Biotechnol. Bioprocess Eng. 2000, 5: 48-52

Characterization of Sulfur Oxidation by an Autotrophic Sulfur Oxidizer, *Thiobacillus* sp. ASWW-2

Eun Young Lee[1], Kyung-Suk Cho[1]*, and Hee Wook Ryu[2]

[1] Department of Environmental Science and Engineering, Ewha Womans University, Seoul 120-750, Korea
[2] Department of Chemical and Environmental Engineering, Soong Sil University, Seoul 156-743, Korea

Abstract An autotrophic sulfur oxidizer, *Thiobacillus* sp. ASWW-2, was isolated from activated sludge, and its sulfur oxidation activity was characterized. *Thiobacillus* sp. ASWW-2 could oxidize elemental sulfur on the broad range from pH 2 to 8. When 5-50 g/L of elemental sulfur was supplemented as a substrate, the growth and sulfur oxidation activity of *Thiobacillus* sp. ASWW-2 was not inhibited. The specific sulfur oxidation rate of strain ASWW-2 decreased gradually until sulfate was accumulated in medium up to 10 g/L. In the range of sulfate concentration from 10 g/L to 50 g/L, the sulfur oxidation rate could keep over 2.0 g-S/g-DCW·d. It indicated that *Thiobacillus* sp. ASWW-2 has tolerance to high concentration of sulfate.

Keywords: autotrophic sulfur oxidizing bacterium, elemental sulfur, tolerance to sulfate, biofilter

INTRODUCTION

Hydrogen sulfide (H_2S) is one of the malodorous compounds most widely emitted from wastewater treatment, petrochemical refining, food preparation, paper and pulp manufacturing, and fuels treatment [1,2]. H_2S not only aggravates the neighborhood but can also cause adverse effects to health [3]. Besides, H_2S has a corrosive property to create a damage and danger of collapse because of the corrosion of pipe and concrete construction [4,5]. To remove this gas, biofiltration is attracting more attention because of low capital and operating costs, low energy requirements and an absence of residual products requiring further treatment or disposal. Biofiltration units are microbial systems incorporating microorganisms grown on a porous solid media like soil, peat, compost or porous ceramics, etc [6-8]. Therefore, it is very important to select effective microorganisms to remove hydrogen sulfide. It has become a common practice to inoculate the filter bed with pure cultures of microorganisms capable of biodegrading H_2S to reduce the adaptation time of the biofilter. For instance, the inoculation of a biofilter with *Thiobacillus* species remarkably reduced the acclimatization period for biodegradation of H_2S [7,9,10].

Diverse autotrophic and heterotrophic microorganisms have been employed to remove H_2S in biofilter systems. In microbial treatment of H_2S, most of microorganisms belong to *Thiobacillus* spp. [7,9-13]. Some chemolitoheterotrophic bacteria such as *Thiothrix*, *Beggiatoa*, and *Hyphomicrobium* can oxidize H_2S to elemental sulfur that will be further oxidized to sulfate [14-16]. Photoautotrophic bacteria including *Chlorobium*, *Chromatium*, *Ectothiorhodospira*, and *Rhodobacter* have been used to convert H_2S to elemental sulfur under anaerobic conditions [17-20]. The major disadvantages of the practical use of photoautotrophic bacteria lie in their anaerobic nature and their need for radiant energy. As for the chemoorganoheterotrophic bacteria, *Pseudomonas* sp. and *Xanthomonas* sp. have also been reported to oxidize H_2S [21,22].

Generally, autotrophic biofilter, employing the autotrophic and sulfur-oxidizing bacteria such as *Thiobacillus* spp., has shown high affinity for H_2S. Besides, it is not necessary to supplement any carbon or/and energy sources into autotrophic biofilter because the autotrophic and sulfur-oxidizing bacteria can utilize CO_2 and H_2S as a carbon and energy source, respectively. However, the final oxidation product of H_2S by the autotrophic sulfur oxidizer is sulfate, and the resulting acidity has adverse effects on microbial activity [7,9]. Therefore, it is important to screen an autotrophic sulfur oxidizer having tolerance to high concentration of sulfate for stable long-term continuous operation of biofilter system. In this study, an autotrophic sulfur oxidizer was isolated from activated sludge, and its sulfur oxidation activity was characterized. In addition, the effect of sulfate concentration on the sulfur oxidation activity of the isolate was studied.

