

## Selection of sulfur oxidizing bacterium for sulfide removal in sulfate rich wastewater to enhance biogas production

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**Abbreviations:** BOD: biochemical oxygen demand  
COD: chemical oxygen demand  
DS: dissolved sulfide  
H<sub>2</sub>S: hydrogen sulfide  
MSM: mineral salts medium  
MPB: methane producing bacteria  
ROW: raw optimized wastewater  
SOB: sulfur oxidizing bacteria  
SOW: sterile optimized wastewater  
SRB: sulfate reducing bacteria  
SRR: a sulfate reduction reactor  
SEM: scanning electron microscope  
TKN: total Kjeldahl nitrogen  
TS: total sulfide  
TSY: thiosulfate yeast extract medium  
UASB: upflow anaerobic sludge blanket  
VFAs: volatile fatty acids

**Sulfur oxidizing bacteria (SOB) were isolated and tested in order to remove sulfide from high sulfate wastewater to reduce the amount of hydrogen sulfide (H<sub>2</sub>S) in the produced biogas. A promising SOB isolate, designated as isolate T307, was selected due to its best sulfide removal (86.7%) in the effluent of a sulfate reduction reactor (SRR) over a 24 hrs incubation. The bacterium was able to grow better as a mixotroph (yeast extract as a carbon source) than as a chemolithoautotroph. In addition, as a heterotroph, the bacterium grew well with yeast extract and peptone. Based on partial 16S rRNA gene sequence, the isolated T307 was an *Alcaligenes* sp. and was able to convert most of sulfide species (total sulfide: TS; dissolved sulfide: DS and H<sub>2</sub>S) into elemental sulfur or sulfate over a 20 hrs period of cultivation by controlling the speed of shaking. In a biogas reactor set, after pre-treating a sulfide medium with *Alcaligenes* sp. T307 there was a much higher specific yield of CH<sub>4</sub> (238 ml CH<sub>4</sub> g<sup>-1</sup>COD removed) and more biogas (154 ml L<sup>-1</sup> d<sup>-1</sup>) was produced with the biogas containing more methane (48.1% CH<sub>4</sub>, 51.5% CO<sub>2</sub> and 0.41% H<sub>2</sub>S) in comparison to a control with a specific yield of CH<sub>4</sub> (72 ml CH<sub>4</sub> g<sup>-1</sup>COD removed) 86 ml L<sup>-1</sup> d<sup>-1</sup> biogas produced with a composition of 35.5% CH<sub>4</sub>, 63.7% CO<sub>2</sub> and 0.86% H<sub>2</sub>S.**

There are numerous latex rubber factories in southern Thailand and anaerobic treatment, particularly in lagoons, is the most commonly used process for treating their wastewater (Kantachote et al. 2005). The anaerobic wastewater system provides many advantages such as, a low cost operation with high efficiency and also with the possibility of producing a useful energy source biogas; however, sulfide is generated during anaerobic treatment of high sulfate wastewater, a characteristic of wastewater from rubber factories. An advanced system such as the sulfate reduction reactor (SRR) has been used to treat sulfate rich rubber wastewater from processes of concentrated latex and skim crape (Tekasakul and Tekasakul, 2006). However, high sulfide levels are produced due to sulfate being used as a terminal electron acceptor in anaerobic respiration. The SRR is a step to reduce sulfate concentration in wastewater prior to transferring the wastewater to an UASB (upflow anaerobic sludge blanket) for obtaining biogas as a by-product. Nevertheless, biogas produced from both the SRR and the UASB do not meet the standard of biogas composition because of their high contaminations of H<sub>2</sub>S. Therefore, the biogas was burnt to remove the very toxic and corrosive H<sub>2</sub>S gas that has a very low odor threshold of 1 µg L<sup>-1</sup> (Oyarzun et al. 2003). Hence, introducing one more step to convert sulfide to sulfur by partial oxidation instead of by completely oxidizing it to sulfate could be an interesting alternative method for solving this problem. It is realized that levels of sulfide oxidation are dependent on oxygen concentration (Gonzalez Sanchez et al. 2005). It has long been recognized that bacteria able to oxidize reduced sulfur compounds can be used to remove contaminating

H<sub>2</sub>S, from either treated wastewater or gaseous systems (Sublette et al. 1998; Cha et al. 1999; Kleerebezem and Mendez, 2002; Chung et al. 2003). Removal of H<sub>2</sub>S from any SRR effluent, would greatly improve the economics of the process, particularly if this could be achieved microbiologically. It is therefore of importance to select a microbe that can grow well at ambient temperatures and neutral pH and oxidize sulfide to sulfur in wastewater. Our previous studies have reported on the potential use of a *Thiobacillus* sp. for treating latex rubber sheet wastewater (Kantachote and Innuwat, 2004). Hence, this work is focused on the isolation and identification of a bacterium able to oxidize sulfide to sulfur in sulfate rich wastewater to achieve our goal of obtaining a better and more efficient production of biogas during the treatment of wastewater from rubber manufacturing plants.

