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Construction of a Biofilter Immobilized with *Rhodococcus* sp. B261 for Removal of H_2S Gas Generated by Livestock

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To explore the optimal conditions for the removal of H_2S gas by biofiltration, various conditions, including inlet H_2S concentration, flow rate, moisture, and cell number, were examined. Heterotrophic bacteria were isolated from the compost of the animal excreta. A strain that effectively removed H_2S was selected and identified as *Rhodococcus rhodochrous* B261 by analysis of its 16S rDNA sequence. A cell number of 10^7 cfu/g·compost was sufficient to dominate the microbiota, and an effective removal was observed at H_2S gas concentrations below 220 mg/L. The moisture content of 33-38% was suitable for activation of the microbial activity and delaying the desiccation. Higher flow rates resulted in lower removal rates of the H_2S gas. Under the conditions of 10^7 cfu/g·compost, H_2S gas concentrations of 220 mg/L, and moisture content of 33-38%, the inlet H_2S gas concentrations of 120 and 400 mg/L were completely removed for 34 and 12 days, respectively. The amount of sulfur removed was 2.99×10^{-9} H_2S-S/cell, which was suggested as the amount of sulfur removed by a single cell. The biofilter consisting of the compost and *R. rhodochrous* B261 could be suitable for a long-term biofilteration for the removal of H_2S and other malodorous compounds.

Key words: *biofilter, compost, hydrogen sulfide, PCR, Rhodococcus rhodochrous B261*

Malodor generated by livestock has become a serious pollution problem in many countries. The main malodorous compounds emitted from the livestock houses are hydrogen sulfide, ammonia, trimethylamine, and volatile fatty acids. High concentrations of these compounds, especially hydrogen sulfide, are harmful to the health of the workers, animals, and local residents [Haggard *et al.*, 1992]. Therefore, elimination or reduction of the sources of malodors is very important from the environmental health standpoint. Several microbial methods for the removal of malodor compounds have been reported in the last two decades [Ottengraf *et al.*, 1986; Leson and Winer, 1991; Wani *et al.*, 1997]. These methods share the common characteristics in that they provide easier operation and lower maintenance costs, and are more environmentally friendly, because no secondary pollution is produced as compared to the chemical and physical methods. There have been a number of studies on the elimination of hydrogen sulfide using biofilters [Furusawa *et al.*, 1984; Cho *et al.*, 1991; Shoda, 1991; Park *et al.*, 1993; Shinabe *et al.*, 1995]. Most of these studies focused on the identification of more effective carriers to prevent the drop in the pressure responsible for reducing the removal efficiency in biofiltration over long periods.

A number of microorganisms with sulfide-oxidizing ability have been reported, including the autotrophic bacteria *Thiobacillus* spp., the photosynthetic bacterium *Chromatium vinosum*, the methylotrophic bacterium *Hyphomicrobium*, and *Xanthomonas* [Fukumori *et al.*, 1979; Zhang *et al.*, 1991; Cho *et al.*, 1992]. Autotrophs use sulfide as an energy source, the mechanism of which has been investigated in a number of studies. However, there have been few studies on the heterotrophs capable of eliminating hydrogen sulfide. *Streptomyces* and *Bacillus* spp. in the livestock waste treatment programs are heterotrophs capable of reducing the level of hydrogen sulfide produced during composting [Nakada

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Abbreviations: PCR, polymerase chain reaction; PE, feces extract medium; PEA, 10% pig feces extract agar medium; PEC, 10% pig feces extract-centrifuged medium; PFE, pig feces extract medium

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and Ohta, 1997; Ohra *et al.*, 1997; Nakada and Ohta, 1999]. Studies have found that rapid composting of the animal excreta by the deodorizing microorganisms removed the main malodorous compounds generated during the composting of the animal excreta, including volatile fatty acids and hydrogen sulfide [Ohta and Ikeda, 1978; Ohta and Kuwada, 1978].

The objective of the present study was to develop biofilters using these microbes for an effective removal of the hydrogen sulfide generated in the livestock houses. Attempts were made to determine the factors required for the effective elimination of the hydrogen sulfide gas.

