Degradation of Hydrogen Sulfide in Stillage as Ethanol Industrial Waste by *Acidithiobacillus thiooxidans* and *Pseudomonas putida* with Aerobic Biofiltration Method in Bioreactor Management Implementation

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***Abstract*— Stillage or vinasse is a by-product or waste from the fermentation-distillation process of the bioethanol industry. Stillage is the bottom product of the ideal distillation column. Stillage has a high sulfur content. In this waste, the sulfur content was 1680 mg/L. This liquid wastes are dangerous if it discharges directly into the environment without pretreatment. For this reason, pretreatment is needed to reduce the sulfur concentration of liquid waste (stillage) using biofiltration method. The objective of this research is to eliminate the content of H2S or sulfur in the wastewater of the bioethanol industry (stillage) by using aerobic bacteria such as *Acidithiobacillus thiooxidans* dan *Pseudomonas putida*. The method of this experimental work used biofiltration which are attached on wood chips by aerobic bacterial to form biofilms in the reactor. The process in this study was conducted in two steps. The first step was carried out by sulfur oxidizing bacteria such as *A.thiooxidans* and *P. putida* with a concentration of 10% and 20% (v/v) that growth on packing to form biofilms in the reactor for 13 days. Furthermore, as the second step the bacteria degraded H2S content of liquid waste with attached bacteria on packing for 15 days in biofiltration reactor. From the preliminary results of this study, reactor with 10% (v/v) *A. thiooxidans* on wood chips packing and 30% (v/v) concentration stillage can degraded H_2S from 4.90 mg/L to 2.61 mg/L (46.73% removal efficiency) and for reactor with 20% (v/v) *A. thiooxidans* can degraded H2S from 4.90 mg/L to 2.43 mg/L (50.41% removal efficiency). Meanwhile, reactor with 10% (v/v) *P. putida* can degraded H2S from 4.90 mg/L to 2.90 mg/L (40.82% removal efficiency) and for reactor with 20% (v/v) *P. putida* can degraded H2S from 4.90 mg/L to 2.84 mg/L (42.04% removal efficiency).**

***Keywords—Acidithiobacillus thiooxidans*, Biofiltration , *Pseudomonas putida*, Stillage, Wood Chips.**

I. INTRODUCTION

Stillage or vinasse is a by-product or waste from the fermentation-distillation process of the bioethanol industry. Stillage is the bottom product of the ideal distillation column. In the bioethanol industry, stillage was produced in large quantities. At present, stillage was used for liquid biofertilizer with a digester process. However, stillage has a high sulfur content. In this waste, the sulfur content was 1680 mg/L [1]. If it used for fertilizer continuously, the sulfur content in the stillage will pollute the environment. So that the wastes must be processed before being discharged into the environment.

There are several processes for removing sulfur in wastes, including physico-chemical methods and biological methods. Physico-chemical methods including absorption, adsorption, and chemical oxidation. While biological methods using microorganisms to remove sulfur including biofilters, biotrickling filters, and bioscrubbers. Each methods have advantages and disadvantages. The advantages of physico-chemical methods were widely used in industry, efficient for a mixture a concentrated gases, and saving energy. While the disadvantages were high chemical disposal, high costs, high energy for several methods, and result in the production of secondary pollutant. For biological methods, the advantaged this methods were more economical, cleaner and greener because of its low operation cost, absence of secondary pollutants and emission of lower amounts of environmentally unfriendly gases compared to other methods. While the disadvantages were removal efficiency can fluctuate depending on operating conditions of pollutant and can only handle waste with low concentrations[2]. For this reason, biofiltration is applied in this study.

Biofiltration is a multi-phase system in which contaminated gas is dissolved and absorbed in the biofilm, and then degraded by microorganisms that are immobilized on packing material forming a thin layer (biofilm)[3]. In biofiltration process, the polluted gas stream passed through the porous media or packing, where the pollutants are absorbed and biodegraded by microorganisms. Microorganisms use pollutants to produce energy and metabolic intermediates and end of the products is CO_2, H_2O, and biomass[2]. Microorganisms that are more suitable to degraded H_2S are chemoautotrophic microorganisms. Among the chemoautotropic bacteria, the sulfur-oxidiing bacteria, which are composed of several general, such as *Thiobacillus*, *Acidithiobacillus*, *Achromatium*, *Beggiatoa*, *Thiothrix*, *Thioplaca*, *Thiomicrospira*, *Thiosphaera*, *Thermothrix*, and others[4].