## MATERIALS AND METHODS

### Isolation and selection of a sulfide oxidizing bacterium

Strains of *Thiobacillus* sp. were isolated from sulfide rich wastewater samples collected from rubber factories in southern Thailand using a thiobacillus isolation medium named thiosulfate mineral salts medium (thiosulfate MSM). The medium composition in 1L of distilled water is 2.0 g KNO<sub>3</sub>, 1.0 g NH<sub>4</sub>Cl, 2.0 g KH<sub>2</sub>PO<sub>4</sub>, 2.0 g NaHCO<sub>3</sub>, 0.8 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 5.0 g Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>·5H<sub>2</sub>O and 1.0 ml trace element solution with the pH adjusted to 6 with 1N KOH. In some situations the thiosulfate was replaced by 5.0 g Na<sub>2</sub>S·9 H<sub>2</sub>O and principle for this replacement is to acclimate the SOB for utilizing sulfide in a SRR effluent. The trace element solution contained in 1L of distilled water: 50 g Na<sub>2</sub>-EDTA, 7.34 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 5.0 g FeSO<sub>4</sub>·7H<sub>2</sub>O, 2.5 g MnCl<sub>2</sub>·4H<sub>2</sub>O, 2.2 g ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, 0.2 g CaSO<sub>4</sub>·5H<sub>2</sub>O and 11.0 g NaOH (DSMZ, 2002). 15 g of agar was added to solidify the medium. Five ml of the wastewater samples were added to a shaking flask containing 50 ml of the MSM and incubated in a rotary shaker (100 rpm) at 30°C for 7 days. The development of turbidity in the medium was assumed to be due to bacterial growth and any flask that also showed a pH drop indicating the growth of thiobacilli was chosen for purification by streaking onto the thiosulfate MSM solid medium, also incubated at 30°C for 7 days. The purified cultures were initially screened by growing in the MSM containing Na<sub>2</sub>S instead of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>. Strains that grew well in sulfide medium as the sole source of energy were further screened by using a SRR effluent as a medium without any supplementation but after it had been centrifuged at 6455 (RCF x g) for 10 min and sterilized at 121°C for 15 min. Parameters of the SRR effluent were (mg L<sup>-1</sup>): BOD 500, COD 2050, TKN 198 (total Kjeldahl nitrogen) and the pH was 7.88. The criteria used for selecting an appropriate sulfide oxidizing bacterium for further study was based on the one producing the highest rate of sulfide removal from

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the SRR effluent. Each experiment in this step was conducted in three replicates.

### Identification of SOB

The SOB selected, isolate T307, was identified using morphological, physiological and biochemical properties according to Bergey's Manual of Systematic Bacteriology, vol. 3 (Kelly and Harrison, 1989). A scanning electron microscope (SEM), model JSM-5800LV, JEOL was used to examine morphology and size of bacterial cells and the protocol used for SEM followed the instruction manuals for the instrument. Bacterial properties investigated were catalase, oxidase, growth conditions at 4 and 42°C and growth as a facultative anaerobe. For these tests bacterial cells were grown in thiosulfate MSM. Heterotrophic growth of a selected strain was determined using the thiosulfate MSM, in which Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> was replaced with each organic compound added at a concentration of 0.1% (w/v). The following organic compounds were tested, yeast extract, peptone, sucrose, fructose, lactose, maltose, mannitol, starch, arginine, sodium acetate and sodium formate. In addition, the API 20E assay (a commercial test kit for the identification of Enterobacteriaceae and other Gram negative bacteria) was carried out in parallel according to the manufacturer's instructions (BioMerieux, Marcy l'Etoile, France) at 30°C. Mixotrophic growth of the selected strain was investigated in the thiosulfate MSM by amending with 0.05% (w/v) yeast extract (Chen et al. 2004) and observing any increase in growth compared with that in the normal thiosulfate MSM. Sulfate production resulting from bacterial growth was observed by measuring the initial and the final sulfate concentration of the culture broth. In order to obtain a high cells density, a thiosulfate medium for *Thiobacillus* sp. IW (Park et al. 2002) was modified by adding 0.1% (w/v) yeast extract instead of 0.2% (w/v) for preparation of the inoculants and this medium was named as thiosulfate yeast extract medium (TSY). This was used for the inoculum to test cultures for their ability to remove sulfide from a SRR effluent and also for identification purposes and for determining the effects of pH (2-9) and temperature (25-40°C) on bacterial growth. The specific growth rate was determined during exponential growth for each pH and temperature. Bacterial growth was measured as turbidity using a spectrophotometer at optical density 660 nm, whereas the standard plate count method was used to count viable cells. 16S rRNA gene sequence (500 base pairs) was also carried out to identify the selected isolate. Genomic DNA was extracted using the standard method and then amplified by GeneAmpPCR system 9600 according to Altschul et al. (1997) followed by a homology search with BLASTn program from the NCBI database. Alignment and production of a phylogenetic tree was obtained using the DNASIS V3.7 program, the unweighted pair-group method using arithmetic averages (UPGMA) was also used.

### Analytical procedures

The following methods used in this study are described in the APHA, AWWA and WPCF (1998). Sulfide was measured in three forms as TS, DS and un-ionized hydrogen sulfide (H<sub>2</sub>S) using an iodometric method, whereas sulfate was determined using a turbidimetric method. BOD<sub>5</sub> was measured by the azide modification method, COD (chemical oxygen demand) by the dichromate reflux method, and TKN using the Kjeldahl method. In addition, volatile fatty acids (VFAs) were examined by the direct titration method and it was calculated in a form of acetic acid. A pH meter was used to measure pH.

### Inoculum preparation

An actively growing culture of a selected SOB isolate, T307, from a thiosulfate MSM agar slant was subcultured in 10 ml TSY in a test tube and incubated under optimum conditions (pH 7, 30°C, 100 rpm) for 24 hrs. The effect of shaking speed on bacterial growth was investigated in our preliminary studies when 100 rpm provided maximal growth and a high cell density. Consequently a 10% inoculum of this culture broth was transferred to 100 ml TSY in an Erlenmeyer flask and incubated under the same conditions for 10 hrs to produce exponentially growing cells and used as inoculants.

### Sulfide removal in the effluent from SRR

Effluent collected from an SRR at Chalong latex industry, Co, Ltd. in Songkhla province, Thailand, was used in this study without amendment, except the effluent was first centrifuged at 6455 (RCF x g) for 10 min and the initial pH was adjusted to 7. Different batches of SRR effluent were used in step of isolation and this experiment. Treatment consisted of 2 sets, of 1 L glass bottles fitted with a plastic cap and filled with 800 ml of either sterile optimized wastewater (SOW) or raw optimized wastewater (ROW) in each bottle. Each treatment set was inoculated with a 10% inoculum of the selected SOB isolate T307, therefore the SOW set contains a pure culture and the ROW set a mixed culture of indigenous microbes and the selected SOB isolate. The culture bottles were incubated at 30°C with 70 rpm (this shaking speed was chosen because it provided partial oxidation of sulfide to sulfur as a yellow precipitation was found in our preliminary study using a shaking speed of 70 rpm). Parameters: BOD (biochemical oxygen demand), COD, VFAs, viable cells, pH, TS, DS, H<sub>2</sub>S and sulfate were monitored at the beginning (t = 0) and the end (20 hrs) of the experiment. The experiment was conducted in three replicates.