Materials and Methods

Strains. Twenty strains were isolated from the compost of pig feces using 10% pig PFE. The strain that showed the highest ability to remove H_2S was selected and used for the experiments.

Identification. The strain showing the highest H_2S removal activity was identified by the morphological and the physiological characterizations [Nakamura and Yoda, 1978; Krieg and Holt, 1984] and by the analysis of its 16S rDNA sequence amplified by PCR.

DNA sequence. The strain was cultured in a nutrient broth at 37°C for 24 h with shaking at 250 rpm, and DNA of the strain was extracted (InstaGen Matrix Catalog 732-6030; Bio-Rad, Hercroules CA) and heated using a block incubator (BI-525, Astech, Fukuoka, Japan) at 100°C for 5 min and quenched on ice. The DNA extract was added to the nuclease P_1 solution (Yamasa Shoyu Co., Tokyo, Japan) and incubated at 50°C for 1 h to induce fragmentation of the DNA. The DNA fragments were analyzed on an L-7400 type HPLC apparatus (Hitachi, Tokyo, Japan) equipped with an ODS column (Cosmoseal 5C18, Nakalai Tesque, Kyoto, Japan). A gradient of ammonium hydrogen phosphate (0.05%) was used, and the absorbance was determined at 265 nm.

16S rDNA analysis. Identification of the strain capable of H_2S removal was performed by NCIMB (Shizuoka Japan).

Compost used. A mixture of 5 kg of fresh pig feces, 2.5 kg of mixed seed culture, and 1.5 kg of rice bran was composted in a treatment box at room temperature for 5 days. The compost was dried to a moisture content of approximately 35%. The dried compost contained 10^9 cells g^{-1} of deodorizing bacteria.

Medium preparation. Ten percent each PE, PEC, and PEA media were used as growth media for *R. rhodochrous* B261. Ten percent pig feces extract was prepared by passage through a cotton filter. All media were autoclaved at 121°C for 20 min, and the pH was adjusted to 7.0 with 10% Na_2CO_3 or 1 N HCl under aseptic conditions.

Culture of strain for biofilter. *R. rhodochrous* B261 was inoculated into 500 mL of PE medium and incubated at 37°C with shaking at 240 rpm for 16 h. A volume of 300 mL of the precultured broth was transferred into a 10-L jar fermentor containing 6 L of fresh PE medium and incubated at 37°C at a flow rate of 3.5 L/min and an agitation speed of 300 rpm for 9 h. The cultured cells were harvested by centrifugation at 17,000×*g* and suspended in distilled saline solution. The suspended cells were mixed with the dried compost and packed into a column.

A deodorization column for H_2S as designed by Furusawa *et al.* [1984] was used. Compressed air at a pressure of 2 kg cm^{-2} was passed through the H_2S-generating column at a specified flow rate controlled by the regulator. Sodium sulfide and HCl solution were passed through the H_2S-generating column at 1 mL/min using a peristaltic pump. Two hundred glass beads (ø 10 mm) were included in the column to ensure effective generation of the H_2S gas. The generated gas was passed through the deodorizing column at the flow rate controlled by a flow meter (Ryutai Kogyo, Tokyo, Japan). The inlet and outlet concentrations of H_2S were determined using an SB type syringe H_2S detector (Kitagawashiki, Tokyo, Japan). The inlet H_2S concentration was controlled at 60-400 mg/L by mixing HCl and sodium sulfide at 1.2-23.6% and 4-10%, respectively.

Samples of 500 g of the mixed and the pure cultures with specified moisture levels were packed into the column. The physical properties of the compost and the operating conditions are shown in Table 1.

Enumeration of viable cells. Viable cells on the PEA medium adjusted to pH 8.0 were counted. In brief, 10 g each sample was diluted in 90 mL of the saline solution. The suspensions were serially diluted to $10^{-1\sim9}$ and inoculated onto the PE plates. The plates were incubated at 37°C for 3 days, and the colonies formed as the viable cells were counted.

Analysis. Moisture contents of the samples were determined by measuring the difference between the wet and dry weights after drying the samples. To maintain the moisture content, an X-ray moisture detector (Kett, F-18, Tokyo, Japan) was used for measuring the moisture content, and the weight of water required to maintain the moisture content was supplied into the column. The water holding capacity was determined as the difference in wet and dry weights of the compost. Porosity of the samples was measured as the difference in volume between the mixture of water and sample, and that of water alone.