MATERIALS AND METHODS

Isolation of a Sulfur-oxidizing Bacterium ASWW-2

Activated sludge, sampled at a wastewater treatment plant, was used as a source for the isolation of sulfur-

* Corresponding author
Tel: +82-2-3277-2393 Fax: +82-2-3277-3275
e-mail: kscho@mm.ewha.ac.kr

oxidizing bacteria. Ten grams of wet activated sludge was inoculated in 100 mL of MW medium in a 500 mL flask, and incubated at 30°C in a shaking incubator with 180 rpm. MW medium is composed of 10 g of S°, 3 g of KH_2PO_4, 0.1 g of NH_4Cl, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of $CaCl_2 \cdot 2H_2O$ in 1 L of distilled water. The pH of medium was adjusted to 4 by using 2N HCl. When the pH of culture broth declined below 1.5 due to the accumulation of sulfate that was the final product of sulfur oxidation, 10 mL of the culture broth was inoculated into 90 mL of fresh medium. After this process was repeated several times, the acclimated culture broth was spread on the MW-agar plate. One dominant colony appearing on a MW-agar plate was isolated, and purified by repeated transfer of the cells to fresh MW medium. The purified bacterium was named as ASWW-2.

Characterization of Sulfur Oxidation

Several factors affected sulfur oxidation activities of the isolate, ASWW-2, were investigated: initial pH of medium, inoculum size, and concentration of substrates. Cells grown in 1 L of MW medium for 3 days at 30°C were harvested by centrifugation (7,500 × *g*, 20 min), washed and suspended in 100 mL of basal mineral medium containing 3 g of KH_2PO_4, 0.1 g of NH_4Cl, 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of $CaCl_2 \cdot 2H_2O$ in 1 L of distilled water (pH 4). The cell suspension was used as inoculum for each experiment, and the amount of initial inoculum was adjusted to 0.05-0.07 of optical density at 660 nm after the cell suspension added into the culture medium.

Experiments were carried out in 500 mL shake flasks. The flasks were charged with 100 mL of the basal mineral medium. To investigate the effect of initial pH on sulfur oxidation rate, 10 g/L of S° was supplemented in the medium as a substrate, and the initial pH of the medium was adjusted to 1, 2, 3, 4, 5, 6, 7, 8 with 2N NaOH or 2N HCl. Also, the effects of substrate concentration (5-50 g/L of S° and 1.8-7.4 g/L of $S_2O_3^{2-}$) on sulfur oxidation rate were investigated. All experiments were carried out at 30°C and 180 ppm on a rotary shaker. Three mL of culture broth from each flask was sampled every 24 h, and the pH, cell density, and sulfate concentration were measured.

Analysis

To monitor the cell growth of strain ASWW-2, the optical density of culture broth was measured by spectrophotometer (Spectronic 20, Milton Roy Company, U.S.A.) at 660 nm. Before the protein assay, the cells were digested with 2N NaOH solution at 100°C for 60 min. The concentrations of thiosulfate and sulfate were analyzed by ion chromatography (Waters 510, U.S.A.). IC-Pak™ anion column (4.6 mm ϕ × 50 mm L, Waters, U.S.A.) and conductivity detector (Waters 432, U.S.A.) were used.

Table 1. Characteristics of strain ASWW-2

Items	Characteristics
Colony on MW-agar plate	Whitish-yellow with sulfur Deposited 1-1.5 mm in diameter
Morphology	Short rod, 0.5 × 1-1.5
Motility	Positive
Gram-staining	Negative
Intracellular sulfur	Negative
Autotrophic growth with	
Hydrogen sulfide	Positive
Elemental sulfur	Positive
Thiosulfate	Positive
Tetrathionate	Positive
Hetrotrophic growth	Negative
Ferrous iron oxidation	Negative
Nitrate respiration	Negative

