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Characterization of thiosulfate-oxidizing *Enterobacter hormaechei* JH isolated from barnyard manure

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Abstract−In an effort to discover sulfur-oxidizing microorganisms, a new bacterium was screened from barnyard manure. This strain was identified as *Enterobacter hormaechei* JH by its morphological, physiological, and biochemical properties, as well as by 16S rRNA gene sequence analysis. *E. hormaechei* JH are Gram-negative rod, non-spore-forming, and aerobic. The pH range for *E. hormaechei* JH growth was 2.0-9.0, and the optimal pH was determined as pH 7.0. The optimal temperature for growth was 30 °C. We compared the growth of this strain with *Thiobacillus delicates* KCTC2851, which is a well known thiosulfate oxidizing microorganism. *E. hormaechei* JH presented greater growth than *T. delicates* KCTC2851. Thus, the above results indicate this strain is a candidate for improving the removal efficiency of biological deodorizing systems.

Key words: Sulfate-oxidation, *Enterobacter hormaechei* JH, Odor Removal, Biological Deodorizing System

INTRODUCTION

Odors from waste water treatment plants and petrochemical plants are highly toxic and harmful to human health. One of the most abundant components present in odor is hydrogen sulfide [1]. Hydrogen sulfide (H_2S) is a highly odorous and toxic gas produced from agricultural and industrial processes such as livestock farming, food and rubber processing, leather manufacturing, wastewater treatment, waste disposal landfills, and natural gas [2,3]. The release of H_2S into the atmosphere brings odor complaints, and can be fatal when it accumulates in confined spaces. The control of H_2S is important for public health and safety, and for environmental protection [4].

To remove H_2S, physicochemical methods are generally used. However, biological methods such as using microbial activity for H_2S removal have drawn attention since they are more efficient and more economical than physicochemical methods, when proper operational conditions are maintained. A number of microbial processes for H_2S removal have been proposed that are based on oxidation by *Thiobacilli* and other sulfur microorganisms. *Thiobacillus* sp. have been used to oxidize H_2S to sulfate in liquid [5], and in packed tower systems such as peat biofilters [6,7]. Mixed cultures of bacteria from the *Beggiatoa* and *Thiothrix* genera [8], and the photosynthetic bacterium *Chlorobium thiosulfatophilum,* have been proposed for use in the oxidation of H_2S to elemental sulfur from gas streams [9]. Most of these are autotrophic bacteria; however, they are often difficult to handle, mainly because their growth rates are significantly lower than those of heterotrophic bacteria. In applying phototrophic microorganisms, the supply of solar or artificial energy is a limiting factor for growth.

The ecology, physiology, and biochemistry of sulfur-oxidizing bacteria were reviewed previously. Among several microorganisms, *Thiobacillus thioparus* [10], *Thiobacillus thiooxidans* [11], *Chlorobium thiosulfatophlium* [12], *Rhodococcus rhodochrous* [13], and *Ralastonia* sp. [14] have been used to oxidize H_2S [15]. The neutrophilic chemolithotrophic bacteria were reviewed by Kelly et al. [16], and the acidophilic sulfur-oxidizing bacteria were reviewed by Harrison [17] and Pronk et al. [18]. In addition, the molecular genetics of *Acidithiobacillus ferrooxidans* was reviewed by Rawlings and Kusano [19]. Finally, the sulfur metabolism of phototrophic bacteria was reviewed by Brune [20] as well as by Trûper and Fischer [21]. The physiology and genetics of both phototrophic and lithotrophic sulfur-oxidizing prokaryotes were also recently discussed [22]. Prokaryotes oxidize hydrogen sulfide, sulfur, sulfate, thiosulfate, and various polythionates under alkaline, neutral, or acidic conditions [23].

The purpose of this study was to investigate the growth characteristics of a hydrogen sulfide removal bacterium newly isolated from barnyard manure. Here, we report on the screening and identification of the thiosulfate-oxidizing strain along with its characteristics.

