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# Characteristics of Sulfur Oxidation by a Newly Isolated *Burkholderia* spp.

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**Abstract** The role of an effective microbial species is critical to the successful application of biological processes to remove sulfur compounds. A bacterial strain was isolated from the soil of a malodorous site and identified as *Burkholderia* spp. This isolate was able to oxidize thiosulfate to sulfate, with simultaneous pH decrease and accumulation of elemental sulfur. The specific growth rate and the sulfate oxidation rate using the thiosulfate basal medium were 0.003 $h^{-1}$ and 3.7 $h^{-1}$, respectively. The isolated strain was mixotrophic, and supplementation of 0.2% (w/v) of yeast extract to the thiosulfate-basal medium increased the specific growth rate by 50-fold. However, the rate of sulfate oxidation was more than ten times higher without yeast extract. The isolate grew best at pH 7.0 and 30°C, and the sulfate oxidation rate was the highest at 0.12 M sodium thiosulfate. In an upflow biofilter, the isolated strain was able to degrade $H_2S$ with 88% efficiency at 8 ppm and 12 l/h of incoming gas concentration and flow rate, respectively. The cell density at the bottom of the column reached $3.2\times10^8$ CFU/(g bead) at a gas flow rate of 12 l/h.

**Key words:** Heterotrophic bacterium, *Burkholderia* spp., sulfur oxidation rate, biodegradation

Hydrogen sulfide ($H_2S$) and other sulfur compounds are produced from industrial processes such as petrochemical refining, wastewater treatment, and food treatment [7]. Removal of these sulfur compounds has become a worldwide environmental issue because of their toxicity, corrosiveness, and malodor, even at low concentrations. Physical and chemical processes utilizing activated carbon adsorption, ozone oxidation, and incineration have been applied to remove $H_2S$ from several waste gases and wastewater [1, 18]. However, these methods have drawbacks, including insufficient removal efficiency, high cost, and the release of byproducts, which lead to secondary pollution problems.

Biological removal of sulfur compounds may offer some advantages in large-scale processes, because of their high removal efficiency without significant secondary contamination [2]. Biological processes can be more successfully applied if effective microbial species are used. Generally, autotrophic and heterotrophic microorganisms can both be used in biological sulfur removal. However, most reports on the bacterial metabolism of $H_2S$ were on autotrophs such as *Thiobacillus* species [3, 9, 10, 11, 12, 16] and *Chlorobium thiosulfatophilum* [6]. Autotrophic and heterotrophic microorganisms are different in their nutritional requirements and abilities to catalyze specific reactions. The autotrophs gain energy from the oxidation of $S^0$ and use $CO_2$ as their carbon source. Some studies were reported on the mechanisms of sulfur oxidation by autotrophs. For instance, the sulfide oxidoreductase from *Thiobacillus ferrooxidans* [17] performs an important role in energy production, and *Paracoccus versutus* [8] has cytochromes $C_{551}$ and $C_{552.5}$ that play a role in sulfide oxidation in electron transport. Usually, these autotrophic bacteria have drawbacks because of their slower growth rate and special nutritional requirements.

Chemoheterotrophic bacteria, such as *Thiotrix*, *Beggiatoa*, and *Hyphomicrobium* genera, can oxidize hydrogen sulfide into elemental sulfur and store it in their cells. The $H_2S$ will be further oxidized to sulfate ($SO_4^{2-}$) by sulfur oxidase, and the sulfate used to synthesize cysteine and methionine [19]. Other reports on the oxidation of $H_2S$ by heterotrophic bacteria include *Streptomyces* spp. [15], *Pseudomonas acidovorans* [22], *Bacillus* spp. [21], and *Xanthomonas* spp. [4].

