Analysis of Sulfur Compounds Produced with Hydrogen Sulfide Removal by *Hyphomicrobium neptunium* ATCC 15444

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Sulfur compounds produced with hydrogen sulfide (H_2S) removal by *Hyphomicrobium neptunium* ATCC 15444 were analyzed. When the loading of H_2S was 24.1 mmol of H_2S per liter-culture per day, the sulfur compounds in the culture after 150 h consisted of 121.4 mM of elemental sulfur and 17.1 mM of thiosulfate. Sulfate and sulfite were not detected. These results demonstrated that the H_2S removal by *H. neptunium* ATCC 15444 resulted from the oxidization of H_2S and the major product with the H_2S removal was elemental sulfur.

Keywords *Hyphomicrobium neptunium*; hydrogen sulfide; deodorization; malodorous component; sulfur; thiosulfate; oxidization

Introduction

Hydrogen sulfide (H_2S) is one of the malodorus compounds produced from waste treatment facilities and factories. To remove this gas, chemical and physical deodorizing methods have been commonly used. Recently, biological and biochemical methods are attracting attention of their low operating costs and high removal abilities.[1-5] In a previous paper,[6] we demonstrated that a culture of *Hyphomicrobium neptunium* ATCC 15444 could effectively remove H_2S gas from air. However, the sulfur products produced with the H_2S removal were not clarified. Thus, in this study, the sulfur compounds produced with H_2S removal by *H. neptunium* ATCC 15444 were analyzed.

Experimental

Bacterial Strain and Growth Condition The bacterial strain used throughout this study was *H. neptunium* ATCC 15444. This strain was pre-cultured at 30 °C in a liquid medium (pH 8.0) containing 0.2% polypeptone (Nihon Seiyaku), 0.1% yeast extract (Difco), 0.1% $CaCl_2 \cdot 2H_2O$ and 0.3% $MgCl_2 \cdot 6H_2O$. In the early stationary phase of growth, a 2 ml-portion of the culture broth was added to 250 ml of the same medium in 500 ml sakaguchi flasks. The flasks were shaken at 30 °C for 18 h, and the bacterial culture, which was in the early stationary phase of growth, was used for a deodorizing test.

Deodorizing Test The deodorizing culture bottle (70 i.d. × 200 mm) was filled with 400 ml of the culture of *H. neptunium* ATCC 15444. Hydrogen sulfide gas was generated by feeding solutions of Na_2S (1%) and HCl (0.12 N) by peristaltic pumps to the top of a H_2S generator. The gas was diluted with air and supplied to the culture bottle. All experiments were carried out at room temperature. The schematic diagram of the deodorizing apparatus used in this study was as described previously.[6] Inlet and outlet concentrations of H_2S in air were measured by gas detector tubes (Gastec). The relative error of the tube to the standard methylene blue method is nominally ± 10%. The lowest detection limit of the tube was 0.2 ppm.