### Biodesulfurization prior to biogas production

At about this time when we were about to confirm our results of the removal of sulfide from a SRR effluent, unfortunately the factory producing the SRR faced a

problem with low efficiency of the SRR and sulfide levels were not sufficiently high. Consequently, the thiosulfate MSM was modified by replacing sodium thiosulfate with sodium sulfide ( $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ :  $5 \text{ g L}^{-1}$ ). Biodesulfurization was performed in 800 ml of the sulfide broth in a bioreactor with a 10% inoculum of the selected SOB inoculant T307 and incubated at 30°C and 70 rpm for 20 hrs. A control abiotic set without inoculation was also conducted. The following parameters: pH, viable cells, TS, DS,  $\text{H}_2\text{S}$  and sulfate were monitored at zero time and after 20 hrs incubation. Four replicates were set in this experiment. In this study, a sulfur balance was not determined due to the volatilization of sulfide as  $\text{H}_2\text{S}$  during the autoclaving that provided sterile conditions.

### Biogas production

1% sodium acetate was added to the sulfide MSM bottles containing the 900 ml of culture broth, pre-treated with the SOB isolate, T307. This was to test for the production of biogas by methane producing bacteria (MPB). 100 ml of seed sediment inoculum from an anaerobic rubber wastewater treatment plant was then added prior to the start of sampling for COD determinations. The inoculum comprised both sulfate reducing bacteria (SRB) and MPB. A control set was prepared at the same but using the uninoculated set bottles from the previous experiment. This biogas fermentation experiment was set up as presented in Figure 1 and the biogas fermentation was conducted at room temperature ( $30 \pm 3^\circ\text{C}$ ). The volume of biogas produced was measured by the water displaced. The Biogas composition was measured from the headspace gas of the biogas fermentation bottles using a portable multi gas detector (Oldham MX 2100) according to the instruction manuals. The biogas fermentation was run for 5 days as at this time gassing had slowed in a control set. The liquid was centrifuged at 6455 (RCF  $\times g$ ) for 15 min and then used to determine the following parameters: pH, TS, DS,  $\text{H}_2\text{S}$ , sulfate, and COD. A specific yield of  $\text{CH}_4$  was calculated based on the COD removed. This experiment was repeated twice and each time in triplicate.

## RESULTS

### Isolation and selection of SOB

A total of 147 isolates were obtained from a variety of latex wastewater treatment plants; however, only 63 isolates (42.9%) were able to oxidize sulfide and following screening by inoculation into a SRR effluent, 22 isolates grew, but only 11 isolates grew well (data not shown). Based on the removal of sulfide, an isolate T307 was chosen as it gave the highest reduction (86.7%) in a sample of the SRR effluent over a 48 hrs cultivation period. Even at 24 hrs the slightly lower percentage reduction was not significantly different from that at 48 hrs (Figure 2).

### Identification of SOB

Isolate T307 was a Gram negative, non motile, short rod, with average dimensions of  $0.5 \times 0.9 \mu\text{m}$  (Table 1) that formed round smooth and white colonies with an average diameter of 0.5 mm on the thiosulfate MSM agar plate. Catalase and oxidase tests were both positive. The isolate T307 grew under both aerobic and anaerobic conditions in which sodium thiosulfate was present. Chemolithotrophic aerobic growth of isolate T307 was found on sulfide and thiosulfate salt. Heterotrophic growth occurred with yeast extract, peptone, urea and gelatin. No growth was observed with any of the other selected single organic compounds except for acetate and formate but growth was poor. Mixotrophic growth was demonstrated on the thiosulfate MSM plus 0.05% yeast extract and as an increased amount of sulfate over the thiosulfate MSM control was detected at the end of growth (data not shown), indicating that thiosulfate was being used as an energy source. The isolate T307 grew better as a heterotroph especially with yeast extract and peptone rather than as a chemolithotroph or mixotroph (Table 2). The pH range for growth was from 5 to 9 (maximal pH tested) with an optimum at pH 7.0 and over a temperature range of 4-42°C with an optimal of 25-30°C. More details of the properties of the isolate T307 are shown in Table 1.

According to the conventional methods used in Table 1, the isolate T307 could be either an *Alcaligenes* sp. or *Thiobacillus delicatus* (Kerstens and Ley, 1984; Kelly and Harrison, 1989). However, some key characteristics of the isolate T307 were different from both organisms such as motility (*Alcaligenes*: motile) and oxidase (*T. delicatus*: negative). Therefore, a partial 16S rDNA analysis was used to help in a decision and its accession number, AY 628412, for the 16s rRNA gene sequence of the isolate T307 was 100% similar to that of *Alcaligenes* sp. YcX-20, and 87.2% to *A. faecalis* strain ITRC EM2. The isolate T307 was therefore named *Alcaligenes* sp. T307 (Figure 3).

### Sulfide removal

*Alcaligenes* sp. T307 was able to remove all forms of sulfide from a SRR effluent: 91.84% TS, 88.75% DS and 95.76%  $\text{H}_2\text{S}$ , whereas with a mixture of indigenous microbes the removal was significantly lower at 56.24% TS, 32.29% DS and 92.11%  $\text{H}_2\text{S}$  (Table 3). Some yellow precipitation was found in both treatments with most in the SOW set. At the end of the experiment, more viable cells were detected (0.2 log cycle) in a ROW set than that in a SOW set even though the initial cell count was lower and the pH was also higher, (pH 8.52 compared with 8.29). This is consistent with the higher growth observed in the presence of yeast extract. In contrast, reduction of VFAs was significantly higher in a ROW set than in a SOW set. There was no significant difference in the BOD/COD ratios

and also the sulfate levels between the SOW and ROW sets at either the start or end of the experiment (Table 3).