The biological role of the sulfide-oxidizing enzyme in heterotrophs was also reported [21]. The hydrogen sulfide oxidase reaction requires $O_2$ and produces mainly elemental sulfur, from which the following two reactions can be deduced:

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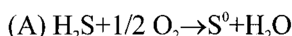
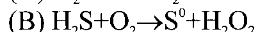

(A) $H_2S+1/2\ O_2 \rightarrow S^0+H_2O$


(B) $H_2S+O_2 \rightarrow S^0+H_2O_2$

The hydrogen sulfide oxidation mechanism of *Bacillus* spp. BN 53-1 [21] is reaction A type. Reaction B type is catalyzed by a hydrogen sulfide oxidase in *Hyphomicrobium* spp. [23]. In this study, we isolated a new strain, *Burkholderia* spp., which was able to grow autotrophically and mixotrophically. The growth and sulfur-oxidizing ability of the strain were characterized at various environmental and nutritional conditions in batch culture and in a biofilter.

## MATERIALS AND METHODS

### Isolation and Cultivation of the Microorganism

The microorganism used in this study was isolated from the soil of a malodorous site near Suwon, Korea. Strains for sulfur oxidation were screened on thiosulfate agar plates containing (g/l): $KH_2PO_4$ 2.0, $K_2HPO_4$ 2.0, $NH_4Cl$ 0.4, $MgCl_2$ 0.2, $FeSO_4{\cdot}7H_2O$ 0.01, and $Na_2S_2O_3{\cdot}5H_2O$ 8.0. Microorganisms showing capability of sulfur oxidation were selected as follows. Ten grams of each soil sample was inoculated into 100 ml of thiosulfate-basal medium (Table 1) in a 250-ml flask, and the suspension was incubated at 30°C in a shaking incubator at 150 rpm (8480 SFN Vision Scientific Co., Ltd., Korea). When the pH of the medium dropped to below 3.5, 10 ml of the culture broth was transferred to 100 ml of fresh thiosulfate medium. Twenty microliters of cell suspension was spread onto solid thiosulfate agar plates, and the plates were incubated at 30°C for 2 days. One strain growing fast on the agar plates was selected and used in this study. To avoid precipitation during autoclaving, sulfur source ($Na_2S_2O_3{\cdot}5H_2O$ and $S^0$) and the yeast extract were autoclaved separately at 121°C for 15 min, and were combined aseptically before adding the other constituents. Glucose solution was sterile-filtered using a 0.45 μm membrane filter (Millipore Bedford, MA, U.S.A.).

### Identification of the Strain Isolated

The isolated strain was examined for its biochemical and morphological properties using Gram Negative Identification Test Panels (Biolog Inc., Hayward, U.S.A.). The suspension of the isolated cells was inoculated on the Microplate™, which had been pre-warmed to 30°C, and was then incubated at 30°C for 24 h. The Microplate™ was read at 590 nm, and the results were analyzed using the Microplate™ software (Biolog Inc.).

**Table 1.** Composition of the medium ($g{\cdot}l^{-1}$).

| | Thiosulfate-basal medium (pH 7.0) | | Modified Waksman (MW) medium (pH 4.0) | |
|---|---|---|---|---|
| Composition | $KH_2PO_4$ | 2.0 | $KH_2PO_4$ | 3.0 |
| | $K_2HPO_4$ | 2.0 | $NH_4Cl$ | 0.1 |
| | $NH_4Cl$ | 0.4 | $MgSO_4{\cdot}7H_2O$ | 0.5 |
| | $MgCl_2$ | 0.2 | $CaCl_2{\cdot}2H_2O$ | 0.3 |
| | $FeSO_4{\cdot}7H_2O$ | 0.01 | $FeSO_4{\cdot}7H_2O$ | 0.01 |
| | $Na_2S_2O_3{\cdot}5H_2O$ | 8.0 | $S^0$ | 10.0 |

### Growth of *Burkholderia* spp.

For batch experiments, the isolated cells were incubated at 30°C in the shaking incubator at 150 rpm. The cells were grown in 250-ml flasks containing 150 ml of thiosulfate-basal medium with 0.8% (w/v) or without sodium thiosulfate.

