Degradation of Thiosulfate by Sulfide-Oxidizing Enzyme Produced by Bacteria Locally Isolated from Effective Microorganism Active Solution (EMAS)

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Abstract

A potential aerobic sulfur-oxidizing bacterium (SOB) that was believed to be sulfide-oxidizing enzyme producing strain was previously isolated from Effective Microorganism Active Solution (EMAS), currently known as SO2. This gram negative bacterium was capable of growing autotrophically in sulfur-oxidizer medium, containing 4mM sodium thiosulfate that serves as an energy sources and electron donor. Sulfate ions were the expected end product of sulfide oxidation catalyzed by extracellular sulfide-oxidizing enzyme. The aims of this study are to identify and characterize the SO2 and to induce the activity of sulfide-oxidizing enzyme in this strain. The activity of sulfide-oxidizing enzyme was determined spectrophotometrically by measuring the increase of sulfate production using $BaCl_2$ solution, while the oxidation of thiosulfate was colorimetrically determined at 460 nm. One unit of sulfide-oxidizing activity was defined as amount of enzyme required to produce 1 µmol sulfate per min per mL (U). The maximum sulfide-oxidizing activity (0.064 U) was achieved when the strain was grown at pH 5.0, 30˚C in medium containing 1% (w/v) peptone as nitrogen sources after 15 hours incubation. The specific growth rate of this strain at this condition was 0.1552 h^{-1}, with doubling time value of 4.47 h.

Key words: sulfur-oxidizing bacteria, thiosulfate, sulfide oxidation

1. Introduction

The removal of hydrogen sulfide (H_2S) from natural or industrial gases is an important concern of environmental technology, since this toxic corrosive gas is extremely hazardous to human health. H_2S is a colorless gas with a strong "rotten egg" smell [1], and their removal is of current interest. It appears naturally as a byproduct of the decomposition process of organic matter or produced in some industries such as pulping site, petroleum refinery plant, drug manufacturing process, sewage treatment facility and livestock raising farm [2].

Conventional physical-chemical processes of H_2S removal are mainly based on oxidation with air or permanganate, precipitation as a metal salt [3] or by direct oxidation by agents like Cl_2, H_2O_2 and NaClO [4]. But, the drawbacks of these methods include the need for specialized facilities, waste disposal, hazardous chemical usage and thus increase the costs of operation due to high energy demand (high pressure and temperature) [5]

Potential biological alternatives for H_2S removal application has been developed by using microorganism that able to oxidize H_2S, producing sulfate or elemental sulfur as a consequence of complete or incomplete metabolism, respectively [4].

A number of microbial processes for H_2S removal have been proposed that are based on oxidation of microorganism that has an ability to oxidize sulfide to sulfate or elemental sulfur. Sulfur-oxidizing bacteria (SOB) are candidates microorganism for accomplishing the removal of H_2S.

Most of the known SOB belongs to the genera *Thiobacillus, Thiothrix, Thiomicrospira, Achromatium and Desulfuromonas* [6]. This autotrophic microorganism utilize reduced inorganic sulfur compounds such as sulfide, thiosulfate, sulfite, tetrathionate or elemental sulfur for the biosynthesis of cellular material or transform these compounds as part of a respiratory energy-generating process [6].

However, oxidation of sulfur compounds is not restricted to the true sulfur bacteria; this process also occurs in heterotrophic bacteria isolated from soil and marine environment [6]. Most of the heterotrophic bacteria belong to the genera *Pseudomonas* [7], *Xanthobacter* [8], or are *Escherichia coli* strains [9].

The oxidation of starting substrate such as sulfide, elemental sulfur or thiosulfate will produce sulfate as the end product, with sulfite or polythionates as the intermediate of sulfur compounds oxidation [10-11]. The oxidation of inorganic sulfur compounds was catalyzed by enzyme generally known as sulfide-oxidizing enzyme [12]. These processes of inorganic sulfur compounds oxidation

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are varying because the mechanism appeared to differ radically among different sulfur bacteria.