Analysis of Sulfur Compounds When sulfide (S^{2-}) such as H_2S is the substrate for oxidation by bacteria, it enters the main pathway ($S^{2-} \rightarrow S \rightarrow SO_3^{2-} \rightarrow SO_4^{2-}$, $S + SO_3^{2-} \rightarrow S_2O_3^{2-}$).[7] Therefore, the quantitative analyses for elemental sulfur, sulfate, sulfite and thiosulfate in the culture medium were conducted. A 3.5 ml-portion of bacterial culture in the deodorizing culture bottle was collected and then centrifuged at 10000 × *g* for 5 min. The supernatant was serially diluted 2-fold, and then analyzed for sulfate, sulfite and thiosulfate ion concentrations. On the other hand, the solid was washed 3 times with distilled water and subjected to elemental sulfur analysis. The quantitative analysis for elemental sulfur was carried out by using the oxygen-flask method of Hozumi and Umemoto.[8] The amount of sulfate ion (SO_4^{2-}) was determined turbidometrically.[9] The $BaCl_2$–polyethyleneglycol (PEG) reagent (1 ml) and 0.1 N HCl (1 ml) were added to 1 ml of the culture supernatant obtained by centrifugation. The resulting white turbidity, due to barium sulfate, was measured by a spectrophotometer at 600 nm. Linearity was obtained between sulfate concentration (1.0—8.0 mM of SO_4^{2-}) and turbidity. The amount of sulfite ion (SO_3^{2-}) was also determined spectrophotometrically. Zinc acetate solution (2%; 1 ml), fuchsin–sulfuric acid solution (0.2 ml), and 1.9% formaldehyde solution (0.2 ml) were added to 1 ml of the culture supernatant obtained by centrifugation. Linearity was obtained between sulfite concentration (0.1—1.0 mM of SO_3^{2-}) and the developed purple color that was measured by a spectrophotometer at 570 nm. The amount of thiosulfate ion ($S_2O_3^{2-}$) was determined by high-performance liquid chromatography (HPLC) of an TSK gel IC-Anion PW column (Tosoh, Tokyo, Japan), which was prepared in 4 mM K_2HPO_4 (pH 9.1). Ethanol (1 ml) was added to 1 ml of the culture supernatant obtained by centrifugation. The mixture was centrifuged at 10000 × *g* for 5 min, and the supernatant was collected, and then passed through a membrane filter (0.22 μm). Then 20 μl of the filtrate was applied to the column, and eluted at a flow rate of 1.2 ml/min and a column temperature of 30 °C. The thiosulfate ion was detected after 10 min of retention time by monitoring the absorbance at 220 nm. The thiosulfate ion concentration was then calculated from the area of the peak. Linearity was obtained between the thiosulfate concentration (0.1—7.0 mM) and the area of the peak.

Results and Discussion

Hydrogen sulfide gas (300 ppm) was supplied at 0.5 l/min to the bacterial culture of *H. neptunium* ATCC 15444. The outlet gas concentration of H_2S was gradually decreased and was less than the detection limit 28 h later (Fig. 1). The effect of complete H_2S removal could continue for 99 h, and the breakdown was observed 127 h later. The absorbance of the culture medium increased with production of yellowish white precipitates according to the H_2S removal. Since it was thought that these precipitates consisted of elemental sulfur or organic sulfur, the pre-

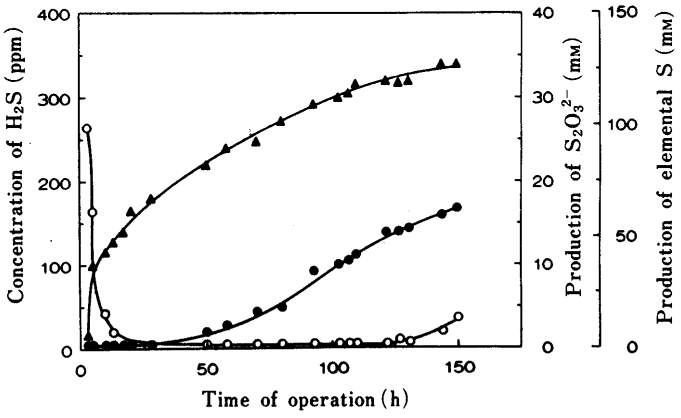

Fig. 1. Analysis of Sulfur Compounds Produced with H_2S Removal by *H. neptunium* ATCC 15444

The inlet H_2S concentration was fixed around 300 ppm at flow rate of 0.5 l/min. Symbols: ○, outlet H_2S concentration; ●, thiosulfate ion concentration; ▲, elemental sulfur concentration.