The effect of several factors on the cell growth was investigated; initial pH (pH 2.0–9.0) of the medium, incubation temperature (10–35°C), substrate concentration (0.016–0.16 M sodium thiosulfate), and supplementation of organic compounds [0.2% (w/v) of yeast extract and 0.2% (w/v) of glucose] with and without sodium thiosulfate. In addition, the possibility of utilizing elemental sulfur was studied by using modified Waksman (MW) medium (Table 1). Samples (5 ml) were taken every 3 h for the measurement of pH and sulfate concentration.

### Biofilter Experiments

A biofilter system including a cylindrical acryl column (3.4 cm i.d.×30 cm height) was used. Twenty-five cm of the column was packed with calcium alginate beads (3.5 mm diameter) (Fig. 1). Initial pH and the cell number were 6.0 and $3.2\times10^4$ CFU/(g bead), respectively. Potassium phosphate buffer solution (100 ml) was supplied from the top of the column using a glass pipet at every 24 h. Hydrogen sulfide was supplied from a gas cylinder. Gas input concentration and flow rate were controlled by mass flow controllers. $H_2S$ removal efficiency was calculated as follows:

$$\text{Removal efficiency} = \frac{\text{inlet gas conc.}}{\text{outlet gas conc.}} \times 100$$

The cell number at three locations (top, middle, and bottom) of the column was determined by dissolving 1 g of bead in 10 ml of sodium citrate solution. The cell

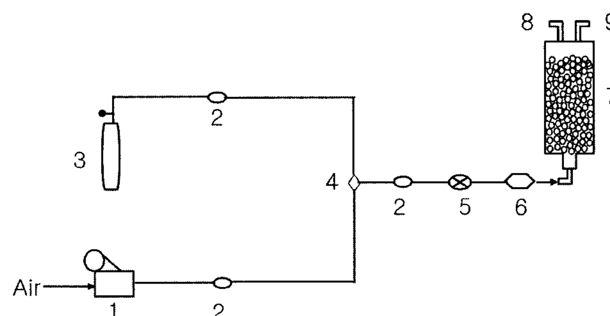



**Fig. 1.** A diagram of the biofilter system for $H_2S$ removal.
(1) Air compressor; (2) flow meter; (3) $H_2S$ gas cylinder; (4) three-way valve; (5) air filter; (6) inlet chamber; (7) acryl column; (8) gas outlet; (9) inlet of buffer solution.

suspension was serially diluted and was spread on the agar plates containing sodium thiosulfate-basal medium.

**Analytical Methods**

For the determination of the cell growth, optical density was measured at 660 nm by using a spectrophotometer (UV-1700 Shimadzu, Tokyo, Japan). To analyze sulfate concentration, 2 ml aliquot was centrifuged (12,000 rpm, at 4°C) and the supernatant was analyzed by an ion chromatograph (DX-500, Dionex U.S.A.) equipped with an Ion-Pac As 12 (anion) column and CD-20 conductivity detector. Hydrogen sulfide was analyzed using a gas chromatograph (HP 5890 series II, U.S.A.) equipped with a detector for thermal conductivity and a column (Alltech, U.S.A.) packed with Super Q 80/100. Helium was used as a carrier gas at a flow rate of 40 ml/min, and the temperatures used were 70°C for the injector, 80°C for the oven, and 90°C for the detector.

## RESULTS

**Isolation and Characterization of a New Strain**

Thirty-six bacterial strains were isolated from the soil of a malodorous site near Suwon city (Korea). Of the five strains capable of degrading thiosulfate, the most efficient strain was characterized (Table 2). The isolated strain was Gram-negative, and it responded positively to catalase and utilized citrate, mannitol, sucrose, fructose, and trehalose. Colonies were white colored and their diameters were 0.5–1 mm. The strain was rod-shaped, motile, and the Biolog data of the strain showed 85.5% similarity to *Burkholderia* spp.