In this paper, a potential aerobic SOB isolated from EMAS has been used throughout this study. The scope of this work was to optimize production of sulfide-oxidizing enzyme by manipulating the physical and environmental factors such as pH, temperature and different nitrogen sources to obtain the optimal condition for enzyme production.

2. Materials and Method

2.1 Media and Cultivation Conditions

Sulfur-oxidizer medium for sulfur-oxidizing bacteria contained (per liter)[13]: 10 g of Bacto-Peptone, 1.5 g of K_2HPO_4, 0.75 g of ferric ammonium citrate and 1.0 g of $Na_2S_2O_3.5H_2O$. The pH was adjusted to 7.0 using 1 M HCl before sterilizing by an autoclave. To prepare solid medium, agar was added to a final concentration of 15 g per liter. The SO2 cultures were incubated at 30˚C at 180 rpm shake, and growth was followed by measuring the turbidity at 660nm using the spectrophotometer.

2.2 Preparation of Crude Enzyme Extract

Freshly grown cells were harvested by centrifugation at 4000 rpm, 4 ˚C for 20 minutes and the supernatant was used as a crude enzyme extract. The crude enzyme was analyzed for sulfide-oxidizing activity, product (sulfate), protein concentration and thiosulfate oxidation.

2.3 Determination of Sulfide-Oxidizing Activity

2.3.1 Reagents Preparation

Sodium sulfide solution 0.02M was prepared by dissolving $Na_2S.xH_20$ in an alkaline solution consisting 0.16 g of NaOH, 0.02 g of ethylenediaminetetraacetic acid disodium salt (EDTA $Na_2.2H_20$), 2 mL of glycerol and 40 mL of distilled water. The Na_2S solution was prepared just before use.

2.3.2 Sulfide-Oxidizing Assay

The sulfide-oxidizing activity was determined by measuring the product of enzymatic reaction, SO_4^{2-} in the reaction mixture. The enzyme assay protocol was a modified version of Hirano *et al.* (1996) enzyme assay [14].

The reaction was initiated by the addition of 0.5 mL Na_2S solution into the reaction mixture that contain 4.5 mL 0.1 M sodium acetate buffer (pH 5.6) and 1 mL crude enzyme. The mixture was incubated for 30 minutes at 30˚C and the reaction was subsequently terminated by addition of 1.5 mL NaOH (1.0 M) followed by thoroughly mixing. The amount of sulfate ion was detected by mixing 2 mL of 10% (w/v) $BaCl_2.2H_2O$ and 2 mL of reactant and the absorbance was measured at 450 nm using spectrophotometer [15]. One unit of sulfide-oxidizing activity was defined as amount of enzyme required to produce 1 μmol sulfate per min per mL (U).

2.4 Analysis of Residual Thiosulfate

The level of thiosulfate in the medium was determined by the colorimetric procedures of Sorbö [16]. This thiosulfate assay reaction occurs at room temperature with the addition of Cu^{2+} ions as catalyst. Thiosulfate was estimated at 460nm after the addition of an acidic solution of ferric nitrate.

2.5 Protein Analysis

Protein was estimated, with crystalline bovine serum albumin as standard, by the method of Lowry *et al.* [17] at 750nm.

2.6 Induction of Sulfide-Oxidizing Enzyme Production

2.6.1 Effect of Various pH

The pH of culture media was adjusted within 4.0 to 9.0 using 1 M HCl or 1 M NaOH. pH adjustment was done before autoclaving process. After autoclaving and cooling, 10% (v/v) inoculum with optical density of 0.6-0.8 was added. The cultures were incubated at 30˚C in 180 rpm-shaking incubator and samples were withdrawn at 3 hours interval for the analysis. The optimum pH of culture medium achieved in this step was fixed for subsequent experiments.

2.6.2 *Effect of Various Temperatures*

The cultures were incubated at various temperatures (30˚C, 37˚C and 45˚C) with pH 5.0. Analysis was performed at 3 hours interval to determine the maximum sulfide-oxidizing activity. The optimum temperature of culture medium achieved in this step was fixed for subsequent experiments.