### Biodesulfurization prior to biogas production

Very little change occurred in the monitored parameters (pH, TS, DS, H<sub>2</sub>S and sulfate) in the uninoculated set (abiotic control). In contrast, there was a big decrease of sulfide species observed in the sulfide medium inoculated with *Alcaligenes* sp. T307, particularly the percentage loss of TS 83.22, DS 71.78 and H<sub>2</sub>S 90.41%. Bacterial cells increased from 8.36 log cfu L<sup>-1</sup> to 9.72 log cfu L<sup>-1</sup> over the 20 hrs of incubation and the pH increased from 7.33 to 8.44 as a result of bacterial growth. There was virtual no increase in sulfate (Table 4).

### Biogas production

1% acetate was added to the sulfide medium pre-treated by *Alcaligenes* sp. T307 (T307) for 20 hrs to remove sulfide, to test for biogas production. The experiment was repeated twice. In both cases the average amount of biogas after treatment with T307 (240 ml L<sup>-1</sup> d<sup>-1</sup>) was significantly higher than that of the control (86 ml L<sup>-1</sup> d<sup>-1</sup>). The biogas composition as a percentage in a set pre-treated by T307 was CH<sub>4</sub> 48.06 ± 7.15, CO<sub>2</sub> 51.53 ± 7.18 and H<sub>2</sub>S 0.41 ± 0.30, whereas in the control set percentage CH<sub>4</sub> was 35.47 ± 12.99, CO<sub>2</sub> 63.67 ± 13.29 and H<sub>2</sub>S 0.86 ± 0.69. Reduction of COD in the biogas reactors pre-treated by T307 was 49.6%, while in the control set it was 36.6%. The specific yield of CH<sub>4</sub> produced per COD removed was 238 ml CH<sub>4</sub> g<sup>-1</sup> in the set pre-treated by T307 whereas in the control set it was only 71.53 ml CH<sub>4</sub> g<sup>-1</sup>. More details on the properties of the sulfide media such as levels of sulfide species and sulfate are shown in Table 5.

## DISCUSSION

### Isolation and selection of SOB

In general, the thiosulfate MSM used in this study is normally used to isolate pure cultures of *Thiobacillus* sp. or other obligate chemolithoautotrophic sulfide oxidizing bacteria. Thiosulfate is stable at neutral pH, easily soluble and non toxic at a high concentration (Kuenen et al. 1992). Although the isolate T307 obtained in this study closely resembled *Thiobacillus delicatus* (Table 1, presently *Thiomonas delicata*, Katayama et al. 2006) it was eventually identified as an *Alcaligenes* sp. based on its rDNA properties and named *Alcaligenes* sp. T307. It was isolated from a sample of wastewater obtained from a rubber treatment plant. The results of this study prove that the ability to oxidize sulfide is a property of *Alcaligenes* sp. T307. There has been other reports that *Alcaligenes* species can grow with sulfur compounds as an energy source (Kerstens and Ley, 1984; Moreira and Amils, 1997) and it was also supported by Potivichayanon et al. (2006) who reported that *Acinetobacter* sp. MU1\_03 and *Alcaligenes faecalis* MU2\_03 in a fixed-film bioscrubber system were

able to remove hydrogen sulfide more than 91% while a mixture of the two strains was capable of 98% hydrogen sulfide removal. Hence based on our results, metabolic capacity, together with rod morphology cannot be considered as useful taxonomic criteria for inclusion of any new species in the genus *Thiobacillus*. This is supported by Moreira and Amils (1997) and Van Trappen et al. (2005) who reported that the mixotrophic *Thiobacillus* organisms form a phylogenetic cluster within the β1 subgroup of the *Proteobacteria* (*Alcaligenes faecalis*, *Alcaligenes* sp., *Burkholderia cepacia*, *Thiobacillus intermedius* and *T. perometabolis*). In addition, the isolate T307 had a 100% similarity index with *Alcaligenes* sp. YcX-20, a methyl-parathion degrading bacterium, as reported by Jiang et al. (2005). The 16S rDNA analysis (500 bp) of the isolate T307 corresponded with that *A. faecalis* strain ITRC EM2 (87.2% homology). It is clear that an apparently close match in 16S rDNA analysis is not sufficient to indicate species identity, but that at least 70% DNA homology is required. Therefore, complete 16S rRNA gene sequence and DNA-DNA hybridization between the selected isolate T307 and related *Alcaligenes faecalis* will be conducted to prove that the isolate T307 may be a novel species of the genus *Alcaligenes*.

### Sulfide removal by *Alcaligenes* sp. T307

As the BOD/COD ratios in the two sets of experiments (SOW and ROW) used to determine the removal of sulfide, both before and after culture, were between 0.20-0.38 (Table 3) this meant that the nutrient composition was suitable for mixotrophic rather than heterotrophic growth. Previously, Bitton, (2005) has established that a BOD/COD ratio in the range of 0.40-0.80 is required to support heterotrophic growth. The increase in viable cells resulting from the addition of yeast extract (Table 2) indicates that *Alcaligenes* sp. T307 can grow as a mixotroph. The observed changes in the different forms of sulfide have therefore occurred during growth of the microbes as mixotrophs. The presence of the organic matter might affect this process when the microbes have to grow as a chemolithotroph.