EXPERIMENTAL

The study samples were collected from a barnyard manure factory in Yangsan, Korea, in July, 2005. Approximately 2 g of each sample was cultured in 100 mL of basic medium consisting of yeast extract (2.0 g/L), KH_2PO_4 (4.0 g/L), NH_4Cl (0.4 g/L), $FeSO_4 \cdot 7H_2O$ (0.01 g/L), K_2HPO_4 (2.0 g/L), $MgCl_2 6H_2O$ (0.2 g/L), and $Na_2S_2O_3 \cdot 5H_2O$ (5.0 g/L), at pH 7.0 and 30 °C and 60 °C for 2 days. This enrichment process was repeated 3 times, and then the samples were smeared on medium with 1.5% agar at 30 °C and 60 °C for 2 days. Finally, the microorganisms on the agar plates were sub-cultured in

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basic medium and isolated as thiosulfate-oxidizing microorganisms.

Temperature and pH were examined to determine the optimum culture conditions for the thiosulfate-oxidizing strain. Here, the cells were aerobically incubated in a pH range of 2-9 and temperature range of 25-60 ºC, in liquid medium. Growth patterns were observed at various thiosulfate concentrations, from 0.032 M to 0.16 M. The carbon sources were glucose, fructose, and galactose. The nitrogen sources were yeast, malt extract, and peptone. The growth was measured by a UV-VIS spectrometer (Mecasys, Optizen 2120UV) at an absorbance of 600 nm.

The identification of the isolate was carried out by morphological observations, conventional biochemical tests, and 16S rRNA gene sequence analysis. The morphological observations were performed with a light microscope (Lieca Optical, Japan: 1,000). Conventional biochemical tests were performed by using the Vitek (Biomerieux, USA) Gram-negative identification card (GNI+ software, version R09.01) according to the manufacturer's instructions. The chromosomal DNA of *E. hormaechei* JH was prepared as described by Sambrook et al. [24]. The 16S rRNA gene of *E. hormaechei* JH was amplified by polymerase chain reactions (PCR) from the genomic DNA. The PCR products were examined by electrophoresis and then isolated and sequenced. A similarity search was performed on the Genbank databases. To characterize *E. hormaechei* JH, a neighbor-joining phylogenetic tree was constructed with the CLUSTAL W program.

Under the optimum conditions, thiosulfate oxidation was examined at various thiosulfate concentrations. The sulfate concentration was measured by a modified version of Kelly's method [16]. Briefly, 2 mL of 10% $BaCl_2 \cdot 2H_2O$ and 2 mL of reactant were added and the absorbance was measured at 420 nm with a UV-VIS spectrophotometer. Finally, we compared the results with *Thiobacillus delicates* KCTC2851, which is a well known thiosulfate-oxidizing microorganism.

Table 1. Morphological, physiological, and biochemical characteristics of the *E. hormaechei* JH strain

Characteristics	Results
Morphological	
Shape	Short rod
Gram stain	-
Swollen spore	-
Physiological	
Aerobic growth	+
Optimum temperature	30
Optimum pH	pH 7.0
Growth pH range	pH 2.0-9.0
Biochemical	
Catalase	+
Gelatinase	+
Chrome oxidase	+
Nitrate reduction	+
Utilization of citrate	+
Starch hydrolysis	
D-Glucose	+
D-Xylose	+
D-Mannose	+
L-Arabinose	+
Sucrose	-
Lactose	-
D-Fructose	-
L-Rhamnose	-
Inositol	-
D-Manintol	-
L-Llysine decarboxylase	+
L-Ornithine decarboxylase	+
L-Arginine dehydrolase	+

RESULTS AND DISCUSSIONS

We screened approximately 46 strains of bacteria that were isolated from barnyard manure collected in Yangsan, Korea. Among them, only one strain was finally selected. This strain had a rapid growth rate and superior thiosulfate-oxidizing ability as compared to the other strains.