RESULTS AND DISCUSSION

Morphological and Physiological Characteristics of Strain ASWW-2

The morphological and physiological properties of strain ASWW-2 were summarized in Table 1. The cell of strain ASWW-2 was short rod, gram negative, and motile. The colony on the thiosulfate-agar medium was small, with a diameter of 0.5-1 mm and whitish yellow by extracellular deposition of sulfur. Fig. 1(a) shows pH and sulfate concentration profiles with cultivation time in the MW medium (pH 4) supplemented with S° as a sole energy source. During the cultivation, the increase of sulfate concentration was accompanied by an increase in the cell mass. The pH value of the culture gradually decreased according to the accumulation of sulfate. The final sulfate concentration was approximately 30 g/L, indicated that elemental sulfur (initial concentration, 10 g/L) was stoichiometrically oxidized to sulfate by strain ASWW-2. In the experiment with MW medium, CO_2 and elemental sulfur were the sole carbon and energy source for the growth of strain ASWW-2, respectively. Therefore, strain ASWW-2 has chemolithotrophically grown by utilizing energy from the oxidation of elemental sulfur. When 8 g/L of $Na_2S_2O_3 \cdot 5H_2O$ was supplemented into the MW medium as an energy source instead of elemental sulfur, strain ASWW-2 could chemolithotrophically grow (Fig. 1(b)). In this medium, visible accumulation of elemental sulfur was found. These results suggested that this strain oxidized thiosulfate to sulfate *via* the accumulation of elemental sulfur as an intermediate [23]. This bacterium could also utilize tetrathionate as an energy source, and oxidize it to sulfate. The strain ASWW-2 could not heterotrophically grow in organic medium (data not shown). Stain ASWW-2 was negative in the oxidation of ferrous and in nitrate respiration. Major cellular fatty acids for stain ASWW-2 were analyzed as uniqinone-8, non-hydroxy 16:0, hydroxy 3-OH 14:0 (data not shown). Based on the description in Bergey's manual [24] and Katayama-F *et al.* [25], strain

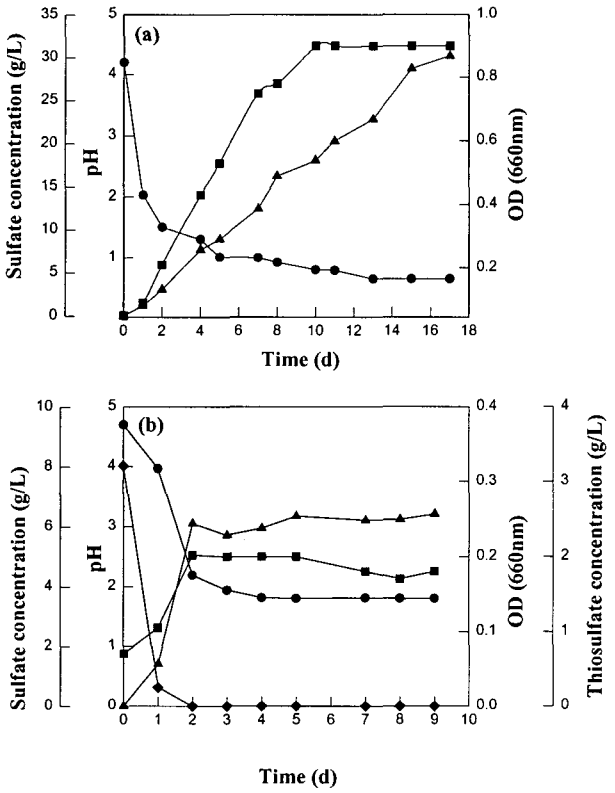

Fig. 1. Growth and sulfur oxidation patterns of strain ASWW-2 in MW medium supplemented with 10 g/L of elemental sulfur (a) and 5.4 g/L of thiosulfate (b): ●, pH; ■, optical density at 660 nm; ▲, sulfate concentration; ◆, thiosulfate concentration.

ASWW-2 was considered to belong to the genus *Thiobacillus* among the colorless sulfur bacteria.