2.6.3 *Effect of Various Nitrogen Sources*

Sulfur-oxidizer liquid medium was amended with varying nitrogen sources. Peptone in the original formulation was substituted with other organic nitrogen sources, particularly yeast extract, urea and casein; other inorganic sources, particularly ammonium chloride, ammonium acetate and potassium nitrate. The concentration of nitrogen sources was remained the same as original recipe, at 1% (w/v). Each medium was adjusted to pH 5.0 and the cultures were incubated at 30˚C. Sample was withdrawn at regular interval for the analysis.

3.0 Results

Effect of Various pH

The effect of initial pH towards sulfide-oxidizing activity was demonstrated in Table 1. Growth of SO2 was studied over a wide range of pH, ranging from 4.0 until 9.0, and the maximal sulfide-oxidizing activity was obtained at pH 5.0. The specific growth rate (μ) and doubling time (t_d) values of SO2 strain in pH 5.0 medium were 0.1552 h^{-1} and 4.47 h, respectively.

The time course of sulfide-oxidizing activity by SO2 strain at initial pH of 5.0 was presented in Figure 1. The highest sulfide-oxidizing activity (0.064 U) was achieved from culture grown at pH 5.0 with production of biomass,

X_{max}, 0.3625 g/L after 15 hours incubation. At this pH, the specific enzyme activity, 0.0241 U/mg was the highest value as compared to other pHs. A drastic decreased in the enzyme activity was observed when the bacteria was grown in the medium with initial pH at 8.0 and 9.0. However, growth of bacteria was not significantly affected by pH. Growth was varied in the biomass ranged of 0.250-0.4625 g/L at pH between 3.0 – 9.0.

Table 1. Effect of Initial pH towards Sulfide-Oxidizing Activity

pH	μ (h^{-1})	X_{max} (g/L)	Activity (U)	[Protein] (mg/mL)	Specific activity (U/mg)
4	0.1296	0.3875	0.037	3.59	0.0102
5	0.1552	0.3625	0.064	2.68	0.0241
6	0.2415	0.2500	0.045	2.70	0.0167
7	0.3035	0.4125	0.042	2.87	0.0148
8	0.1687	0.4625	0.031	3.34	0.0092
9	0.1004	0.4625	0.024	3.10	0.0077

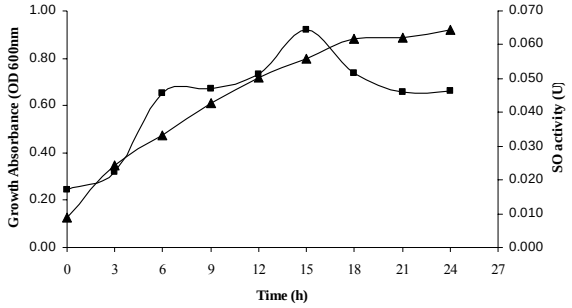

Figure 1. Time Course of Sulfide-Oxidizing Activity (SO Activity) by SO2 Strain at Initial pH of 5.0. (▲: Bacterial Growth; ■: Sulfide-Oxidizing Activity).

Effect of Various Temperatures

The effect of temperature was studied by incubated the cultures at different temperature such as 30˚C, 37˚C and 45˚C and the result was illustrated in Table 2**.** From the results, it is clearly shown that microbial growth of SO2 strain at 30˚C resulted the highest sulfide-oxidizing activity. The specific growth rate (μ) and doubling time (t_d) values at 30˚C are 0.1552 h^{-1} and 4.47 h, respectively. The maximum biomass (0.3625 g/L) was produced at this temperature. Temperature higher than 30˚C resulted in the decrease of sulfide-oxidizing activity and also the specific growth rate of SO2 strain. Growth at 45˚C was not favorable with a relatively low biomass (0.1500 g/L) and slow doubling time value, 0.1155 h^{-1}.

The time course of sulfide-oxidizing activity by SO2 strain at 30˚C was expressed in Figure 2**.** The increase of growth showed a corresponding increase of sulfide-oxidizing activity when cells were in log phase. The maximum sulfide-oxidizing activity (0.052 U) and specific activity (0.0167 U/mg) were attained after 15 hours incubation.