The results presented in Table 3 indicate that *Alcaligenes* sp. T307 in pure culture was better at removing all forms of sulfide than when it was present together with indigenous microbes. A possible explanation could be that in the ROW set, T307 has to compete with some indigenous microbes less effective sulfide oxidizing microbes (e.g. Table 1) for some essential nutrient. The indigenous microbes may also be able to utilize the VFAs for growth whereas the ability of T307 is restricted (Table 3). This may also account for the increased removal of VFAs (28 mg L<sup>-1</sup> SOW, 107 mg L<sup>-1</sup> for ROW) and growth observed in a ROW set (0.61 cfu ml<sup>-1</sup> in SOW compared to 0.80 cfu ml<sup>-1</sup> for ROW). Removal of sulfide and VFAs from the medium will cause a rise in pH but this requires the presence of bacteria. The increase in pH was slightly bigger with ROW than SOW due mainly to the bigger loss of VFAs. It is well known that

pH alters the forms of sulfide (< 7: H<sub>2</sub>S, 7-9: HS<sup>-</sup> and > 8: S<sup>2-</sup>) (Markl, 1999), so this also may have some effect on the process. As mentioned above sulfide removal was satisfactorily high for SOW and not for ROW. However, due to the fact that the most toxic form of sulfide is H<sub>2</sub>S, the results showed the isolate T307 gave a high efficiency to remove H<sub>2</sub>S (92-96%) both in ROW and SOW. Therefore, the less toxicity of sulfide, the more MPB could grow. In both cases some precipitation of elemental sulfur occurred, slightly more in SOW. This is due to the partial oxidation of reduced S compounds as there was virtually no change in the sulfate levels. As there was no precipitation in the control, without added organisms, its formation was also being catalyzed by the bacteria. The increased amount in the SOW set could be due to a higher population of T307, a lower pH or the presence of slightly more O<sub>2</sub>. In both cases it is likely that O<sub>2</sub> levels were limiting. It is possible that the shaking speed of only 70 rpm used in this experiment may limit the amount of oxygen, thereby restricting complete oxidation to sulfate. It is known that the oxidation of several sulfide species to elemental sulfur and sulfate can be controlled by oxygen levels (Gonzalez Sanchez et al. 2005).

Comparing the growth in the SRR-SOW set with that in the sulfide medium, over the 20 hrs incubation (Table 3 and Table 4) viable cells increased by 1.36 log cfu ml<sup>-1</sup> in the sulfide medium, whereas in the SRR - SOW set, the increase was only 0.61 log cfu ml<sup>-1</sup>. This means some growth inhibitors may have been present in the SRR effluent. Therefore, rates of sulfide removal in the sulfide medium were significantly higher (3 mg L<sup>-1</sup> h<sup>-1</sup>) than that in the SRR-SOW set (0.90 mg L<sup>-1</sup> h<sup>-1</sup>) due to a higher viable cell of the isolate T307 in the sulfide medium.

### Biogas production

After removal of sulfide by pre-treating sulfide medium with T307, biogas production was significantly increased and contained a higher proportion of methane. During the 5 days incubation period, there are 3 parameters that have to be considered as likely to affect biogas production: changes in sulfide species, sulfate levels and COD (Table 5). It has long been known that in a biogas reactor the relative amounts of SRB and MPB affect the amount and composition of biogas (Santegoeds et al. 1999). Sulfate is utilized as a terminal electron acceptor by SRB and the generation of sulfide inhibits MPB due to sulfide toxicity (Khanal and Huang, 2003). The higher levels of sulfide species in the control set could inhibit MPB causing a 12 hrs delay in biogas production (data not shown) with the MPB able to catalyze only weak methanogenic activity. The lower amount of sulfate found in the control set, after 5 days, indicates that some sulfate was being consumed by SRB, and initially these may be the dominant organisms (Table 4 and Table 5). As the percentage of COD removal in the control was lower (13%) than after the treatment with T307 this may indicate that the higher sulfide levels produced by SRB also inhibited COD removal.

In conclusion, results in this study indicate that MPB were strongly inhibited by sulfide produced by SRB in a sulfate rich wastewater. Therefore biodesulfurization is an important step for the effluent from wastewater with high sulfate prior to a step for producing biogas. *Alcaligenes* sp. T307 has the potential to use in a step of biodesulfurization.

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### REFERENCES

- ALTSCHUL, S.F.; THOMAS, L.M.; ALEJANDRO, A.S.; JINGHUI, Z.; ZHENG, Z.; WEBB, M. and LIPMAN, D.J. Gapped BLAST and PSI-BLAST: a new generation of protein databases search program. *Nucleic Acids Research*, 1997, vol. 25, no. 17, p. 3389-3402.
- APHA, AWWA and WPCF. *Standard Method for Examination of Water and Wastewater* 20<sup>th</sup> ed. Washington, D.C. American Public Health Association, 1998, 1325 p. ISBN 0-87553-235-7.
- BITTON, Gabriel. *Wastewater Microbiology* 3<sup>rd</sup> ed. Chichester, John Wiley & Sons, 2005. 768 p. ISBN 978-0-471-65071-3.
- CHA, J.M.; CHA, W.S. and LEE, J.H. Removal of organo-sulphur odour compounds by *Thiobacillus novellas* SRM, sulphur-oxidizing microorganisms. *Process Biochemistry*, 1999, vol. 34, no. 6-7, p. 659-665.
- CHEN, X.G.; GENG, A.L.; YAN, R.; GOULD, W.D.; NG, Y.L. and LIANG, D.T. Isolation and characterization of sulphur-oxidizing *Thiomonas* sp. and its potential application in biological deodorization. *Letters in Applied Microbiology*, 2004, vol. 39, no. 6, p. 495-503.
- CHUNG, Y.C.; HO, K.L. and TSENG, C.P. Hydrogen sulfide gas treatment by a chemical-biological process: chemical absorption and biological oxidation steps. *Journal of Environmental Science Health Part B*, 2003, vol. 38, no. 5, p. 663-679.
- DSMZ. List of media. Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures) Germany, 2002.
- GONZALEZ SANCHEZ, A.; ALCANTARA, S.; RAZO-FLORES, E. and REVAH, S. Oxygen transfer and consumption in a thiosulfate oxidizing bioreactor with sulfur production. *Letters in Applied Microbiology*, 2005, vol. 41, no. 2, p. 141-146.