**Table 2.** Biochemical characteristics of *Burkholderia* spp.

| Characteristics | Reaction | Characteristics | Reaction | Characteristics | Reaction |
|---|---|---|---|---|---|
| α-Cyclodextrin | V | Acetic acid | + | L-Leucine | + |
| Dextrin | + | cis-Aconitic acid | + | L-Ornithine | + |
| Glycogen | + | Citric acid | + | L-Phenylalanine | + |
| Tween 40 | + | Formic acid | + | L-Proline | + |
| Tween 80 | + | D-Galactonic acid | + | L-Pyroglutamic acid | – |
| N-Acetyl-D-galactosamine | + | D-Galacturonic acid | + | D-Serine | + |
| N-Acetyl-D-glucosamine | + | D-Gluconic acid | + | L-Serine | + |
| Adonitol | + | D-Glucosaminic acid | + | L-Threonine | + |
| L-Arabinose | – | D-Glucuronic acid | + | D,L-Carnitine | + |
| D-Arabitol | – | α-Hydroxybutyric acid | + | γ-Amino butyric acid | + |
| Cellobiose | + | β-Hydroxybutyric acid | + | Urocanic acid | + |
| Erythritol | + | γ-Hydroxybutyric acid | + | Inosine | + |
| D-Fructose | + | p-Hydroxyphenylacetic acid | + | Uridine | + |
| L-Fucose | + | Itaconic aicd | – | Thymidine | + |
| D-Galactose | + | α-Ketobutyric acid | + | Phenyl ethylamine | + |
| Gentiobiose | + | α-Ketoglutaric acid | – | Putrescine | – |
| α-D-glucose | + | α-Ketovaleric acid | + | 2-Amino ethanol | + |
| Inositol | + | D,L-Lactic acid | + | 2,3-Butanediol | + |
| α-D-Lactose | – | Malonic acid | + | Glycerol | + |
| Lactulose | – | Propionic acid | + | D,L-α-Glycerol phosphate | + |
| Maltose | + | Quinic acid | + | Glucose-1-phosphate | + |
| D-Mannitol | + | D-Saccharic acid | + | Glucose-6-phosphate | + |
| D-Mannose | + | Sebacic acid | V | | |
| D-Melibiose | – | Succinic acid | + | | |
| β-Methyl-D-glucoside | V | Bromo succinic acid | + | | |
| D-Psicose | + | Succinamic acid | + | | |
| D-Raffinose | – | Glucuronamide | + | | |
| L-Rhamnose | – | Alaninamide | + | | |
| D-Sorbitol | + | D-Alanine | + | | |
| Sucrose | + | L-Alanine | + | | |
| D-Trehalose | + | L-Alanyl-glycine | + | | |
| Turanose | – | L-Asparagine | + | | |
| Xylitol | – | L-Aspartic acid | + | | |
| Methyl pyruvate | + | L-Glutamic acid | + | | |
| Mono-methyl-succinate | + | Glycyl-L-aspartic acid | – | | |
| | | Glycyl-L-glutamic acid | + | | |
| | | L-Histidine | – | | |
| | | Hydroxy L-proline | + | | |

+: Positive; –: negative; V: variable.

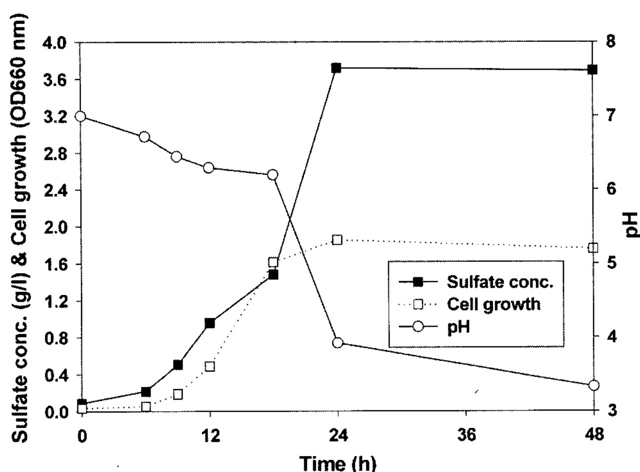



**Fig. 2.** Growth of *Burkholderia* spp. and sulfate concentration in thiosulfate-basal medium supplemented with 8 g·l$^{-1}$ of sodium thiosulfate.