Hydrogen sulfide and sulfide compounds. One

Table 1. Physical properties of compost and operating conditions for H_2S removal

Compost	Density	0.33 g/cm^3
	Porosity	77.8%
	Water-holding capacity	65%
	Packed weight	500 g
Column	Size (ϕ×height)	7×50 cm
	Packed volume	1530 mL
Operating conditions	Flow rate	0.1-1.2 L/min
	Column initial pH	7.5-8
	Column temperature	37°C
	Inlet concentration	100-400 mg/L
	Initial cell number	10^{4-9} CFU/g-ceramic beads
	Moisture content	33-38, 40-45, 55-60, 50-55%

hundred grams each of the samples were transferred into 500-mL flasks, made air-tight with rubber tubings, and incubated at 40°C for 1 h. The headspace gas in the flask was sampled by an air-tight microsyringe (Hamilton, Reno, NV) and injected into a gas chromatograph (GC-14B, Shimadzu, Tokyo, Japan) equipped with an Flame Photometric Detector (Shimadzu, Tokyo, Japan). A column packed with polyphenyl ether (5-rings) OS-124 (10%, 60-80 mesh, Shimadzu) was used. Nitrogen was used as a carrier gas, and hydrogen was used as a fuel at a flow rate of 40 mL/min. The levels of sulfur, sodium thiosulfate, sodium sulfite, and sulfate in the compost were determined by the method of Tachihana [1985]. Ten grams each of the chemicals were diluted with 100 mL of distilled water and extracted using a Soxhlet apparatus. The extracts were used in the sulfate barium precipitation and the ureic acid titration.

Results and Discussion

Identification of strain. Strain B261 was identified as *Rhodococcus rhodochrous* B261 based on its morphological, biological, and chemical properties, as well as through the analysis of its 16S rDNA sequence amplified by PCR (Fig. 1). Moreover, in the neighbor joining phylogenic tree, a significantly high similarity (98.19%) was observed with the 16S rDNA sequence of *R.* B261 in the database using MicroSeq™ Identification System Software V.1.4.1 and BLAST (National Center for Biotechnology Information, Bethesda, MD).

Comparison of pure and mixed cultures. At the flow rate of 250 mg/L, H_2S gas was removed completely for 12 days in the column packed with the pure culture (Fig. 2). From day 13, only 3 mg/L was detected from the outlet port of the column and resulted in a decrease in H_2S removal rate. In the case of mixed cultures, H_2S was removed completely for 8 days. The removal rate of H_2S in the columns of the autoclaved pure and mixed cultures decreased after 4 and 2 days, respectively. This result clearly showed that the pure culture had higher removal capacity than the mixed culture. In addition, the degrees of removal of H_2S were significantly different between the autoclaved and non-autoclaved cultures, indicating that the living deodorant microorganisms are responsible for the removal of H_2S. The removal of H_2S by the autoclaved cultures was suggested to be due to the abiotic adsorption or the remaining viable cells (about 10^3 cells/g).

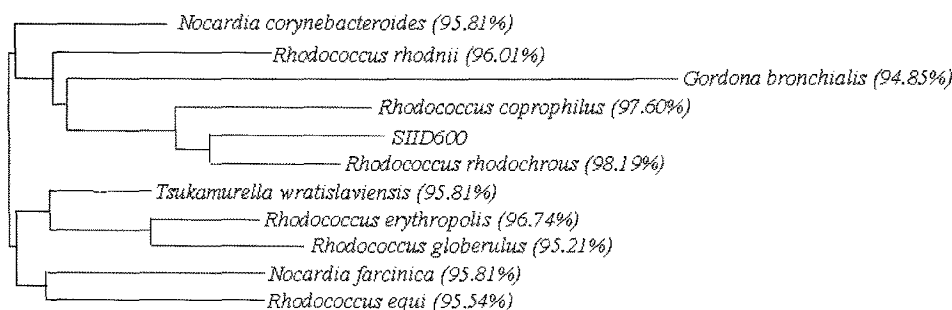

Fig. 1. Neighbor-joining phylogenic tree of *Rhodococcus* sp.B261. The values in parentheses show similarity with strain B261 as determined by 16S rDNA sequence analysis.