The morphological, physiological, and biochemical characteristics of the *E. hormaechei* JH strain are given in Table 1. The selected strain is a Gram-negative rod, and non-spore-forming. It is aerobic and non-fermentative, and utilized glucose oxidatively; the strain utilized D-glucose, D-xylose, D-galactose, D-mannose, and L-arabinose, but not sucrose, lactose, D-fructose, L-rhamnose, inositol, or D-mannitol. The test results for chrome oxidase, catalase, gelatinase, L-lysine and L-ornithine decarboxylases, and L-arginine dehydrolase were positive, but the strain did not hydrolyze starch. Finally, the denitrification and citrate utilization tests were positive.

To further confirm the strain's identification, its 16S rRNA gene sequence (Fig. 1) was compared to different strains obtained from the NCBI database by using the neighbor-joining method. The results showed that this strain had highest similarity (99%) to *E. hormaechei* (Fig. 2). The type species was *E. hormaechei*. Therefore, the strain

CAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAACAGGAAGCAGCTT
GCTGCTTCGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGG
GATAACTACTGGAAACGGTAGCTAATACCGCATAANGTCGCAAGACCAAAGAGGGGGACCTT
CGGGCCTCTTGCCATCGGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACC
TAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGTC
CAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCC
ATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGAT
AAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCA
GCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGC
AGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACT
GGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGA
TCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAA
AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTG
GAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGT
ACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGT
GGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTACCAGAG
ATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGT
GAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTAGGCC
GGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCAT
CATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCT
CGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACT
CCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCC
TTGTACACACCGCCCGTCC

Fig. 1. The 16S rRNA gene sequence of *E. hormaechei* JH.

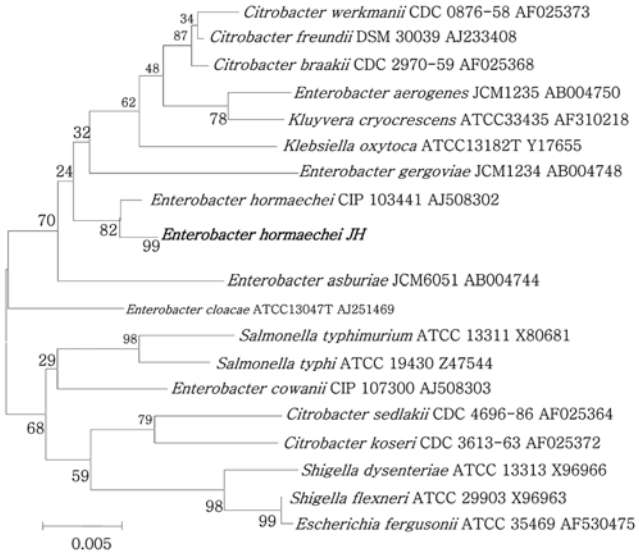

Fig. 2. Neighbor-joining tree showing the phylogenetic positions of *E. hormaechei* JH and its nearest neighbors based on 16S rRNA gene sequence analysis.

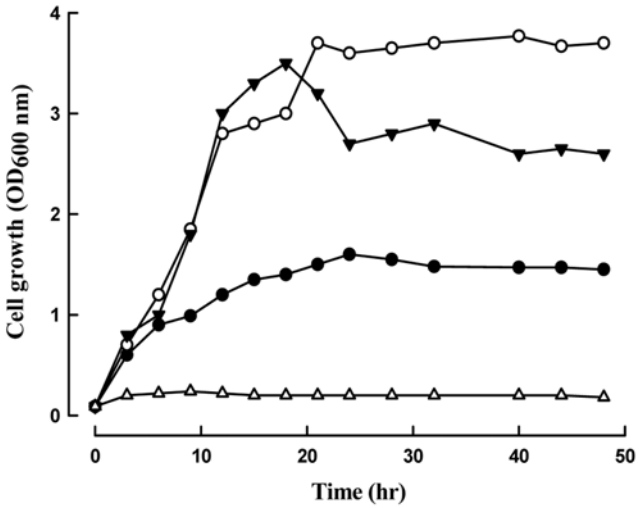

Fig. 3. Effect of temperature on *E. hormaechei* JH cell growth: ●, 25 °C; ○, 30 °C; ▼, 35 °C; △, 60 °C.