cipitates were washed with distilled water and the quantitative analysis of sulfur in the precipitates was performed by the oxygen-flask method. The amount of sulfur in the precipitates gradually increased from time zero, and the sulfur of 121.4 mM were detected at 150 h in the bacterial culture medium. Furthermore, the precipitates were extracted with carbon disulfide at room temperature for 1 h. After that the evaporation of the solvent residue was recrystallized from acetone. The deposited sulfur was identified as rhombic sulfur by measurement of the melting point and X-ray analysis. Also, the sulfur compounds were not detected in the organic residue. The supernatant of the bacterial culture that eliminated the sulfur precipitates was also analyzed for the quantitative analysis of sulfate, sulfite and thiosulfate ion concentrations. The sulfate ion and sulfite ion were not detected even 150 h after the H_2S loading. However, the thiosulfate ion was detected after 50 h of the H_2S loading, and the thiosulfate ion concentration at 150 h increased to 17.1 mM. In a control experiment using only culture medium, the concentrations of elemental sulfur and thiosulfate ion were 3.1 mM and 0.6 mM at 150 h, respectively. These results demonstrated that the majority of H_2S removal by *H. neptunium* ATCC 15444 was dependent on the oxidization to elemental sulfur.

We have previously demonstrated that the ability of H_2S removal was strongly related to the existence of Mg^{2+} or Ca^{2+} in the culture medium.[6] Therefore, we tried to clarify the relation between amounts of sulfur compounds produced and the ability of H_2S removal. *H. neptunium* ATCC 15444 was grown in the medium including either Mg^{2+} or Ca^{2+}, and each bacterial culture was used for H_2S removal. Hydrogen sulfide gas (300 ppm) was supplied to each bacterial culture at 0.5 l/min. As shown in Fig. 2, *H. neptunium* ATCC 15444 grown in the Mg^{2+}-free medium could completely remove H_2S gas from air at 63 h of H_2S loading. The effect could continue for only 15 h, and then breakdown was gradually observed. Since the yellowish white precipitates were also produced gradually with the H_2S removal, the amount of sulfur in the precipitates was similarly estimated. The concentration of sulfur in the precipitates at 150 h was 78.6 mM, and its concentration was much lower than that shown in Fig. 1. Furthermore, the thiosulfate was detected after 63 h, and the ion concentration at 150 h was 1.41 mM. The sulfate ion and sulfite ion were not detected even after 150 h of H_2S loading. On the other hand, *H. neptunium* ATCC 15444 grown in the Ca^{2+}-free medium could not completely remove H_2S gas from air at any time during the loading (Fig. 3). The maximum H_2S removal was observed at 35 h, and the removal rate was about 73.3%. This effect could continue for only 35 h, and the breakdown was gradually observed. The sulfate ion and sulfite ion were not detected at any time, but the productions of sulfur precipitates and thiosulfate ion were observed with the H_2S removal, and the sulfur and ion concentrations were 71.4 and 5.7 mM at 150 h, respectively. The sulfur precipitates shown in Figs. 2, 3 were also extracted with carbon disulfide, and it was confirmed that these sulfur precipitates consisted of elemental sulfur and did not include organic sulfur. In control experiments using only culture medium including either Mg^{2+} or Ca^{2+}, the concentrations of elemental sulfur and thiosulfate ion were 3.0 and 0.5 mM at 150 h, respectively. These results suggested that the total

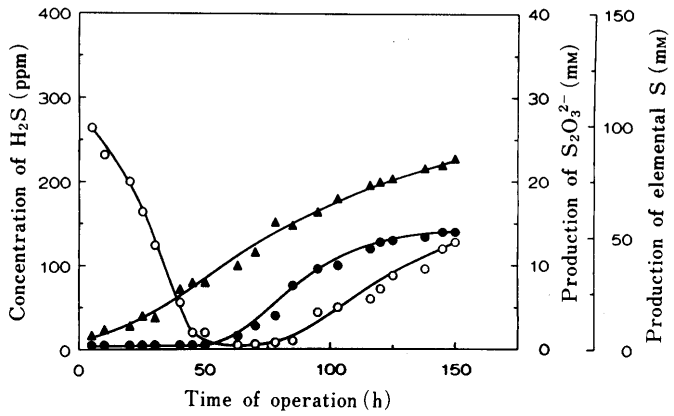

Fig. 2. Analysis of Sulfur Compounds Produced with H_2S Removal by *H. neptunium* ATCC 15444 Grown in Mg^{2+}-Free Medium

The inlet H_2S concentration was fixed around 300 ppm at flow rate of 0.5 l/min. Symbols: ○, outlet H_2S concentration; ●, thiosulfate ion concentration; ▲, elemental sulfur concentration.