Effect of Initial pH on Sulfur Oxidation Rate

Strain ASWW-2 could oxidize sulfur on the broad range from pH 2 to 8 (data not shown). The growth of strain ASWW-2 was observed without a lag period at pH below 6, but it at relatively higher pH of 7 and 8 showed a lag period of 2 and 8 days, respectively. However, when the pH of medium decreased from 7 - 8 to 6 due to the accumulation of sulfate produced during lag time, the growth of strain ASWW-2 was obviously accelerated. It means that strain ASWW-2 is an acidophilic sulfur-oxidizing bacterium.

Table 2 shows the sulfur oxidation rate of strain at different initial pH of medium. The sulfur oxidation rates were calculated from the slopes of the sulfate con-centration versus time curves at the exponential growth phase. The sulfur oxidation rate increased with increasing pH values up to pH 4, but it decreased with increasing pH value in the range of pH 5 and 8. The maximum sulfur oxidation rate, 0.61 g-S/L · d, was obtained at pH 4.

Table 2. Sulfur oxidation rate of strain ASWW-2 at different initial pH of medium

Initial pH	Sulfur oxidation rate (g-S/L · d)
2	0.50
3	0.58
4	0.61
5	0.49
6	0.46
7	0.46
8	0.39

Effect of the Substrate Concentration on Sulfur Oxidation Rate

Sulfur oxidation rates were compared on each condition that 5-50 g/L of elemental sulfur was supplemented as a substrate. Fig. 2 shows the typical patterns of growth and sulfur oxidation of strain ASWW-2 at elemental sulfur of 50 g/L. The sulfate concentration, the final product of sulfur oxidation, increased with constant rate until the accumulated sulfate concentration became to be 45 g/L, but the accumulation rate of sulfate decreased when sulfate accumulated in the medium over 45 g/L. It was considered that the reduction of sulfur oxidation rate was caused by a high ionic strength of sulfate. Although the growth of strain ASWW-2 was reached the stationary phase after 8 days of cultivation and pH in the culture medium dropped to 0.7, the sulfur oxidation activity was not diminished. Compared with the specific sulfur oxidation rates calculated from the respective sulfur concentration, the rate was increased with increasing sulfur concentration upto about 30 g/L (Fig. 3). There was no inhibition of substrate to the growth and sulfur oxidation activity of strain ASWW-2 when elemental sulfur was used as a substrate. The specific sulfur oxidation rate well describes as follow a Monod equation (Fig. 3) and the values for K_m and V_m determined by a Lineweaver-Burk plot were 3.8 g-S/g-DCW·d and 14.3 g/L, respectively.

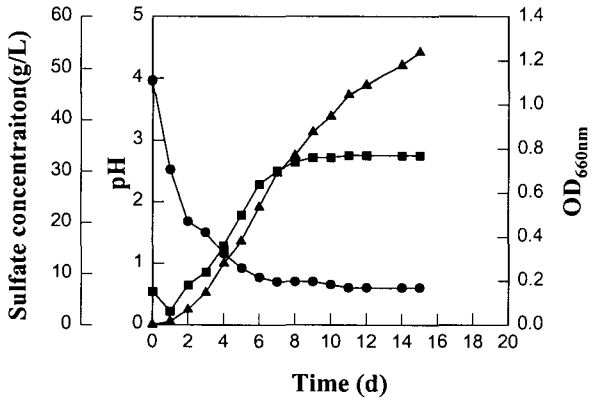

Fig. 2. Growth and sulfur oxidation patterns of strain ASWW-2 in MW medium supplemented with 50 g/L of elemental sulfur: ●, pH; ■, optical density at 660 nm; ▲, sulfate concentration.

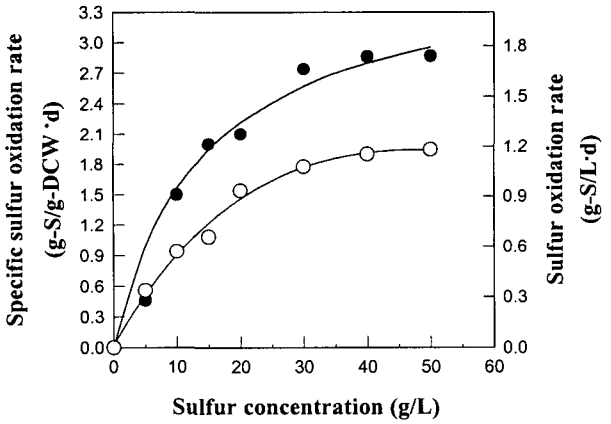

Fig. 3. Effect of elemental sulfur concentration on the sulfur oxidation rate of strain ASWW-2: ●, specific sulfur oxidation rate; O sulfur oxidation rate.