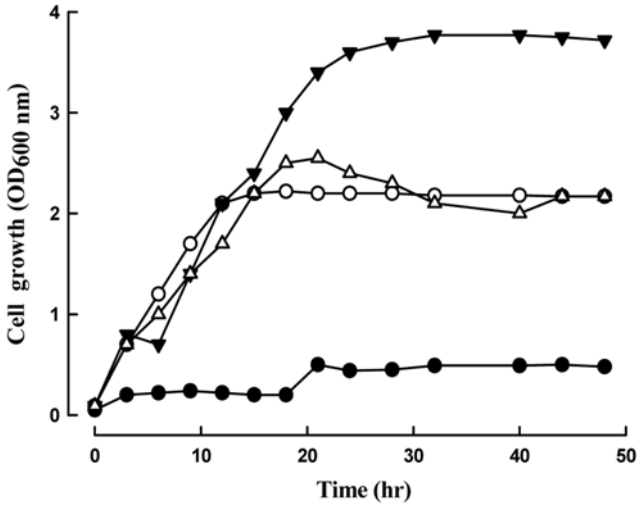

Fig. 4. Effect of initial pH on *E. hormaechei* JH cell growth: ●, pH 2; ○, pH 5; ▼, pH 7; △, pH 9.

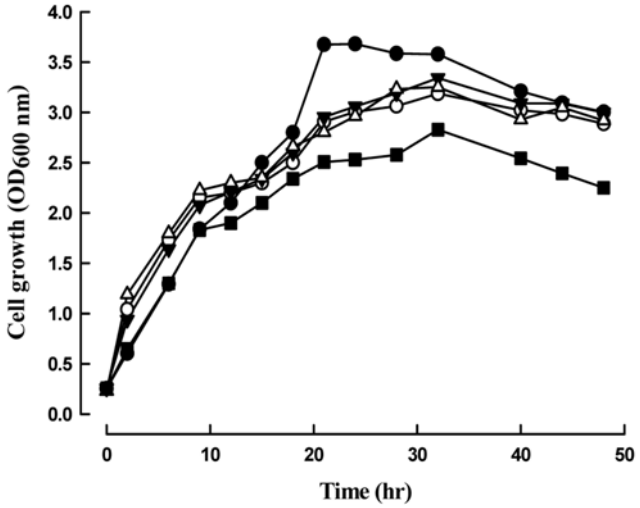

Fig. 5. Effect of thiosulfate concentrations on *E. hormaechei* JH cell growth: ●, 0.032; ○, 0.06 M; ▼, 0.08 M; △, 0.12 M; ■, 0.16 M.

was named *E. hormaechei* JH. Diverse microorganisms capable of H_2S oxidation have previously been reported: colorless sulfur bacteria such as *Thiobacillus* spp. [5,10,11], *Thiothrix* sp. [8], and *Beggiatoa* sp. [8]; phototrophs such as *Chlorobium* sp. [12], *Rhodococcus* sp. [13]; and cyanobacteria. However, there have been few reports on H_2S oxidation by aerobic heterotrophic microorganisms, and this is the first extensive report on H_2S removal by a heterotropic bacterium, *Enterobacter hormaechei*.

E. hormaechei JH grew at temperatures between 25-60 °C, and the optimal growth temperature was 30 °C (Fig. 3). As shown in Fig. 4, the optimal pH was 7.0. The optimal growth temperature and pH were similar to typical soil bacterium's optimal growth temperature and pH. These conditions are normal conditions which are widespread in the environment. Therefore, *E. hormaechei* JH is major bacteria able to degrade H_2S in the neutral pH region and in normal temperature environment.