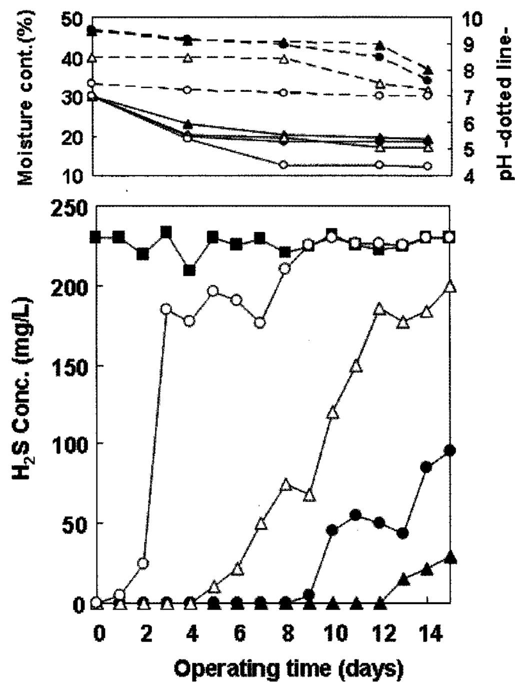

Fig. 2. Comparison of H_2S removal by biofilters packed with pure, mixed, autoclaved pure, and autoclaved mixed cultures (a) and removal rates (b). Symbols: (■), Inlet H_2S concentration; (▲), Pure culture; (●), Mixed culture (△), Autoclaved pure culture; (○), Autoclaved mixed culture.

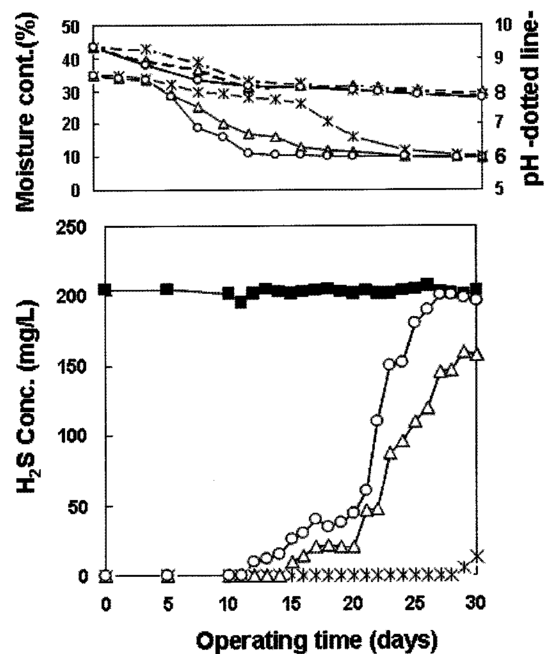

Fig. 3. Effect of flow rate on H_2S removal by the biofilter packed with pure culture at an inlet concentration of 200 mg/L. Symbols: (■), Inlet H_2S concentration; (✳), 4.8 m^3/kg/day; (△), 9.6 m^3/kg/day; (○) 14.4 m^3/kg/day.

Table 2. Characteristics of No.B261 strain by NCIMB[a]

Items	Characteristics
Gram	+
Spores	-
Mobility	-
Colonial morphology	Round Smooth Low convex Orange pink 1-1.5 mm in diameter
Temperature growth - 37°C	+
- 10°C	-
Catalase	+
Oxidase, Kovacs	-
O-F glucose	- (alkaline)
First, stage identification	*Rhodococcus* sp.

[a]National Collection of Industrial Bacteria Torry Research Station.