Table 2. Effect of Temperature towards Sulfide-Oxidizing Activity

Temp. (˚C)	μ (h^{-1})	X_{max} (g/L)	Activity (U)	[Protein] (mg/mL)	Specific activity (U/mg)
30	0.1544	0.3625	0.052	3.09	0.0167
37	0.1477	0.3000	0.042	3.14	0.0135
45	0.1155	0.1500	0.035	3..07	0.0115

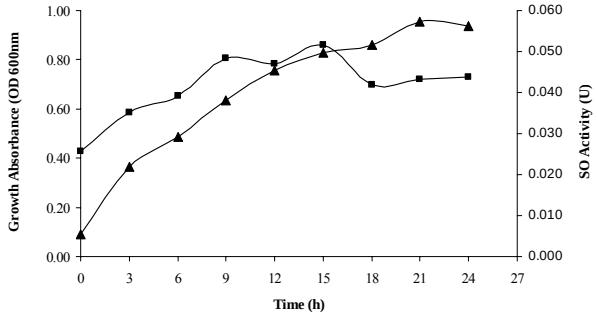

Figure 2. Time Course of Sulfide-Oxidizing Activity (SO Activity) by SO2 Strain at 30˚C *▲: Bacterial Growth; ■: Sulfide-Oxidizing Activity).*

Effect of Various Nitrogen Sources

Several kinds of organic and inorganic nitrogen sources were used in these studies as replacement of peptone in the original media formulation. Summarized results for the effect of various nitrogen sources towards sulfide-oxidizing activity are shown in Table 3.

From the experiment, it showed that culture growing in medium supplement with peptone as nitrogen source showed the highest sulfide-oxidizing activity of 0.047 U compared to others, whilst ammonium chloride exhibited the lowest sulfide-oxidizing activity (0.030 U). However, the highest specific activity (0.0437 U/mg) was obtained when urea was used as sole nitrogen source, due to low protein concentration (0.82 mg/mL) produced in this culture. But, growth with urea was not favorable due to poor concentration of biomass produced (0.1375 g/L). The highest biomass level obtained when yeast extract was used in the culture media with value of 1.8625 g/L. Besides that, casein, ammonium chloride and potassium nitrate also promoted good growth of SO2 strain with their respective specific growth rate, μ were 0.1936 h^{-1}, 0.0889 h^{-1} and 0.113 h^{-1}.

The time course of sulfide-oxidizing activity by SO2 strain supplemented with peptone as nitrogen source was demonstrated in Figure 3**.** Sulfide-oxidizing activity started

to increase as cells were in exponential phase, reaching the maximum level at 15 hours and decrease slowly beyond this point. The amount of protein and specific activity liberated at this time were 3.21 mg/mL and 0.0146 U/mg, respectively.

Table 3. Effect of Various Nitrogen Sources towards Sulfide-Oxidizing Activity

Nitrogen Sources	μ (h^{-1})	X_{max} (g/L)	Activity (U)	[Protein] (mg/mL)	Specific Activity (U/mg)
Peptone	0.1447	0.5125	0.047	3.21	0.0146
Yeast extract	0.1841	1.8625	0.038	1.94	0.0196
Urea	0.0676	0.1375	0.036	0.85	0.0437
Casein	0.1936	1.1125	0.032	3.17	0.0101
Ammonium chloride	0.1011	0.2250	0.030	0.79	0.0433
Ammonium acetate	0.0424	0.1250	0.037	0.98	0.0381
Potassium nitrate	0.1130	0.2250	0.044	0.87	0.0512

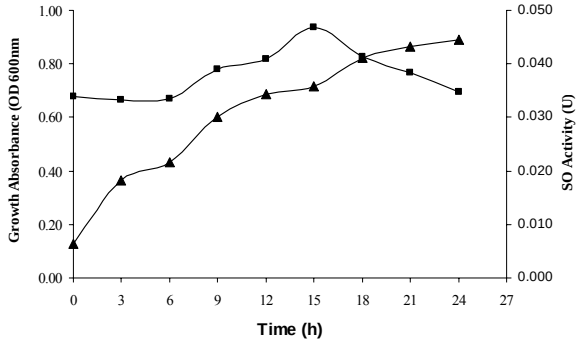

Figure 3. Time Course of Sulfide-Oxidizing Activity (SO Activity) by SO2 Strain Supplemented with Peptone as Nitrogen Source. (▲: Bacterial Growth; ■: Sulfide-Oxidizing Activity).