- JIANG, H-X.; WANG, S-H.; XUE, Q-J. and YAN, Y-C. Isolation, identification and characterization of a methyl-parathion degrading bacterium. *Journal of Agro-Environment Science*, 2005, vol. 24, p. 962-965.
- KANTACHOTE, D. and INNUWAT, W. Isolation of *Thiobacillus* sp. for use in treatment of rubber sheet wastewater. *Songklanakarin Journal of Science and Technology*, 2004, vol. 26, no. 5, p. 649-657.
- KANTACHOTE, D.; TORPEE, S. and UNSAKUL, K. The potential use of anoxygenic phototrophic bacteria for treating latex rubber sheet wastewater. *Electronic Journal of Biotechnology*, 2005, vol. 8, no. 3, p. 314-323.
- KATAYAMA, Y.; UCHINO, Y.; WOOD, A.P. and DONOVAN, P.K. Confirmation of *Thiomonas delicata* (formerly *Thiobacillus delicatus*) as a distinct species of the genus *Thiomonas* Moreira and Amils 1997 with comments on some species currently assigned to the genus. *International Journal of Systematic and Evolutionary Microbiology*, 2006, vol. 56, p. 2553-2557.
- KELLY, D.P. and HARRISON, A.P. *Genus Thiobacillus*. In: STALEY, J.T. ed. *Bergey's Manual of Systematic Bacteriology*. Baltimore, Williams & Wilkins, 1989, vol. 3, p. 1842-1858.
- KERSTERS, K. and LEY, J.D. *Genus Alcaligenes*. In: KRIEG, N.R. ed. *Bergey's Manual of Systematic Bacteriology*. Baltimore, Williams & Wilkins, 1984. vol. 1, p. 361-373.
- KHANAL, S.K. and HUANG, J.C. Anaerobic treatment of high sulfate wastewater with oxygenation to control sulfide toxicity. *Journal of Environmental Engineering*, December 2003, vol. 129, no. 12, p. 1104-1111.
- KLEEREBEZEM, R. and MENDEZ, R. Autotrophic denitrification for combined hydrogen sulfide removal from biogas and post-denitrification. *Water Science and Technology*, 2002, vol. 45, no. 10, p. 349-56.
- KUENEN, J.G.; ROBERTSON, L.A. and TUOVINEN, O.H. The genera *Thiobacillus*, *Thiomicrospira* and *Thiosphaera*. In: BALOWS, A.; TRUPER, H.G.; DWORKI, M.; HARDER, W. and SCHLEIFER, K. ed. *The Prokaryotes*. New York. Springer-Verlag 1992, vol. 3, p. 2636-2657.
- MARKL, H. Modeling of biogas reactors. In: WINTER, J. ed. *Biotechnology*. 2<sup>nd</sup> New York: A Wiley Company, 1999, vol. 11a, p. 527-560.
- MOREIRA, D. and AMILS, R. Phylogeny of *Thiobacillus cuprinus* and other mixotrophic Thiobacilli: proposal for *Thiomonas* gen. nov. *International Journal of Systematic Bacteriology*, April 1997, vol. 47, no. 2, p. 522-528.
- OYARZUN, P.; ARANCIBIA, F.; CANALES, C. and AROCA, G.E. Biofiltration of high concentration of hydrogen sulphide using *Thiobacillus thioparus*. *Process Biochemistry*, 2003, vol. 39, p. 165-170.
- PARK, D.H.; CHA, J.M.; RYU, H.W.; LEE, G.W.; YU, E.Y.; RHEE, J.I.; PARK, J.J.; KIM, S.W.; LEE, I.W.; JOE, Y.I.; RYU, Y.W.; HUR, B.K.; PARK, J.K. and PARK, K. Hydrogen sulfide removal utilizing immobilized *Thiobacillus* sp. IW with Ca-alginate bead. *Biochemical Engineering Journal*, 2002, vol. 11, no. 2-3, p. 167-173.
- POTIVICHAYANON, S.; POKETHITIYOOK, P. and KRUATRACHUE, M. Hydrogen sulfide removal by a novel fixed-film bioscrubber system. *Process Biochemistry*, 2006, vol. 41, no. 3, p. 708-715.
- SANTEGOEDS, C.M.; DAMGAARD, L.R.; HESSELINK, G.; ZOPFI, J.; LENS, P.; MUYZER, G. and BEER, D. Distribution of sulfate-reducing and methanogenic bacteria in anaerobic aggregates determined by microsensor and molecular analyses. *Applied Environmental Microbiology*, October 1999, vol. 65, no. 10, p. 4618-4629.
- SUBLETTE, K.L.; KOLHATKAR, R. and RATERMAN, K. Technological aspects of the microbial treatment of sulfide-rich wastewaters: a case study. *Biodegradation*, 1998, vol. 9, no. 3-4, p. 259-271.
- TEKASAKUL, P. and TEKASAKUL, S. Environmental problems related to natural rubber production in Thailand. *Journal of Aerosol Research*, 2006, vol. 21, no. 2, p. 122-129.
- VAN TRAPPEN, S.; TAN, T.L.; SAMYN, E. and VANDAMME, P. *Alcaligenes aquatilis* sp. nov., a novel bacterium from sediments of the Weser Estuary, Germany, and a salt march on Shem Creek in Charleston Harbor, USA. *International Journal of Systematic Evolutionary Microbiology*, November 2005, vol. 55, no. 6, p. 2571-2575.

## APPENDIX FIGURES



Figure 1. An experiment set of biogas production and gas collection apparatus.