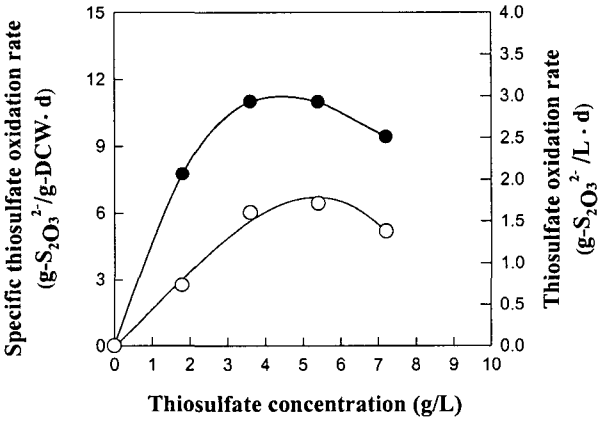

Fig. 4. Effect of thiosulfate concentration on the sulfur oxidation rate of strain ASWW-2: ●, specific sulfur oxidation rate; O, sulfur oxidation rate.

The strain ASWW-2 could have used elemental sulfur as an energy source and oxidized it to sulfate. The attack of sulfur particles by this strain is believed to be initiated by the adhesion of the cells to the sulfur surface [26,27]. The outer surface of the sulfur particle is available for bacterial colonization and subsequent metabolism. Therefore, higher sulfur concentration is considered to facilitate faster sulfur oxidation because of higher surface area of the sulfur. The activity of sulfur oxidation of strain ASWW-2 is similar to those of several strains of *T. thiooxidans* (0.53 to 0.95 g-S/L · d) [28, 29].

The effect of thiosulfate concentration on the sulfur oxidation rate of strain ASWW-2 was also investigated (Fig. 4). The sulfur oxidation rate was increased with increasing thiosulfate concentration below 3.6 g-$S_2O_3^{2-}$/L. However, the sulfur oxidation rate was remarkably decreased at 5.4 g-$S_2O_3^{2-}$/L. The maximum specific sulfur oxidation rate were 16.9 g-$S_2O_3^{2-}$/g-DCW·d at 3.6 g-$S_2O_3^{2-}$/L of thiosulfate concentration. It was considered

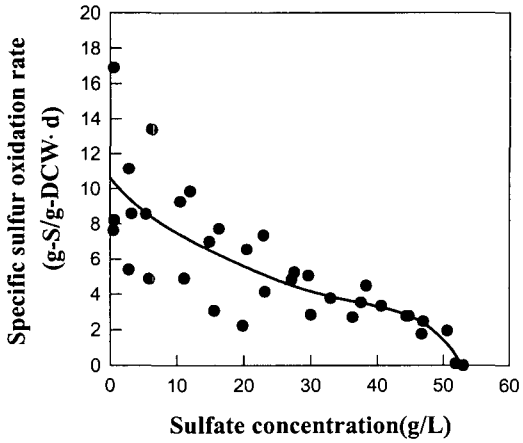

Fig. 5. Relationship between the sulfur oxidation rate of strain ASWW-2 and sulfate concentration.

that high concentration of thiosulfate had suppressed the growth and sulfur oxidation rate because thiosulfate could be dissolved easily. However, as can be seen in Fig. 3, elemental sulfur did not display inhibitory effect due to its insoluble property in water.

Sulfur Oxidation Rate at Various Sulfate Concentration

Fig. 5 shows the specific sulfur oxidation rate as the function of the concentration of sulfate formed in each condition of the concentration of elemental sulfur, 30, 40, and 50 g/L. In the range of sulfate concentration from 10 g/L to 50 g/L, the specific sulfur oxidation rate gradually decreased with increasing concentration of sulfate accumulated, and the sulfur oxidation rate could be maintained over 2.0 g-S/g-DCW·d although the sulfur oxidation rate was very variable. When sulfate was accumulated in medium over 50 g/L, the sulfur oxidation rate remarkably decreased and the strain ASWW-2 was completely inhibited. On the other hand, as can be seen in Fig. 2, the sulfur oxidation activity of strain ASWW-2 could be maintained at low pH below 0.8. Therefore, the decrease in the sulfur oxidation activity of strain ASWW-2 at the sulfate concentration over 50 g/L is may be due to a high ionic strength of sulfate.