The initial pH values of pure and mixed cultures were almost the same, *i.e.*, pH 9.6, as composting progressed for 4 days. However, after autoclaving, both the pure and the mixed cultures showed decreases in pH to about 8.3 and 7.6, respectively. The pH decreased with the increasing rate of H_2S removal. Finally, the pH values of pure cultures, mixed cultures, autoclaved pure cultures, and autoclaved mixed cultures on day 14 were 8.1, 7.7, 7.3, and 7.1, respectively, indicating that alkaline pH is associated with the removal of H_2S. Moisture contents adjusted to 35% respectively decreased to 18.3, 17.1, 16.4, and 13.0% in the pure cultures mixed cultures, autoclaved pure cultures, and autoclaved mixed cultures on day 14.

Effect of flow rate on H_2S removal. At the flow rate of 4.8 m^3/kg/d, H_2S gas was removed completely for 28 days (Fig. 3). At flow rates of 9.6 and 14.4 m^3/kg/d, complete removal of H_2S gas was maintained for 14 and 11 days, respectively. The pH decreased gradually from the initial value of 9.4 to 7.9 at the flow rate of 4.8 m^3/kg/d, to 8.0 at 9.6 m^3/kg/d, and to 7.8 at 14.4 m^3/kg/d. At higher flow rates, the moisture content decreased rapidly reaching a final stable value of 10-13%. At the flow rate of 4.8 m^3/kg/d, removal of H_2S gas remained at 100% for 7 days even after the moisture content decreased to about 12%. In addition, it was clear that lower moisture content, below 12%, at the higher flow rate of 14.4 m^3/kg/day caused a reduction in the H_2S removal rate.

Effect of moisture content on H_2S removal. At 60 mg/L of the inlet H_2S concentration, H_2S gas was completely removed for 23 days (Fig. 4), and then the inlet H_2S concentration increased to 120 mg/L. When the moisture content was maintained at 40-45%, 3 mg/L of

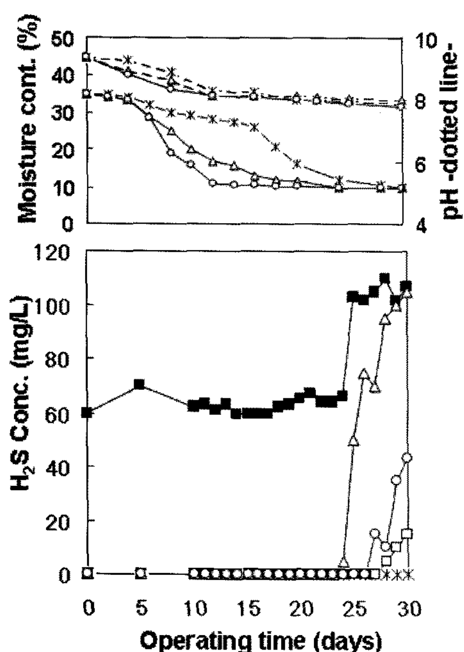

Fig. 4. Effect of moisture content on H_2S removal by the biofilter at a flow rate of 14.4 m^3/kg/day. Symbols: (■), Inlet H_2S concentration; (✳), 33-38%; (△), 40-45%; (○), 55-60%; (□), 50-55%.

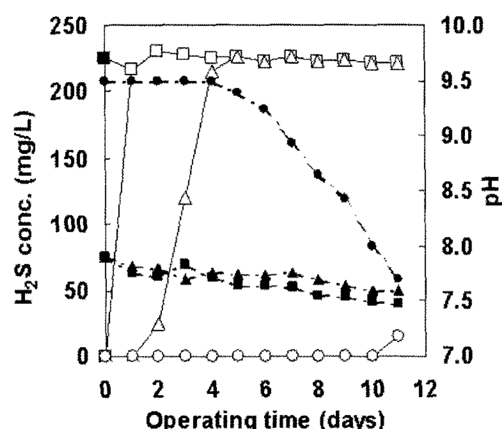

Fig. 5. Effect of cell number on H_2S removal by the biofilter with the most suitable moisture content. Symbols: (■), Inlet H_2S concentration; (□) 3.2×10^5; (△) 3.1×10^6; (○) 3.3×10^7.

H_2S was emitted from the column on day 24. At the moisture contents of 55-60 and 50-55%, 14 and 1 mg/L of H_2S were emitted from days 26 and 28, respectively. However, in the column maintained at a moisture content of 33-38%, H_2S was removed completely until the end of the experimental period. In particular, the color of the culture in the column with a moisture content of 40-45% changed to white after 1 week. Based on these results, the optimum initial moisture content for H_2S removal was determined to be 33-38%.