Metabolism of Thiosulfate

Thiosulfate is the common oxidizable substrate that is most suitable for investigations of sulfur-oxidizing bacteria processes. In this study, thiosulfate acts as electron donor and energy sources for the sulfur-oxidizing bacteria. The sulfur-oxidizer media was supplemented with 4 mM sodium thiosulfate as substrate for microbial activity. At this concentration, the oxidation of thiosulfate by SO2 strain was low. The maximum oxidation of thiosulfate that can be achieved at this stage of experiment is when peptone was used as nitrogen source. In medium containing peptone under optimized condition, the thiosulfate oxidation percentage by SO2 strain was 6.65% with highest sulfide-oxidizing activity (0.047 U) and considerable amount of biomass (0.5125 U/mg) (Table 3). The results of thiosulfate oxidation by SO2 strain, at pH 5.0 and 30˚C at various nitrogen sources was summarized in Table 4.

Table 4. Percentage of Thiosulfate Oxidation by SO2 Strain at Various Nitrogen Sources

Nitrogen Sources	Thiosulfate Oxidation (%)
Peptone	6.65
Yeast extract	3.33
Urea	2.30
Casein	2.30
Ammonium chloride	3.33
Ammonium acetate	2.30
Potassium nitrate	6.43

4.0 Discussion

A possible sulfide-oxidizing enzyme that oxidize sulfide to sulfate was detected in the cell-free supernatant of SO2 strain, based on the enzyme activity obtained. However, the activity of this enzyme was relatively low. Kransil'nikova *et al*., (2004) also demonstrated low activity of enzyme in his work with value of 0.3 nmol/mg protein/min and 1.2 nmol/mg protein/min for strain N1 and strain SSO that grown in presence of elemental sulfur, respectively [18]. It should be noted that activity of enzymes involved in the metabolism of inorganic sulfur compounds varied depending on type of sulfur compounds added to the medium.

This SO2 strain use thiosulfate in the growth medium that serves as energy substrate, and electron donor for the oxidation of sulfur compounds. Thiosulfate was uses in the growth medium because it is the common oxidizable substrate and more readily soluble in water compared to elemental sulfur.

Induction of sulfide-oxidizing enzyme. Most natural environment has pH values between 5.0 and 9.0, and organisms with optima in this range are most common. Growth of SO2 strain was observed under acid, neutral and alkaline conditions. This indicated that the SO2 strain were tolerant to acid and alkaline condition. Optimum pH for the microbial growth in this work was at pH 5.0. From literatures, the optimum pH for sulfur-oxidizing bacteria was varied, depending on the microbial habitat. The haloalkaliphilic *Thioalkalivibrio* isolated from soda lakes growth optimally at pH 10.0 [19] while *Thiobacillus caldus* found in environment such as coal spoil heaps was able to grow down to pH 1.0 [20].

As Table 1 shows that the sulfide-oxidizing activity of SO2 strain was affected by pH changes. The sulfide-oxidizing activity increase until reaches the optimum pH at 5.0 and start to decrease above the optimum pH. When the pH deviates appreciably from optimum pH, denaturation occurs leading to reduction of catalytic properties.