## Cell Growth Pattern and Sulfate Oxidation Rate

During the first 24 h of cell growth in the thiosulfate-basal medium (initial pH 7.0) supplemented with yeast extract, the pH decreased to 3.9 and the sulfate concentration increased up to 3.7 g/l. The specific growth rate and sulfate oxidation rate were 0.15 h$^{-1}$ and 0.2 g-S/l/h, respectively (Fig. 2). This result indicates that the strain is capable of oxidizing thiosulfate to sulfate. This is the first report on the use of sulfur compounds by *Burkholderia* spp., which is known to degrade aromatic and chloroaliphatic hydrocarbons [13].

After another 24 h of cultivation, the cell density and sulfate concentration remained almost constant, and the pH dropped to 3.3. Elemental sulfur was visibly accumulated in the medium. This suggests that the strain was capable of oxidizing thiosulfate to sulfate with concurrent accumulation of elemental sulfur. *Burkholderia* spp. was able to grow on

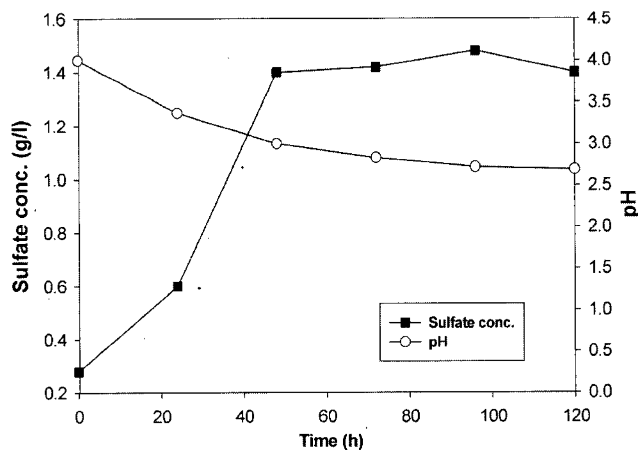



**Fig. 3.** Sulfate concentration in modified Waksman (MW) medium supplemented with 10 g·l$^{-1}$ of elemental sulfur.

the modified Waksman (MW) medium containing 10 g/l of elemental sulfur. The maximum sulfate concentration was 1.5 g/l, and the pH dropped to 2.7 (Fig. 3).

## Effect of Initial pH and Temperature on Cell Growth

The maximum specific growth rate was 0.15 h$^{-1}$ at pH 7 in thiosulfate-basal medium with 0.2% (w/v) of yeast extract. However, in the pH range of 5.0–7.0, the specific growth rate was similar (0.14–0.15 h$^{-1}$). In the pH range of 2.0–9.0 studied, the cells did not grow at pH 2.0, 3.0, and 9.0. For the pH experiments, temperature was fixed at 30°C, and the concentrations of sodium thiosulfate and yeast extract were 8 g/l (0.03 M) and 0.2% (w/v), respectively. The effect of temperature was studied at 10°C–35°C. The cell growth rate was the best at 30°C (0.16 h$^{-1}$), followed by 35°C (0.12 h$^{-1}$) and 20°C (0.08 h$^{-1}$). The cells did not grow at 10°C. For the temperature experiments, the initial pH was 7.0 and the concentrations