The growth curve of *E. hormaechei* JH at various thiosulfate concentrations is shown in Fig. 5. The cell growth reached a stationary phase after 20 h, and *E. hormaechei* JH presented its highest cell growth pattern at the 0.032 M thiosulfate concentration. This indicates that oxidation reduced the pH of the medium, and the lower pH influenced *E. hormaechei* JH's growth. Thus, a proper thiosulfate concentration is required for *E. hormaechei* JH.

The *E. hormaechei* JH strain showed similar growth patterns on glucose, fructose, and galactose (Fig. 6A). Among these carbon sources, galactose utilization was stable after 20 h. *E. hormaechei* JH presented different growth patterns on yeast, malt extract, and peptone (Fig. 6B). Among these nitrogen sources, peptone utilization was lowest and yeast utilization highest. Therefore, the *E. hormaechei* JH is able to degrade H_2S in environmental conditions where the concentration of organic matter is high.

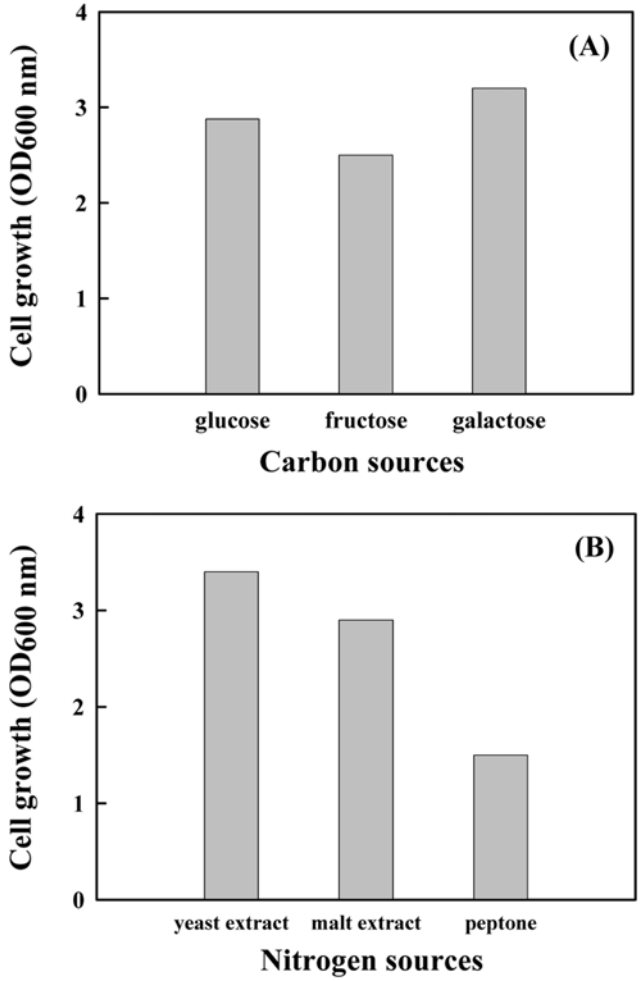

Fig. 6. Growth characterization of *E. hormaechei* JH in thiosulfate medium supplemented with Carbon-sources (A) and Nitrogen-sources (B). The growth conditions of the thiosulfate medium were incubation at 30 °C and 250 rpm for 24 h.