In the previous study conducted by Aita et al. (2015) where *Acidithiobacillus thiooxidans* can degrade H_2S obtained from biogas that contained H_2S and the other previous study, H_2S has obtained from pure H_2S gas. In this study, H_2S has obtained from wastewater of bioethanol industry (stillage).

According to Aita et al. (2015) about *Acidithiobacillus thiooxidans*, these bacteria which were immobilized in wood chips could degrade H_2S for 37 days with and average H_2S

removal efficiency of 97%. It is because *Acidithiobacillus thiooxidans* bacteria had the ability to degraded sulfur or H_2S. The objective of this research is to eliminate the content of H_2S or sulfur in the wastewater of the bioethanol industry (stillage) by using aerobic bacteria such as *Acidithiobacillus thiooxidans* and *Pseudomonas putida*.

II. MATERIALS AND METHODS

A. Sampling and Preparation of Stillage

Stillage wastes were obtained from PT Energi Agro Industri, Mojokerto, East Java, Indonesia. Stillage was prepared by separating the impurities. Then the sampels were sterilized by autoclave at 121°C and 1.2 atm for 15 minutes.

B. Preparation of Medium Acidithiobacillus thiooxidans and Pseudomonas putida

1) Media for Acidithiobacillus thiooxidans

Liquid media was prepared by mixing 40 grams of nutrient broth, 15 grams of glucose, 2 grams of $(NH_4)_2SO_4$, 20 grams of KH_2O_4, 1.25 grams of $CaCl_2$, 25 grams of $Na_2S_2O_3$, 2.5 grams of $MgSO_4$, and 0.05 grams of $FeSO_4$ into 5 liters of aquadest. Then the liquid media was sterilized by autoclave at 121°C and 1.2 atm for 15 minutes.

2) Media for Pseudomonas putida

Liquid media was prepared by mixing 40 grams of nutrient broth and 15 grams of glucose into 5 liters of aquadest. Then the liquid media was sterilized by autoclave at 121°C and 1.2 atm for 15 minutes.

C. Preparation of Packing Media

400 grams of wood chips used as packing media were removed by impurities (stones, leaves, etc.). After that, wood chips was washed with warm water at 40°C and dried. Then wood chips was sterilized by autoclave at 121°C and 1.2 atm for 15 minutes.

D. Preparation of Biofilm on Packing

Bacterial media that have been prepared as much as 5 liters are accommodated in a biofiltration reactor. After that, 500 and 1000 ml of *Acidithiobacillus thiooxidans* and *Pseudomonas putida* isolates were added to biofiltration reactor with concentration of 10% and 20% (v/v) to the total bacterial media volume for inoculum and aerated at 30°C to form biofilms with a thickness of ± 2 mm or around 13 days.

E. Biofiltration of Stillage

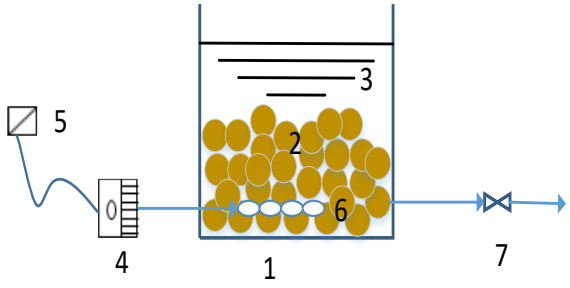


Figure 1. Biofiltration Reactor

Notes :
1. Bioreactor
2. Packing that has been immobilized by bacteria
3. Wastewater (Stillage)
4. Aerator
5. Electricity Source
6. Sparger
7. Valve

8 liters of sterilized stillage with 30% (v/v) concentration was put into the bioreactor containing biopacking that has been immobilized by bacteria. Air from aerator was flowed into the reactor to maintain aerobic conditions. The reactor volume used was 16 liters. Biofiltration was performed in batch reactor with aeration for 15 days (Figure 1). Every 3 days, sampels were taken from bioreactors and analyzed for H_2S concentration, BOD (Biochemical Oxygen Demand), and microbial population during the experiment.