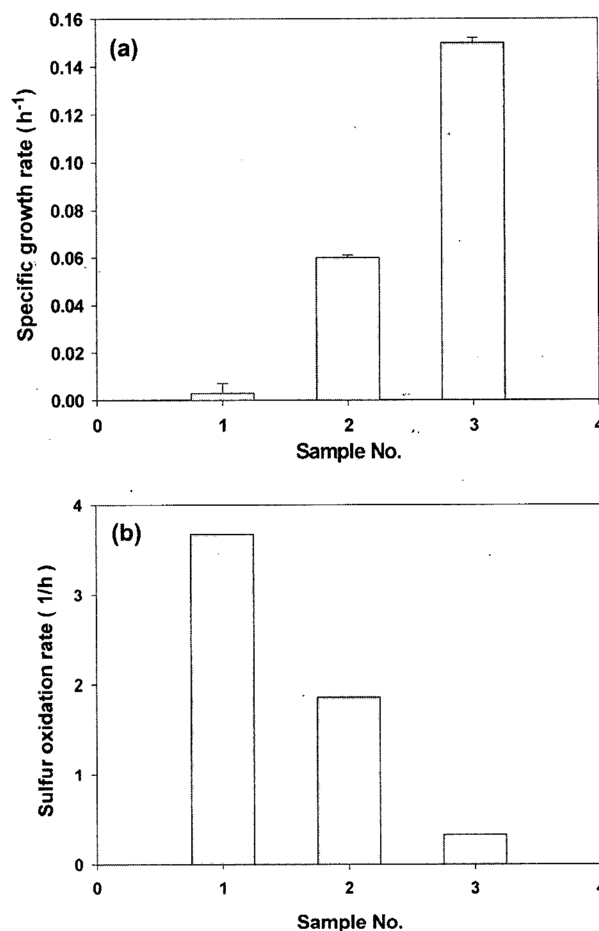



**Fig. 4.** Effect of organic compound supplementation on the cell growth (a) and sulfur oxidation rate per g of dry cell weight (b) of *Burkholderia* spp. in thiosulfate-basal medium.

(a): (1) sodium thiosulfate; (2) glucose+sodium thiosulfate; (3) yeast extract+sodium thiosulfate. (b): (1) sodium thiosulfate; (2) glucose+sodium thiosulfate; (3) yeast extract+sodium thiosulfate.

of sodium thiosulfate and yeast extract were 8 g/l and 0.2% (w/v), respectively.

### Effect of Organic Compounds on the Cell Growth and Sulfate Oxidation Rate

Supplementation of organic compounds (glucose and yeast extract) to the thiosulfate-basal medium increased the growth rate of *Burkholderia* spp. [Fig. 4(a)]. Yeast extract was a better supplement than glucose. The specific growth rate in the thiosulfate-basal medium was 0.003 $h^{-1}$. When 0.2% (w/v) of glucose was added to the thiosulfate-basal medium, the specific growth rate was 0.06 $h^{-1}$. The presence of both yeast extract and sodium thiosulfate in the medium synergistically stimulated the cell growth, and the specific growth rate increased substantially to 0.15 $h^{-1}$, which was 50-fold higher than that on sodium thiosulfate alone. The growth of the isolated strain on the mixture of sodium thiosulfate and organic compounds was similar to reports in the literature on the growth of *Pseudomonas* strains in the media supplemented with organic compounds (glucose and yeast extract) [5, 18].

The new strain that we isolated grew at an extremely low rate on sodium thiosulfate, when organic carbon sources were not supplemented. Addition of glucose or yeast extract increased the cell growth rate by 20-fold and 50-fold, respectively. These results suggest that the strain can grow autotrophically by obtaining some energy from the sulfur oxidation and by using $CO_2$ as its carbon source. However, this autotrophic metabolism appeared far less efficient for the production of energy and carbon skeleton materials, as compared to heterotrophic metabolism. The higher total sulfur oxidation rate with organic compound supplemention was due to the higher cell mass formation. Sulfate oxidation rate per dry cell weight was more than ten times higher for