These results indicated that strain ASWW-2 could oxidize the reduced sulfur compounds such as elemental sulfur and H_2S at the strong acidic condition. Generally, it gives rise to the trouble to decrease the efficiency of deodorization, because the activities of deodorizing microorganisms were inhibited by pH decline. Therefore, strain ASWW-2 is considered to be an adequate candidate for the improvement of removal efficiency in biodeodorization system.

Acknowledgements This research was funded by the G-7 project from the Ministry of Environment in 1996.

REFERENCES

[1] Eikum, A. S. and R. Storhang (1986) Odour problems related to waste water and sludge treatment. pp. 12-18. In: V. C. Neilsen, J. H. Voorgurg, and P. L. Hermite, (Eds.), *Odour Prevention and Control of Organic Sludge and Livestock Farming*, Elsevier Applied Science Publishers, London.

[2] Yang, Y. and E. R. Allen (1994) Biofiltration control of hydrogen sulfide 1. Design and operational parameters. *J. Air Waste Magm. Assoc.* 44: 863-868.

[3] Vanhoorne, M., A. Rouck, and D. de. Bacquer (1995) Epidemiological study of eye irritation by hydrogen sulphide and/or carbon disulphid exposure in viscose rayon workers. *Ann. Occup. Hyg.* 39: 307-315.

[4] Mori, T., M. Koga, Y. Hikosaka, T. Nonaka, F. Mishina, Y. Sakai, and J. Koizumi (1991) Microbial corrosion of concrete sewer pipes, H_2S production from sediments and determination of corrosion rate. *Wat. Sci. Tech.* 23: 1275-1282.

[5] Cho, K. S. and T. Mori (1995) A newly isolated fungus participates in the corrosion of concrete sewer pipes. *Wat. Sci. Tech.* 31: 263-271.

[6] Shoda, M. (1991) Methods for the biological treatment of exhaust gases in biological degradation of waste. pp. 31-46. In: A. M. Martin (ed.), *Biological Degradation of Wastes,* Elsevier Applied Science Publishers, London.

[7] Cho, K. S., L. Zhang, M. Hirai, and M. Shoda (1991) Removal characteristics of hydrogen sulfide and methanethiol by *Thiobacillus* sp. isolated from peat in biological deodorization. *J. Ferment. Bioeng.* 71: 44-49.

[8] Rands, M. B., D. E., Cooper, C. P. Woo, G. C. Fletcher, and K. A. Rolfe (1981) Compost filters for H_2S removal from anaerobic digestion and rendering exhausts. *J. Water Pollut. Control Fed.* 53: 185-189.

[9] Cho, K. S., M. Hirai, and M. Shoda (1991) Degradation characteristics of hydrogen sulfide, methanethiol, dimethyl sulfide and dimethyl disulfide by *Thiobacillus thioparus* DW44 isolated from peat biofilter. *J. Ferment. Bioeng.* 71: 384-389.

[10] Cho, K. S., M. Hirai, and M. Shoda. (1992) Enhanced removal efficiency of malodorous gases in a pilot-scale peat biofilter inoculate with *Thiobacillus thioparus* DW44. *J. Ferment. Bioeng.* 73: 46-50.

[11] Sublette, K. L. and N. D. Sylvester (1987) Oxidation of hydrogen sulfide by *Thiobacillus denitrificans* : desulfurization of natural gas. *Biotechnol Bioeng.* 29: 249-257.

[12] Kanagawa, T. and E. Mikami (1989) Removal of methanethiol, dimethyl sulfide, dimethyl disulfide, and hydrogen sulfide from contaminated air by *Thiobacillus thioparus* TK-m. *Appl. Environ. Microbiol.* 55: 555-558.

