Factors Influencing Degradation of Mercaptans by *Thiobacillus thioparus* TK-m (1)

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**ABSTRACT:** Degradation of methylmercaptans by *Thiobacillus thioparus* TK-m was influenced by pH of the reaction medium. Ratios of headspace concentrations in empty vials and those of acidified buffer solutions were less than 1.0. 95% of the H$_2$S was in headspace with the remaining 5% in solution upon acidification. The values for MM were 80% in headspace and 20% in solution. Different buffer solutions also influence the rate of removal of mercaptans from reaction vessels. For methylmercaptan oxidase, using Tris/HCl buffer, the highest level of oxygen uptake was recorded at pH 8.5. Mild changes in the pH levels were recorded at the end of the reactions. Compared to Tris/HCl buffer, phosphate buffer supports a significantly lower reactivity of MM-oxidase towards methylmercaptan. *@JASEM*

Thiols and hydrogen sulphide (H$_2$S) are malodorous compounds which exceed the odour threshold at low concentrations (Leonardos et al., 1969). They are produced by the wood-pulping industry, manure and sewer systems as exhaust gases. They constitute health problems as some are known to be toxic to both man and animals at very low concentrations.

Thiols are among the major volatile organic sulphur compounds produced in natural environments (Krouse and McCready, 1979). They can arise from the breakdown of sulphur-containing amino acids and lignin, and can be produced by algae, and in soils, freshwater, anaerobic lagoons and oceans (Loveluck et al., 1972; Sivela and Sundman, 1975; Zinder and Brock, 1978; Banwart and Brenner, 1976; Young and Maw, 1958). Thiols also present a major odour problem in the wood-pulping industry (Sivela and Sundman, 1975). Little work has been published on the microbial degradation of other thiols with the exception of methanethiol.

The biogenesis of methylsulphides provides a principal input of volatile sulphur to the atmosphere. This contribution has significant effects on the sulphur cycle and on global geochemistry (Taylor and Kiene, 1989). Dimethylsulphide (DMS) is photochemically oxidized in the atmosphere to methanesulphonic and sulphuric acids. These strong acids contribute, along with nitric and organic acids, to the natural acidity of precipitation (Taylor and Kiene, 1989). Recent problems with acid rain have aroused interest in the anthropogenic and natural sources of volatile sulphur compounds. In addition to affecting the pH of precipitation, the emission of DMS has been linked with the regulation of global climate. There is at present little knowledge available on the microorganisms involved in the biodegradation of methylsulphides. DMS has been reportedly degraded by *Thiobacillus* sp. strain MS1 (Sivela, 1980), *Thiobacillus thioparus* TK-m (Kanagawa et al., 1982; Kanagawa and Kelly, 1986; Kanagawa and Mikami, 1989; Tanji et al., 1989; Gould and Kanagawa, 1992) *Hyphomicrobium* sp. strain S (deBont et al., 1981) and *Hyphomicrobium* sp. strain EG (Suylen and Kuenen, 1986; Suylen et al., 1986; Suylen et al., 1987; Smith and Kelly, 1988).

This study aims at ascertaining the degradation of higher chain-length thiols by intact cells of *Thiobacillus thioparus* TK-m.

**MATERIALS AND METHODS**

**Enzyme:** Purified methyl mercaptan oxidase used for this assay was obtained from cells of *Thiobacillus thioparas* TK-m and supplied by Dr. T. Kanagawa (National Institute of Bioscience and Human-Technology, Tsukuba, Japan) and maintained as slants on medium C (Kanagawa and Kelly, 1986).

**Stability of mercaptans in acid solutions:** Into each of phosphoric acid coated serum vials (68.8 ± 0.6ml vol) was put 3ml of Tris/HCl buffer (pH 8.2) containing 0.2mM EDTA. These were then sealed with Teflon-cated rubber stoppers and 3ml headspace gas replaced by 3ml of the following gas combinations (in duplicates): 3ml methylmercaptan (MM); 2.5ml of MM + 0.5ml of H$_2$S; 1.5ml of MM + 1.5ml of H$_2$S; 0.5ml of MM + 2.5ml of H$_2$S; 3ml of H$_2$S.

