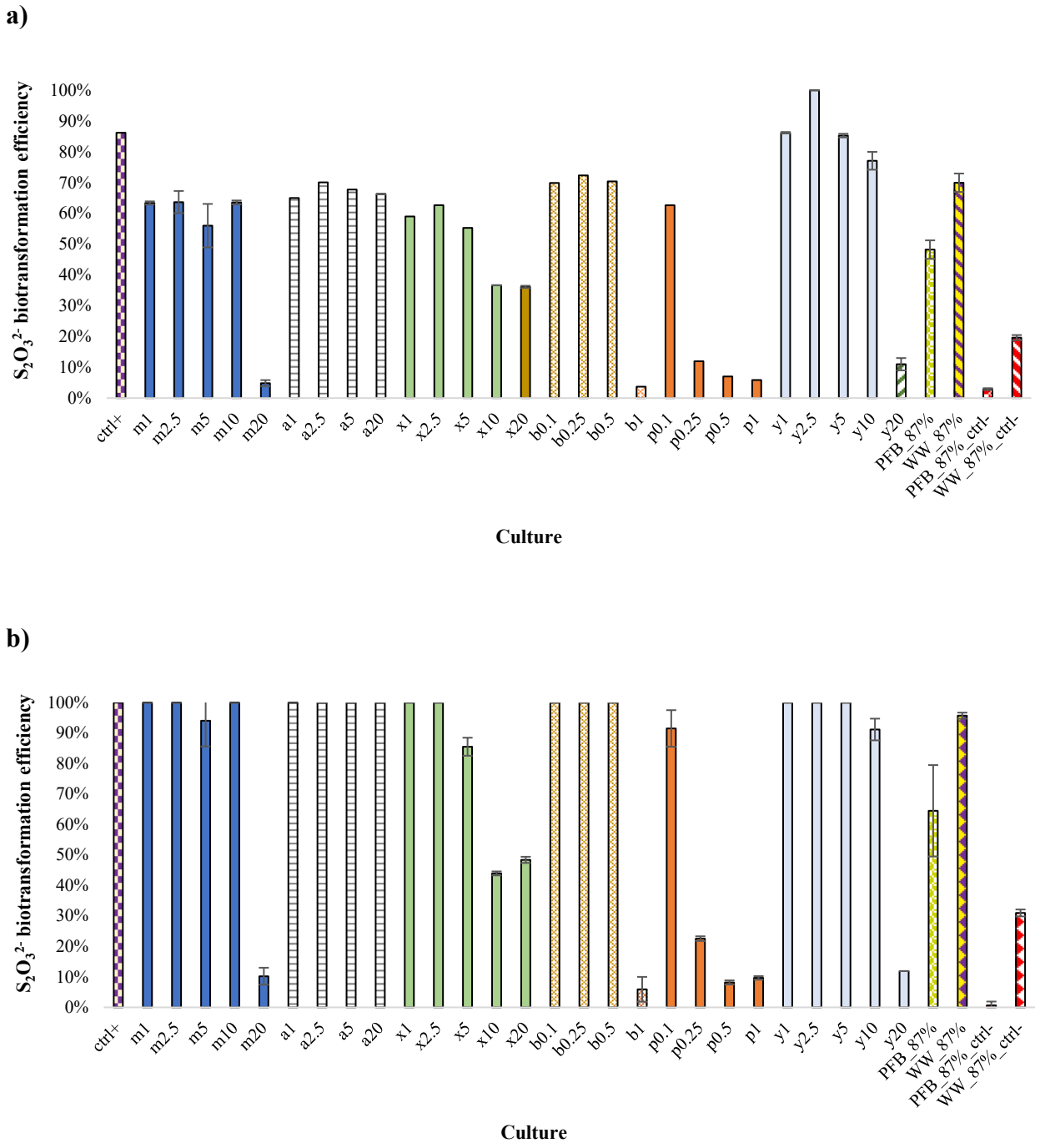

Fig. 8. Thiosulphate biotransformation efficiency by *T. versutus* in the presence of different organic compounds and wastewaters in 7 (a) and 10 (b) days. Some of the results were calculated, thus the standard deviations are missing. In the names, the letter indicates the compound, and the number is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

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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.

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Appendix A. Supplementary data

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