The metabolic activities of microorganism were followed by changes in pH of their environment as growth proceeds. The pH profile of SO2 culture grown in medium with pH 5.0 was determined to be constant around pH 7.0-8.0. The pH rises in this culture of sulfur-oxidizing bacteria, might be due to formation of polythionate as products of thiosulfate oxidation rather than the formation of sulfate, according to Reaction 1 [21]. The product of complete oxidation of thiosulfate is sulfate or sulfuric acid, results in lowering of pH and acidification of medium (Reaction 2).

$$2S_2O_3^{2-} + 0.5O_2 + H_20 \rightarrow S_4O_6^{2-} + 2OH^- \quad (1)$$

$$S_2O_3^{2-} + 2O_2 + H_20 \rightarrow 2SO_4^{2-} + 2H^+ \quad (2)$$

Temperature is one of the most important environmental factors affecting growth and activity of microorganisms. In this work, the optimum temperature for sulfide-oxidizing enzyme production by SO2 strain was 30˚C. Literatures also reported that sulfur-oxidizing bacteria such as *Thiobacillus novellus* and *Thiobacillus ferrooxidans* were found to be grown optimally at 30˚C [15, 22].

Sulfide-oxidizing enzyme production was greatly associated with growth, and was found to be affected by microbial growth. Therefore, growth was essential for ensuring a greater sulfide-oxidizing activity. The activity of sulfide-oxidizing enzyme was increase with an increase in temperature, until the optimum temperature was reached at 30˚C, and it starts to decrease beyond that temperature. At 45˚C, growth started to reduce eventually and activity of enzyme was low. This might be due to denaturation of enzyme that leads to changes in the configuration of enzyme active site and loss of catalytic properties [23]. SO2 strain was a mesophile microorganism and most protein from mesophiles is inactivated by temperatures above 40˚C [23].

Nitrogen is a major element in proteins, nucleic acid and several other constituents in the cell. Nitrogen can be found in nature as both organic and inorganic forms. In the study of various nitrogen sources effect, organic nitrogen sources that were used are peptone, yeast extract, casein and urea while the inorganic nitrogen sources was supplied by ammonium chloride, ammonium acetate and potassium nitrate.

All selected nitrogen sources except urea and ammonium acetate supported the growth of SO2 strain. Yeast extract and casein produced the higher concentration of biomass, and the specific growth rate was high compared to other nitrogen sources. Yeast extract contain various type of amino acid, while casein contain amino nitrogen which is enough to support the bacterial growth.

Peptone was found to be the best nitrogen sources for sulfide-oxidizing enzyme production, which results in the highest sulfide-oxidizing activity (Table 3) compared to others. The order of nitrogen sources preferential was peptone > potassium nitrate > yeast extract > ammonium acetate > urea > ammonium chloride > casein. The highest specific activity was obtained in urea-containing medium, due to low protein concentration produced (Table 3). However, urea was not a promising nitrogen sources due to insignificant growth of cells in the medium. In literature, urea has been shown to serve as a nitrogen source for other thiobacilli [24]. Ammonium chloride exhibited the poorest level of sulfide-oxidizing activity. This might be due to inhibitory effect of chloride ions. As reviewed, increased concentration of chloride ions in basal medium would inhibit the oxidation activity [25] .

Metabolism of Thiosulfate. Oxidation of thiosulfate with oxygen as electron donor has been demonstrated in SO2 strain. However, low rate of thiosulfate oxidation was observed with this strain. The SO2 strain only uses 6.65% (w/v) of thiosulfate. Low thiosulfate oxidation might be due to substrate inhibition at high concentration of thiosulfate.

Conclusion

The microorganism known as SO2 strain that has been used in this work was able to grow optimally at pH 5.0 and 30˚C. The specific growth rate (μ) and doubling time (t_d) for this mesophile microorganism were 0.1552 h^{-1} and 4.47 h, respectively. The maximum sulfide-oxidizing activity of SO2 strain (0.064 U) was achieved by employing peptone as nitrogen source under optimized pH and temperature at pH 5.0 and at 30˚C, respectively. The highest oxidation rate of thiosulfate has been achieved in the same condition, at initial concentration of thiosulfate, 4 mM with oxidation percentage by SO2 strain at 6.65%. The highest sulfide-oxidizing activity was obtained during the exponential growth of SO2 strain, and based on the growth profile, sulfide-oxidizing enzyme could be categorized as growth-associated metabolite or primary metabolite.

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