The serum vials containing the gases were shaken at 25°C and the headspace gas concentrations determined after 5, 10 and 15 minutes to ascertain equilibrium between gas and liquid phases. After 15 minutes, 100 µl of 4N-H$_2$SO$_4$ was added to one vial of a set and shaken by hand for 5 seconds. The headspace gas was then analysed. The vial was further shaken and headspace gas reanalysed after 5, 10, 20, 30 and 60 minutes. Two empty phosphoric acid-coated vials were sealed with Teflon-coated...
rubber stoppers and 3ml of headspace gas replaced with 3ml of MM in one vial and the other with 3ml of 
H₂S. These were then shaken for 10 minutes and the 
gas concentrations determined.

Influence of pH on MM oxidase from Thiobacillus thioparus: Buffer solutions: For this assay two sets of buffers were used. Phosphate buffers (pH 6.0, 6.5, 
7.0, 7.5, 8.0) were prepared by mixing 50mM 
K₂HPO₄ and 50mM KH₂PO₄ and the pH of the 
solution was determined by the use of a pH 
meter (D-12 Horiba). One hundred millilitres of each 
set was prepared and 1ml of 20mM EDTA solution 
(pH 8.1) added. Tris buffers (pH 7.5, 8.0, 8.5 and 9.0) 
were prepared by mixing 5ml of 1M Tris and 1ml of 
20mM EDTA (pH 8.1). To the resulting solution was 
added 85ml distilled water and then 1M HCl 
dropwise to make buffers of the pH range stated 
elier. The resulting buffers were made up to 100ml 
with distilled water.

Potassium biphthalate buffer was prepared by first 
making a 0.1M solution of C₆H₄(COOH)(COOK) in 
distilled water. Fifty millilitres (50ml) of the 
biphthalate solution was then put in 100ml beakers 
and 1N NaOH added dropwise to make buffers of pH 
4.0, 4.5, 5.0, 5.5 and 6.0. The resulting solutions were 
then made up to 100ml with distilled water. Glycine 
buffer was prepared by first making 50mM each of 
glycine plus sodium chloride and sodium hydroxide. 
The 50mM NaOH solution was then added to the 
50mM glycine + NaCl solution to give buffers of pH 
8.5, 9.0, 9.5, 10.0, 10.5 and 11.0.

Preparation of methylmercaptan solution: Into each 
of clean serum vials was put 50ml of 50mM phosphate buffer, pH 7.5, containing 0.2mM EDTA. 
The vials were then sealed with Teflon-coated rubber 
stoppers and N₂ gas bubbled into the solution for 30 
mins. Methylmercaptan (MM) gas (2,000ppm in N₂) 
was then bubbled for 30 minutes also.

Analysis: The influence of pH on methylmercaptan 
oxidase was determined by measuring oxygen uptake 
during degradation of methylmercaptan in an oxygen 
electrode chamber using a digital DO/O₂/Temp meter 
(UC-12, Central Kagaku Co. Ltd, Japan). The 
reaction was initiated by first pipetting 2ml each of 
the sets of buffers and 8.2µl of methylmercaptan 
solution. To the reaction vessel was then added 
alloquid of the MM-oxidase. Increased concentrations 
of enzyme were also applied. Reaction was stopped 
after 6 minutes.

Stoichiometry of methylmercaptan oxidation: Serum 
vials (68.8 ±0.6ml vol.) were soaked in 0.1N HCl for 
24h, washed with distilled water and dried at 105°C. 
The inside of each vial was then coated with phosphoric acid using 150mM H₂PO₄ in acetone. To 
each vial 3.0ml of the appropriate buffers containing 
0.2mM EDTA (pH 8.1) was added and the vials 
sealed as described earlier (vial volume reduced by 
0.4ml). To the sealed vials 239 nMol MM gas was 
added by replacing 3.0ml of headspace gas with 
3.0ml MM gas (2mol L⁻¹ of nitrogen) and then the 
vials shaken for more than 10 minutes at 25°C. The 
amount of MM in headspace was then determined. 
The reaction was initiated by the injection of 10µl of 
enzyme solution and the vials shaken at 25°C for 10 
minutes. The reaction was terminated by the injection 
of 0.1ml 2N H₂SO₄. Methylmercaptan and H₂S in 
headspace were then determined.