The initial cell number of 1.85×10^9 cells · g^{-1} was not significantly changed in any of the columns with moisture contents of 33-38, 40-45 or 50-55% on day 7 (Table 3). However, at the end of the experimental period, the viable cell number was reduced to about 10^8 cells · g^{-1} in all columns. The columns showed contamination rates of 21.9, 51.4, 47.8, and 30.4% and the contamination rate in the column decreased in the order of moisture contents of 40-45>50-55>55-60>33-38%. The extent of contamination was related to the emission of H_2S from the column (Fig. 4). Based on the results of inhibition of the H_2S removal activity by *R. rhodochrous* B261, the reduction of removal rate in the columns with moisture contents maintained above 33-38% may have been due to contamination. However, when the moisture content was maintained below 30%, H_2S removal was also reduced because of the significant decrease in the viable cell number (Table 3). Therefore, a moisture content of 33-38% is needed for a long-term removal of H_2S gas.

Effect of cell number on H_2S removal. In the columns containing 10^5 and 10^6 cells/g, H_2S gas was completely removed after 3 h and 1 day, respectively (Fig. 5). However, in the column containing 10^7 cells/g, complete removal of H_2S gas was maintained until the end of the experimental period. The period of complete removal of H_2S was clearly extended in proportion to the *R. rhodochrous* B261 cell number. The initial pH values in the columns with cell numbers lower than 10^7 were not

Table 3. Cell numbers of *R. rhodochrous* B261 in pure cultures under controlled moisture content conditions during H_2S removal

Moisture content of pure culture (%)	Microbial number (cells/g-dry weight)		
	0 day	7 day	29 day
33-38	1.85×10^9	1.27×10^9 (15%)	1.60×10^8 (22%)
40-45	1.85×10^9	1.32×10^9 (14%)	3.71×10^8 (51%)
50-55	1.85×10^9	1.24×10^9 (16%)	4.43×10^8 (48%)
55-60	1.85×10^9	1.25×10^9 (14%)	4.62×10^8 (30%)

The numbers in parentheses show contamination ratios. (Total microbial cell number-*R. rhodochrous* B261 cell number/total microbial cell number)×100.

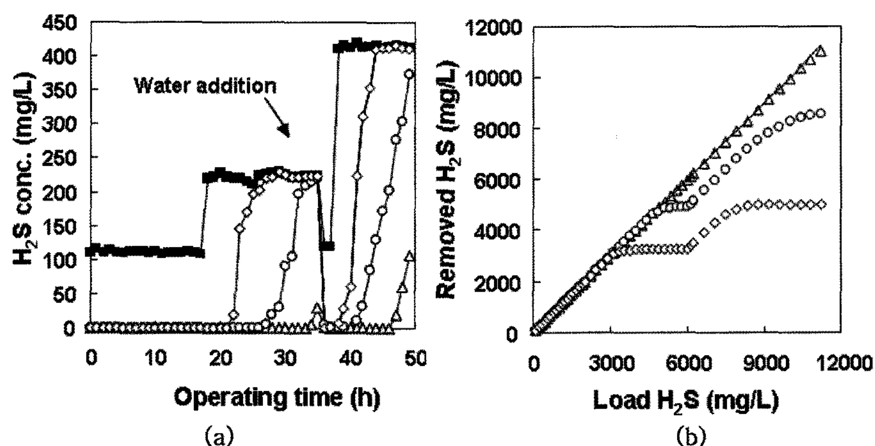

Fig. 6. Effect of inlet concentration of H_2S on removal in biofiltration at different flow rates (a) and removal rates (b) at a moisture content of 35%. Symbols: (a), (■), Inlet H_2S concentration; (△) 4.8 m^3/kg/day; (◇) 9.6 m^3/kg/day; (O) 14.4 m^3/kg/day; (b) (—), 100% removal.

significantly reduced. However, in the columns with cell numbers of 10^7 or higher, the pH of 9.5 did not change for 4 days and declined rapidly to pH 7.7 for 10 days.