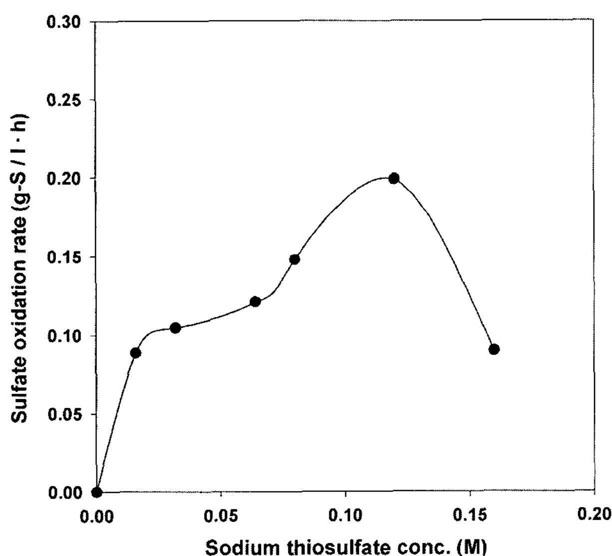



**Fig. 5.** Effect of sodium thiosulfate concentration on the sulfate oxidation rate by *Burkholderia* spp.

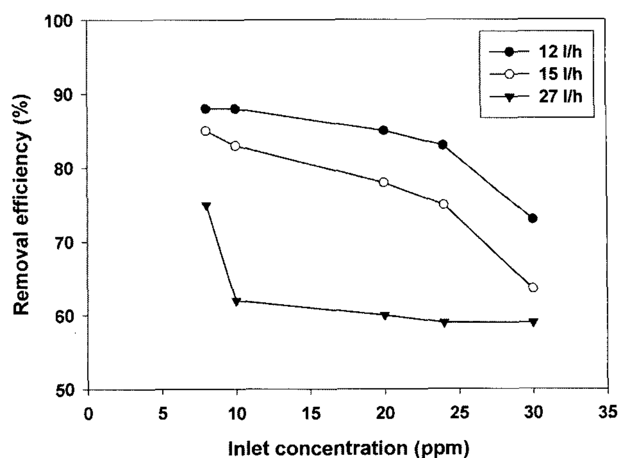



**Fig. 6.** Dependence of $H_2S$ removal efficiency on the inlet $H_2S$ concentration at different flow rates (12, 15, and 27 $l{\cdot}h^{-1}$).

autotrophically grown cells [Fig. 4(b)]. These results suggest that, when cells were able to grow chemotrophically on glucose or yeast extract, the autotrophic metabolism using sulfur compounds was significantly inhibited.

### Effect of Sodium Thiosulfate Concentration on Sulfate Oxidation Rate

The dependence of sulfate oxidation rate on the concentration of sodium thiosulfate (without yeast extract) followed substrate inhibition kinetics. The rate reached a maximum of 0.2 $h^{-1}$ at 0.12 M sodium thiosulfate concentration and decreased at higher concentrations of sodium thiosulfate (Fig. 5).

### Effect of Inlet Concentration and Flow Rate on $H_2S$ Removal in a Biofilter

$H_2S$ removal efficiency was 88% at 8 ppm of inlet $H_2S$ concentration and 12 l/h of gas flow rate. Removal efficiency decreased slowly up to 20 ppm and then decreased to 73% at 30 ppm of $H_2S$ concentration. This decrease of removal efficiency was also similar for 15 l/h. However, at a flow rate of 27 l/h, the removal efficiency decreased sharply from 75 to 62%, when the inlet concentration was increased from 8 ppm to 10 ppm (Fig. 6).

At 12 l/h of gas flow rate, the number of cells in the column was $3.2\times10^8$ CFU/(g bead) and $6.6\times10^7$ CFU/(g bead) at the top and the bottom, respectively. These cell concentrations were $10^4$ and $4.8\times10^3$ times larger than the initial loading of the cells in the column. At 27 l/h, the number of cells in the column was $3.9\times10^4$ CFU/(g bead) and $7.4\times10^3$ CFU/(g bead) at the top and the bottom, respectively.

## DISCUSSION

The overall sulfur oxidation rate of the *Burkholderia* spp. increased, when organic compounds were supplemented to

the thiosulfate medium. This increase was mainly due to higher biomass formation, as evidenced by the ten-fold decrease in the sulfur oxidation rate per unit cell mass with organic compound supplementation. This suggests that the cells that are forced to fully utilize the sulfur oxidation system under autotrophic conditions switch the substrate to organic compounds because the metabolic process is more energy efficient. Under this condition, the induction of the sulfur oxidation system seemed to be significantly repressed by the organic compounds. One possible reason for why the cells still used the less efficient energy system in the presence of organic compounds may be that this sulfur oxidation system serves other functions in the cell as well. However, it needs to be explored by further investigation.

## Acknowledgment

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