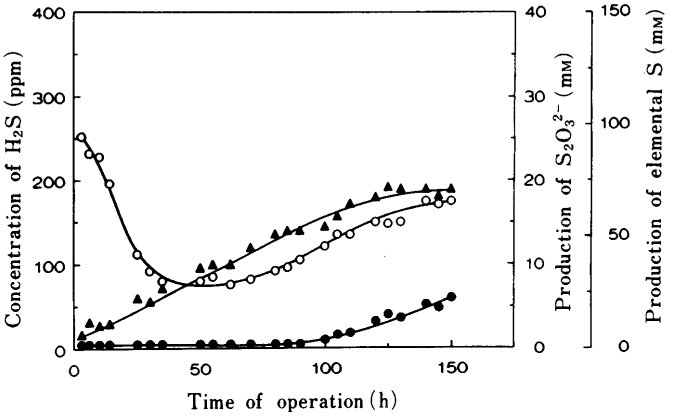

Fig. 3. Analysis of Sulfur Compounds Produced with H_2S Removal by *H. neptunium* ATCC 15444 Grown in Ca^{2+}-Free Medium

The inlet H_2S concentration was fixed around 300 ppm at flow rate of 0.5 l/min. Symbols: ○, outlet H_2S concentration; ●, thiosulfate ion concentration; ▲, elemental sulfur concentration.

amount of production of elemental sulfur, which was the major product produced with H_2S removal, paralelled with the ability of H_2S removal of the bacterial culture.

In this study, the H_2S gas was loaded for 150 h at the rate of about 1 mM/h. *H. neptunium* ATCC 15444 grown in the medium including both Mg^{2+} and Ca^{2+} could degrade about 93.3% (140 mM) of the total loaded H_2S gas after 150 h (150 mM). The result of quantitative analysis showed that the 140 mM of H_2S degraded was oxidized to 121.4 mM of elemental sulfur and 17.1 mM of thiosulfate ion. On the other hand, *H. neptunium* ATCC 15444 grown in the medium including only Ca^{2+} could degrade about 66.7% (100 mM) of the total loaded H_2S gas after 150 h (150 mM). The 100 mM of H_2S degraded was oxidized to 78.6 mM of elemental sulfur and 14.1 mM of thiosulfate ion. *H. neptunium* ATCC 15444 grown in the medium including only Mg^{2+} could degrade about 53.3% (80 mM) of the total loaded H_2S gas after 150 h (150 mM). The 80 mM of H_2S degraded was oxidized to 71.4 mM of elemental sulfur and 5.7 mM of thiosulfate ion. These results suggested that the H_2S gas degraded was independently of the ability of the H_2S removal oxidized to elemental sulfur and thiosulfate ion. Also, our calculation showed that about 80 to 90% of the H_2S degraded was oxidized to elemental

sulfur and the other was oxidized to thiosulfate ion. In the previous papers, two species of bacteria, *Thiobacillus intermedius*[10] and *Thiobacillus thioparus* TK-m,[11,12] have been reported as the bacteria which effectively remove H_2S gas from air. These bacteria produce sulfuric acid as a final product with the H_2S removal, and lower the pH in the medium, while *H. neptunium* ATCC 15444 did not produce any concentration of sulfuric acid. This fact suggested that *H. neptunium* ATCC 15444 might oxidize H_2S with a different mechanism as compared with *Thiobacillus* species.

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