Effect of inlet concentration on H_2S removal. Figure 6 shows the removal of H_2S in the columns with the moisture content maintained at 33-38%, inlet H_2S concentration of 100, 200 or 400 mg/L, and at different flow rates of 4.8, 9.6, and 14.4 $m^3 \cdot kg^{-1} \cdot d^{-1}$ for 48 days. In all columns, H_2S gas, at an inlet concentration of 100 mg/L, was removed completely for 18 days.

The columns were autoclaved and used to determine the abiotic removal under the same conditions as those of the controls (data not shown). At the flow rates of 4.8, 9.6, and 14.4 m^3/kg/d, no H_2S gas was emitted from the columns from days 1, 3, and 6, respectively, after autoclaving. The results at 4.8 m^3/kg/d showed that the surviving cells remaining in the column after autoclaving were responsible for the maintenance of complete removal of H_2S gas for 6 days.

With the increasing inlet concentration from 100 to 200 mg/L, H_2S gases from the columns were detected at 4.8, 9.6, and 14.4 m^3/kg/d at days 21, and 26, and 34, respectively. Moisture contents of all columns decreased to below 15%, and this decrease was responsible for the observed decrease in the H_2S removal. Therefore, to reactivate the microorganisms, water was added to bring up the moisture content of all columns to 35%. Inlet concentration decreased to 100 mg/L from days 36 to 37. In the columns with the flow rates of 9.6 and 14.4 m^3/kg/d, the 99% removal rate of H_2S was maintained after the addition of water. Increasing the inlet concentration of H_2S from 100 to 400 mg/L decreased the H_2S removal at days 2 and 6 at the flow rates of 9.6 and 14.4 m^3/kg/d, respectively. However, the removal in the column supplied with 400 mg/L H_2S at the flow rate of 4.8 m^3/kg/d was maintained for almost twice as long as that at the flow rate of 9.6 m^3/kg/d with the addition of water. However, the moisture content showed almost no effect on the physicochemical H_2S removal activity. H_2S gas dissolved in the water was suggested to be degraded by *R. rhodochrous* B261, resulting in the increased removal rate. In addition, the decrease in pH was delayed with the addition of water. These results indicated that the higher removal activity was due to not only increases in the level of the dissolved H_2S in the water phase, but also the physiological activation of *R. rhodochrous* B261 in the pure culture columns.

Changes in forms of sulfur compounds. The concentrations of single-sulfur, thiosulfate-S, sodium sulfite-S, and sulfate-S increased with the progressive removal of H_2S (Fig. 7), indicating that hydrogen sulfide is oxidized first into a single-sulfur, then into thiosulfate-S, and finally into sulfite-S and sulfate-S. The decrease in pH was also suggested to be caused by the accompanying increase in the sulfur level.

Use of the compost biofilters mixed with microorganisms capable of degrading the malodorous compounds is a promising and economical method for the removal of these compounds, which are produced not only by livestock and fisheries, but also by various kinds of industrial processing plants, such as those used for the sewage treatment, and chemical and fertilizer industries. This is because compost provides well-acclimatized microorganism communities, high bulk density, high specific surface area, and good water-holding capacity and moisture content [Van Groenestjin and Hesselink, 1993]. Smet *et al.* [1996] also reported that compost is a better carrier material for biofiltration.

Flow rate and pH are the major factors regulating the effective removal of hydrogen sulfide [Cho *et al.*, 2000;