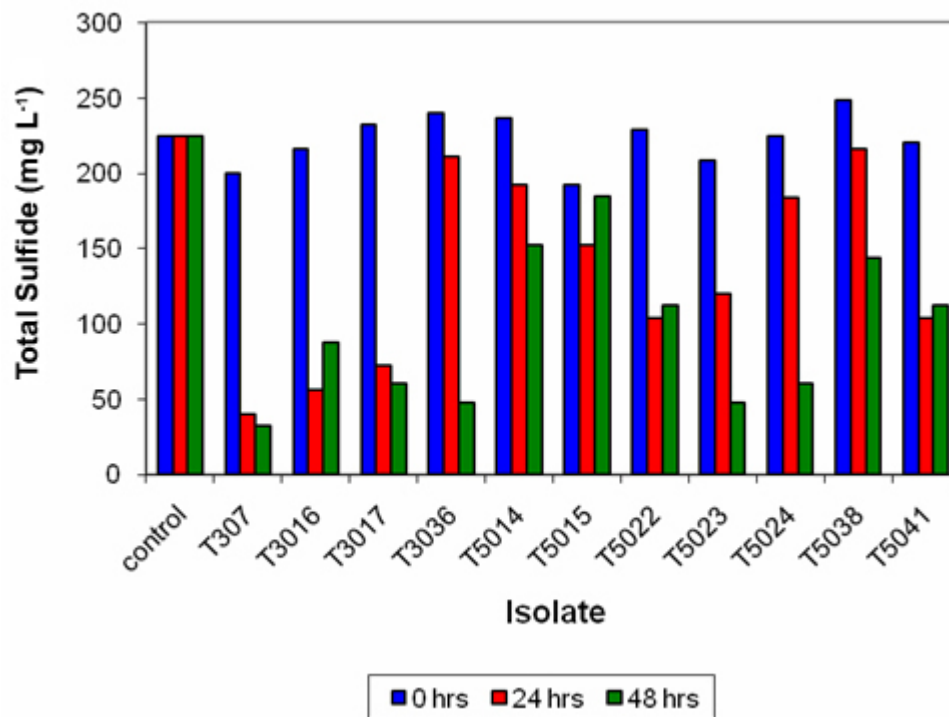


Figure 2. Reduction of total sulfide in effluent from a sulfate reduction reactor by sulfur oxidizing bacteria.



Selection of sulfur oxidizing bacterium for sulfide removal in sulfate rich wastewater to enhance biogas production

Sequences producing significant alignments:		SCORE	E VALUE
AY628412	<i>Alcaligenes</i> sp. YcX-20	991	0.0
AF155147	<i>Alcaligenes faecalis</i>	991	0.0
DQ435021	<i>Alcaligenes faecalis</i> strain ITRC EM2	983	0.0
AY548384	<i>Alcaligenes faecalis</i> strain WM2072	975	0.0
AY346140	<i>Alcaligenes</i> sp. IS-67	975	0.0

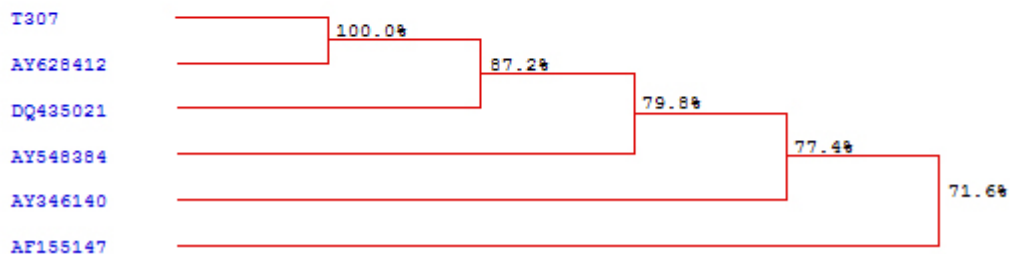


Figure 3. Phylogenetic tree inferred from a comparison of 16S rRNA gene sequences, showing relationships between the isolate T307 and other species of *Alcaligenes* sp.

Table 1. Characteristic of sulfur oxidizing bacterium isolate T307.

Characteristic	T307	<i>Alcaligenes</i> sp. <sup>a</sup>	<i>Thiobacillus delicatus</i> <sup>b</sup>
Cell shape (size: $\mu\text{m}$ )	Short rod (0.5 x 0.9)	Rod (0.5-1.0 x 0.5-2.6)	Short rod (0.4-0.6 x 0.7-1.6)
Cell arrangement	single, rarely in pairs	Single, rarely in pairs	Single, rarely in pairs
Motility	Non motile	Motile	Non motile
Catalase	+	+	+
Oxidase	+	+	-
Facultative anaerobe <sup>c</sup>	+	V	V
Chemolithotroph	+	+	+
Mixotroph	+	+	+
Chemoorganotroph	+	+	+
Nitrate reduction	-	V	-
Growth at 4 and 42°C	+/+	+/+	-/+ (15-42°C)
Optimum temperature (°C)	25-30	20-37	30-35
Growth at pH 2-4	-	ND	-
Growth range: pH	5-9	ND	5.0-7.0
Optimum pH	7.0	7	5.5-6
Fructose	(+)	V	(+)
Maltose	-	V	(+)
Sucrose	-	V	ND
Lactose	-	V	ND
Mannitol	-	V	ND
Arginine	-	V	ND
Hydrolysis of starch	-	V	ND
Sodium acetate	+	+	ND
Sodium formate	+	ND	ND
<b>API 20E, Biomerieux</b>			
Citrate	-	+	+
Indole	-	-	ND
Voges-Proskauer	-	ND	ND
H <sub>2</sub> S production	-	-	ND
Urease	+	V	+
Gelatin	+	V	ND
O-F (glucose)	-/-	V	-/-
Mannose	-	V	ND
Arabinose	-	V	ND
Amygdalin	-	-	ND
Saccharose	-	-	ND
Rhamnose	-	V	ND
Sorbitol	-	V	ND
Inositol	-	V	ND
Ornithine decarboxylase	-	V	ND
Lysine decarboxylase	-	V	ND
Tryptophan deaminase	-	V	ND

<sup>a</sup>Code from Kerster and Ley, 1984, <sup>b</sup>Code from Kelly and Harrison, 1989, <sup>c</sup>mixotrophic and chemolithotrophic media with thiosulfate, (+) = weakly positive, ND = not determined, V = Variable depending on species/strain.