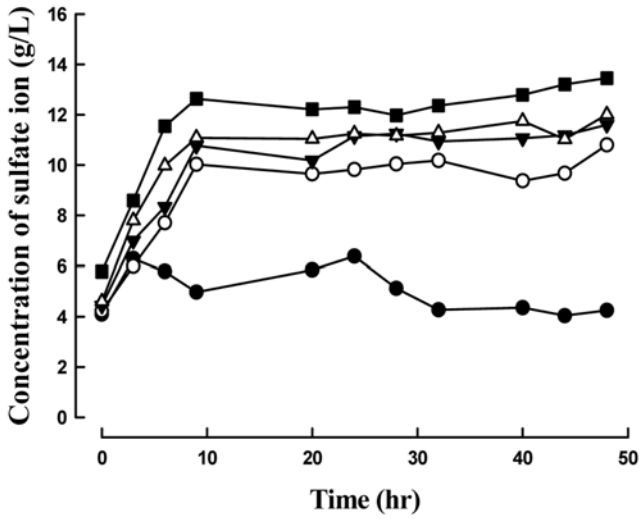

Fig. 7. Effect of thiosulfate concentration on the sulfur oxidation of *E. hormaechei* JH: ●, 0.032; ○, 0.06 M; ▼, 0.08 M; △, 0.12 M; ■, 0.16 M.

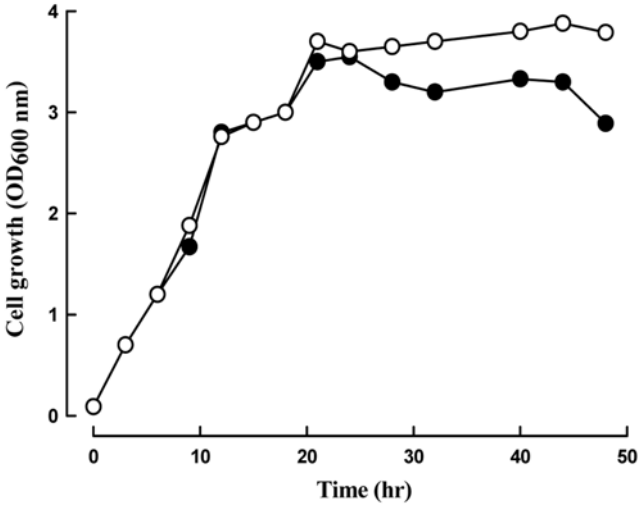

Fig. 8. Cell growth comparison of thiosulfate-oxidizing bacteria: ○, *E. hormaechei* JH; ●, *T. delicates* KCTC2851.

Under the optimum conditions, thiosulfate oxidation was examined at various thiosulfate concentrations (Fig. 7). *E. hormaechei* JH oxidized thiosulfate to sulfate. We determined the sulfate quantity in order to identify the thiosulfate-oxidizing level. As the concentration of thiosulfate increased, the thiosulfate oxidizing rate also increased.

In addition, we compared the growth of this strain to that of *Thiobacillus delicates* KCTC2851, which is a well known thiosulfate-oxidizing microorganism (Fig. 8). *E. hormaechei* JH presented greater growth than *Thiobacillus delicates* KCTC2851. Therefore, *E. hormaechei* JH would be more effective for improving the removal efficiency of biological deodorizing systems. Thus, we suggest that *E. hormaechei* JH is a prospective candidate for use in bio-deodorization, and has the potential to be effective in biological deodorizing systems if a suitable and proper combination with other bacteria is employed.

CONCLUSION

The selected strain was an aerobic gram negative rod-shaped bacterium. The optimal pH and temperature for cell growth were pH 7.0 and 30 °C, respectively. The conventional biochemical tests were performed by using the Vitek, and its 16S rRNA gene sequence was analyzed. This strain had highest similarity to *Enterobacter hormaechei*, 99%. The selected strain was named *Enterobacter hormaechei* JH, and it converted from thiosulfate to sulfate at 0.16 M highest. This strain very well utilized galactose and yeast extract for carbon and nitrogen source individually. *Enterobacter hormaechei* JH has more effective growth compared to *Thiobacillus delicates* KCTC2851. We suggest that this strain will be a good candidate for maintaining high H_2S removability in biological deodorizing systems.

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