RESULTS AND DISCUSSION
Stability of mercaptans in acid solutions: Analysis of 
final headspace gas concentrations before and after 
injecting 100ul of 4N- H₂SO₄ revealed that both MM 
and H₂S were stable in acid solutions. However, 
when headspace concentrations in empty vials and 
those of acidified buffer solutions were compared, it 
was observed that ratios of vials containing solutions 
were less than 1.0 (see Table 1). For H₂S, a ratio 
(B/A where A = theoretical concentration in vial, and 
B = determined in vial containing buffer) of about 
0.95 was obtained while for MM the corresponding 
value was 0.80. The results obtained indicated that 
only about 95% of the H₂S was in headspace with the 
remaining 5% in solution upon acidification. The 
values for MM were 80% in headspace and 20% in 
solution.

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<thead>
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<th>Table 1: Stability of mercaptans and hydrogen sulphide in acid solutions</th>
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<td><strong>Gas Combination</strong></td>
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<tr>
<td>Empty Bottle + 3ml Substrate</td>
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<td>Buffer + 3ml Substrate + Acid</td>
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<tr>
<td>Buffer + 2.5ml H₂S + 0.5ml MM</td>
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**Influence of pH on MM oxidase from Thiobacillus thioparus:** Figures 1 to 3 show the uptake of oxygen by methylmercaptan oxidase at varying pH levels using tris/HCL and phosphate buffers. From the figures it could be deduced that in the absence of substrate or enzyme there was a slight oxygen uptake in Tris buffer at pH 8.0. This was a sharp contrast to the more than 6-fold increase in oxygen uptake recorded when substrate was added at the same pH. This was due to auto-oxidation of MM, a reaction that takes place even in the absence of MM-oxidase. Also, the concentration of the enzyme in the reaction mixture was found to influence the level of O$_2$ uptake. Due to reduced activity of the enzyme extract, an enzyme volume of 100µl was found to have the highest O$_2$ uptake when compared with other levels tested.

![Fig 1: Influence of varying methylmercaptan (MM) oxidase titres on degradation of MM at pH 8.0](image1)

![Fig 2: Influence of pH levels on degradation of methylmercaptan (MM) by MM-oxidase](image2)

![Fig 3: Influence of pH on methylmercaptan oxidase using phosphate buffer](image3)
For methylmercaptan oxidase, using Tris/HCl buffer, the highest level of oxygen uptake was recorded at pH 8.5. Mild changes in the pH levels were recorded at the end of the reactions. Compared to Tris/HCl buffer, Figure 3 shows that phosphate buffer supports a significantly lower reactivity of MM-oxidase towards methylmercaptan. The highest level of O$_2$ uptake recorded for phosphate buffer (2.95 mg/l) is less than the levels recorded for Tris/HCl buffer (3.3 to 4.3 mg/L). The results generally show that the choice of buffer and the pH level are crucial factors influencing the degradation of methylmercaptan by MM-oxidase.

Figure 4 shows that at the pH levels tested, mole ratios of MM consumed and H$_2$S produced were stoichiometrically equivalent. Similarly, Figure 5 also shows that at all enzyme titres used, concentrations of MM consumed corresponded to H$_2$S production.

Preliminary results on the degradation of different thiols by enzyme extracts of *Thiobacillus thioparus* TK-m in Liquid Medium are shown in Figure 6. The data show that enzyme extracts of the test strain could degrade other thiols apart from C$_1$ and C$_2$.
As expected, the degradability rates depended on the chain-length of the thiol, a consistently higher rate was recorded for \( \text{C}_1 \) compared to \( \text{C}_2 \). Much lower activities were recorded with propanethiol, butanethiol, pentanethiol and haxanethiol (0.069, 0.068 and 0.090 µmol\( \text{mg}^{-1}\text{protein} \cdot \text{min}^{-1} \) respectively). Only very minimal degradation of secondary thiols was recorded. The degradation of thiols by a strain of \( T. \text{thioparus} \) has been reported previously (Smith and Kelly, 1988).

**Conclusion:** Results obtained show that mercaptans and their biodegradation product, hydrogen sulphide, are fairly stable in acid solutions. However, the degradation rate of mercaptans by MM-oxidase is influenced by the pH of the reaction medium and choice of buffer solution. Also, lower chain length primary mercaptans are more easily degraded than their corresponding secondary counterparts.

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