Selection of sulfur oxidizing bacterium for sulfide removal in sulfate rich wastewater to enhance biogas production

**Table 2. Specific growth rates of isolate T307 at different values of yeast extract, pH and temperature.** TSY medium was used for investigation effects of pH and temperature on bacterial growth.

Variable Factor					
%Yeast extract in thiosulfate MSM	$\mu$ (h <sup>-1</sup> )	pH <sup>a</sup>	$\mu$ (h <sup>-1</sup> )	Temperature (°C) <sup>b</sup>	$\mu$ (h <sup>-1</sup> )
0	0.015 ± 0.001	6.0	0.120 ± 0.004	25	0.137 ± 0.004
0.05	0.096 ± 0.002	6.5	0.126 ± 0.002	30	0.138 ± 0.002
0.10	0.138 ± 0.003	7.0	0.138 ± 0.001	35	0.118 ± 0.002
0.15	0.179 ± 0.002	7.5	0.133 ± 0.002	40	0.100 ± 0.009
0.20	0.234 ± 0.001	8.0	0.119 ± 0.003		
		8.5	0.117 ± 0.004		
		9.0	0.113 ± 0.006		

<sup>a</sup>Temperature incubation at 30°C, <sup>b</sup>Initial pH at 7.  
 $\mu$ : mean value and standard deviation of three determinations.

**Table 3. Removal of sulfide in the effluent from SRR by *Alcaligenes* sp. T307.**

Parameter (mg L <sup>-1</sup> )*	Sterile optimized wastewater (SOW: pure culture)		Raw optimized wastewater (ROW: mixed cultures)	
	0 hr	20 hrs	0 hr	20 hrs
BOD	503 ± 12	483 ± 10	553 ± 15	575 ± 17
COD	2500 ± 24	1533 ± 18	2450 ± 21	1533 ± 15
BOD/COD	0.20 ± 0.003	0.32 ± 0.003	0.23 ± 0.004	0.38 ± 0.007
VFAs	222 ± 8.6	194 ± 7.6	210 ± 7.3	103 ± 5.6
pH	6.96 ± 0.18	8.29 ± 0.21	6.97 ± 0.15	8.52 ± 0.22
Viable cells (cfu ml <sup>-1</sup> )	8.41 ± 0.21	9.02 ± 0.24	8.16 ± 0.19	8.96 ± 0.22
Total sulfide	19.6 ± 1.4	1.6 ± 0.14 (91.84%)	18.9 ± 1.2	8.27 ± 0.32 (56.24%)
Dissolved sulfide	8.27 ± 0.7	0.93 ± 0.08 (88.75%)	8.27 ± 0.5	5.6 ± 0.13 (32.29%)
Hydrogen sulfide	1.18 ± 0.11	0.05 ± 0.001 (95.76%)	1.14 ± 0.09	0.09 ± 0.002 (92.11%)
Sulfate	498 ± 9.5	487 ± 8.7	485 ± 7.9	491 ± 8.5

\*Unless stated, mean value and standard deviation of three determinations.

Table 4. Removal of sulfide in sulfide medium prior to biogas production.

Parameter (mg L <sup>-1</sup> )*	Abiotic control		<i>Alcaligenes</i> sp. T307	
	0 hrs	20 hrs	0 hrs	20 hrs
pH	7.30 ± 0.23	7.32 ± 0.26	7.33 ± 0.18	8.44 ± 0.21
Viable cells (cfu ml <sup>-1</sup> )	0	0	8.36 ± 0.15	9.72 ± 0.18
Total sulfide (TS)	71.1 ± 1.3	72 ± 1.5	72.1 ± 1.4	12.1 ± 0.15 (83.22%)
Dissolved sulfide	61.6 ± 0.56	62.1 ± 0.61	60.6 ± 0.47	17.1 ± 0.31 (71.78%)
Hydrogen sulfide (H <sub>2</sub> S)	0.74 ± 0.09	0.63 ± 0.07	0.73 ± 0.08	0.07 ± 0.002 (90.41)
Sulfate	456 ± 6.8	468 ± 7.3	442 ± 5.7	449 ± 5.5

\*Unless stated, mean value and standard deviation of four determinations

Table 5. Effect of sulfide removal by *Alcaligenes* sp. T307 in sulfide medium on biogas production over 5 days incubation.

Parameter (mg L <sup>-1</sup> )*	Control	Pre-treated by T307
Effluent pH	8.76 ± 0.28	8.37 ± 0.10
Effluent total sulfide	70.8 ± 1.70	12.1 ± 0.20
Effluent dissolved sulfide	14.7 ± 0.57	10.0 ± 0.27
Effluent H <sub>2</sub> S	1.0 ± 0.15	1.1 ± 0.16
Effluent sulfate	100.5 ± 6.36	143 ± 4.24
Influent COD	5790 ± 61.47	4890 ± 49.1
Effluent COD	3670 ± 56.57	2465 ± 39.5
COD reduction (%)	36.62 ± 0.98	49.60 ± 1.01
Biogas produced (ml L <sup>-1</sup> d <sup>-1</sup> )	85.5 ± 32.96	240 ± 24.86
Biogas composition		
% CH <sub>4</sub>	35.47 ± 12.99	48.06 ± 7.15
% CO <sub>2</sub>	63.67 ± 13.29	51.53 ± 7.18
% H <sub>2</sub> S	0.86 ± 0.69	0.41 ± 0.3
Specific yield of CH <sub>4</sub> (ml g <sup>-1</sup> COD removed)	71.53 ± 18	237.81 ± 17

\*Unless stated, mean value and standard deviation of six determinations