[13] Chung, Y. C., C. Huang, and C. F. Li (1997) Removal characteristics of H_2S by *Thiobacillus novellus* CH13 biofilter in autotrophic and mixotrophic environments. *J. Environ. Sci. Health.* A32: 1435-1450.

[14] Larkin, J. M. (1980) Isolation of *Thiotrix* in pure culture and observation of a filamentous epiphyte on *Thiotrix*. *Curr. Microbiol.* 4: 144-158.

[15] Nelson, D. C. and H. W. Jannasch (1983) Chemoautotrophic growth of a marine *Beggiatoa* in sulfide-gradient cultures. *Arch. Microbiol.* 136: 262-269.

[16] Zhang, L., M. Hirai, and M. Shoda (1991) Removal characteristics of dimethyl sulfide, methanethiol and hydrogen sulfide by *Hyphomicrobium* sp. 155 isolated from peat biofilter. *J. Ferment. Bioeng.* 72: 392-396.

[17] Steinmetz, M. A. and U. Fisher (1982) Cytochromes of the green sulfur bacterium *Chlorobium vibrioforme f. thiosulfatophilum*. Purification, characterization and sulfur metabolism. *Arch. Microbiol.* 131: 19-26.

[18] Gray, G. O. and J. G. Knaff (1982) The role of a cytochrome *c*-552-cytochrome *c* complex in the oxidation sulfide in *Chromatium vinosum*. *Biochim. Biophys. Acta* 680: 290-296.

[19] Then, J. and H. G. Truper (1983) Sulfide oxidation in *Ectothiorhodospira abdelmalekii*. Evidence for the catalytic role of cytochrome *c*-551. *Arch. Microbiol.* 135: 254-258.

[20] Brume, D. C. and H. G. Truper (1986) Noncyclic electron transport in chromatophores from photolithotrophically grown *Rhodobacter sulfidophilis*. *Arch. Microbiol.* 145: 295-301.

[21] Chung, Y. C., C. Huang, and C. P. Tseng (1996) Biodegradation of hydrogen sulfide by a laboratory-scale immobilized *Pseudomonas putida* CH11 biofilter. *Biotechnol. Prog.* 12: 773-778.

[22] Cho, K. S., M. Hirai, and M. Shoda. (1992) Degradation of hydrogen sulfide by *Xanthomonas* sp. strain DY44 isolated from peat. *Appl. Environ. Microbiol.* 58: 1183-1189.

[23] Kelly, D. P. (1985) Physiology of the thiobacilli: elucidating the sulphur oxidation pathway. *Microbiol. Sci.* 2: 105-109.

[24] Kuenen, J. G. (1989) Colorless sulfur bacteria. pp. 1834-1871. In: J. T. Staley, M. P. Bryant, N. Pfenning, and J. G. Holt, (eds.), *Bergey's Manual of Systematic Bacteriology*, Vol. 3, The Williams Wilkins Co., Baltimore.

[25] Katayama-F., Y., N. Tsuzaki, and H. Kuraishi (1982) Ubiquinone, fatty acid and DNA base composition determination as a guide to the taxonomy of the genus *Thiobacillus*. *J. Gen. Microbiol.* 128: 1599-1611.

[26] Takakuwa, S., T. Fujimori, and H. Iwasaki (1979) Some properties of cell sulfur adhesion in *Thiobacillus thiooxidans*. *J. Gen. Appl. Microbiol.* 25: 21-29.

[27] Takeuchi, T. L. and I. Suzuki (1997) Cell hydrophobicity and sulfur adhesion of *Thiobacillus thiooxidans*. *Appl. Environ. Microbiol.* 63: 2058-2061.

[28] Konishi, Y., S. Asai, and N. Yoshida (1995) Growth kinetics of *Thiobacillus thiooxidans* on the surface of elemental sulfur. *App. Environ. Microb.* 61: 3617-3622.

[29] Porro, S., S. Ramirez, C. Reche, G. Curutchet, A. Alonso-Romanowski, E. Donati (1997) Bacterial attachment : its role in bioleaching processes. *Process Biochem.* 32: 573-578.

[Received August 20, 1999; accepted December 30, 1999]