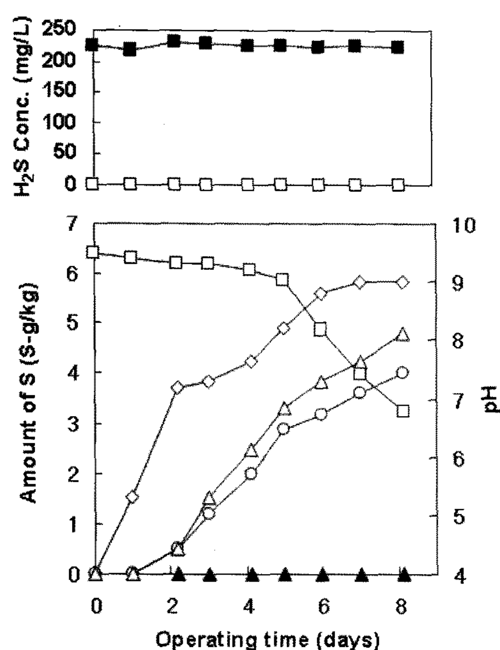

Fig. 7. Changes in the form of sulfur compounds during H_2S removal by the biofilter. Symbols: (■), Inlet H_2S concentration; (□) Outlet H_2S concentration; (◇) Amount of S in the biofilter; (▲) S_2O_3-S; (○) SO_3-S; (△) SO_4-S; (□) pH.

Park *et al.*, 2002]. Neutral or weak alkaline pH (pH 7-9) has been shown to be suitable for achieving the maximum removal of hydrogen sulfide [Yun and Ohta, 1998; Cho *et al.*, 2000; Park *et al.*, 2002]. Cho *et al.* [2000] obtained the maximum removal capacity of 428 g-S/m^{-3}/h at SV 300 h^{-1}; the removal capacity was reduced at higher space velocity. However, they found the inlet H_2S concentrations from 300 to 500 mg/L have no effect on the removal capacity at SV 300 h^{-1}. In addition, Park *et al.* [2002] obtained the maximum inlet H_2S concentration necessary for maintaining the maximum removal capacity in biofilters containing the immobilized *Thiobacillus* sp. IW using the Ca-alginate beads. However, in the present study removal efficiency of H_2S was affected by both the flow rate and the inlet H_2S concentration above 200 mg/L. To maintain a constant H_2S removal capacity, the inlet H_2S concentration should be $\leq$100 mg/L at flow rates below 8 m^3/kg/d.

In general, removal of malodorous compounds, such as H_2S and ammonia, is affected by the moisture content and the water-holding capacity due to the solubility of these compounds in water and the requirement of microbial activity [Wani *et al.*, 1997; Atlas and Bartha, 1997]. Cho *et al.* [2000] reported the removal of H_2S using biofilters consisting of porous lava packed with the autotrophic bacterium, *Thiobacillus thiooxidans*. They suggested that increasing the water-holding capacity increases the deodorization efficiency. However, in the present study, a lower moisture content of 33-38% resulted in the maximum removal rate. The low removal rate of hydrogen sulfide was suggested to be due to the drop in the pressure caused by the high moisture content, which reduces the solubility of hydrogen sulfide in water. However, the pressure drop was not detected in the present study. Therefore, 33-38% is suggested to be a suitable initial moisture content to facilitate the microbial activity, especially that of *R. rhodochrous* B261, and the column pass-through.

The amount of sulfur removed by the *R. rhodochrous* B261 cells in the biofilter was calculated by the following equation:

$$C=2.99\times10^{-9}\times N=1.437\times10^{-3}\times FR\times HC\times T$$

Where C=removal capacity (g-S/kg), N=*R. rhodochrous* B261 cells number (cells/kg), FR=flow rate (m^3/kg//d), HC=H_2S concentration (mg/L), and T=time required for H_2S removal.

The amount of sulfur removed was 2.99×10^{-9} H_2S-S/cell, which corresponded to the value reported previously for the amount of sulfur removed by a single cell [Zhang *et al.*, 1991]. The removal capacity of H_2S was proportional to the cell number, whereas the removal time was inversely proportional to the flow rate and the concentration of the inlet H_2S.

The removal capacity of H_2S in the box sealed hermetically with the immobilized *R. rhodochrous* B261 was not significantly different from the results obtained with the active carbon (data not shown). However, active carbon is more expensive than the immobilized *R. rhodochrous* B261 biofilter. In addition, the heterotrophic bacterium *R. rhodochrous* B261 is capable of degrading volatile fatty acids, which are one of the major malodorous compounds generated by the livestock, especially swine [Yun and Ohta, 1998]. As the compost is already at alkaline pH during composting, *R. rhodochrous* B261 can dominate the microbiota, resulting in the increased removal capacity. Therefore, compost with *R. rhodochrous* B261 might be suitable for the long-term biofiltration in the removal of H_2S and other malodorous compounds.

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