F. Analysis of H_2S

Sampel from biofiltration process then analyzed of H_2S concentration by iodometric titration method according to SNI 6989.75:2009 at Politeknik Kesehatan Surabaya. H_2S concentration were measured every 3 days for 15 days. The percentage of H_2S concentration was calculated by using equation 1.

$$Degradation\ (\%) = \frac{[H_2S]_0 - [H_2S]_n}{[H_2S]_0} \times 100 \qquad (1)$$

G. Analysis of BOD

Sampel from bifiltration process then analyzed of BOD or Biochemical Oxygen Demand using DO meter according to SNI 6989.72:2009 at Wastewaster Treatment Laboratory, Chemical Engineering Department, Institut Teknologi Sepuluh Nopember, Surabaya. BOD were measured every 3 days for 15 days.

H. Total Cell Counts

Total cell counts were determined during the biofiltration using counting chamber . 1 ml of slurry (biofilm) was diluted by 9 ml aquadest. Dilution was put in haemacytometer. Cell counts were observed and counted under microscope. Cell counts were done three times for a week. The result was plotted in graphic, compared with time and decrease of H_2S substances.

III. RESULTS AND DISCUSSION

A. Composition of Stillage

Table 1.
Composition of 30% (v/v) Stillage from PT Energi Agro Industri

Parameter	Characteristic
Color	Brown
pH	4.23
Temperature (°C)	30
BOD (mg/L)	649.240
COD (mg/L)	1021.325
Concentration of H_2S (mg/L)	4.9
Fenol (mg/L)	291.378

B. Effect of Addition of Acidithiobacillus thiooxidans and Pseudomonas putida Bacteria on Stillage

The addition of bacteria on the stillage waste spill accelerate the process degradation of sulfur. Addition of *Acidithiobacillus thiooxidans* and *Pseudomonas putida* was supposed to decrease the sulfur or H_2S concentration that was contained in the stillage.

The results of the chemical analysis related to the H_2S concentration in four bioreactors were shown in the table 2. Chemical analysis after biodegradation showed decreased H_2S concentration in all bioreactors.

Table 2.a.
Result of H_2S degradation by 10% *Acidithiobacillus thiooxidans*

10% (v/v) Acidithiobacillus thiooxidans		
Days	**H_2S Concentration (mg/L)**	**Percent Degradation (%)**
0	4.9	0
3	4.41	10
6	3.99	18.57
9	3.51	28.37
12	2.95	39.8
15	2.61	46.73

Table 2.b.
Result of H_2S degradation by 20%*Acidithiobacillus thiooxidans*

20% (v/v) *Acidithiobacillus thiooxidans*		
Days	**H_2S Concentration (mg/L)**	**Percent Degradation (%)**
0	4.9	0
3	4.01	18.16
6	3.89	20.61
9	3.31	32.45
12	2.69	45.1
15	2.43	50.41

Table 2.c.
Result of H_2S degradation by 10% *Pseudomonas putida*

10% (v/v) *Pseudomonas putida*		
Days	**H_2S Concentration (mg/L)**	**Percent Degradation (%)**
0	4.9	0
3	4.61	5.92
6	4.09	16.53
9	3.94	19.59
12	3.32	32.24
15	2.9	40.82

Table 2.d.
Result of H_2S degradation by 20% *Pseudomonas putida*

20% (v/v) *Pseudomonas putida*		
Days	**H_2S Concentration (mg/L)**	**Percent Degradation (%)**
0	4.9	0
3	4.59	6.33
6	4.01	18.16
9	3.88	20.82
12	3.11	36.53
15	2.84	42.04

Tabel 1 a-d showed that the addition of *Acidithiobacillus thiooxidans* and *Pseudomonas putida* bacteria affected to H_2S concentration. Concentration of H_2S decreased day by day for all bioreactors. Bacteria in stillage could decreased H_2S concentration if supported by operating conditions, such as aeration, temperature, and nutrition.

In bioreactor A, with addition of 10% (v/v) *Acidithiobacillus thiooxidans* with wood chips packing, at the end process the total H_2S concentration was decreased from 4.90 mg/L become 2.61 mg/L. In bioreactor B, with the addition of 20% (v/v) *Acidithiobacillus thiooxidans*, decreasing of H_2S concentration from 4.90 mg/L become 2.43 mg/L. While in the bioreactor C, with the addition of 10% (v/v) *Pseudomonas putida* with wood chips packing, H_2S concentration was decreased from 4.90 mg/L become 2.90 mg/L. In bioreactor D, with the addition of 20% (v/v) *Pseudomonas putida* was decreased H_2S concentration from 4.90 mg/L become 2.84 mg/L.

Percent degradation increase day by day for all bioreactors, and at the end process, percent degradation reached 46.73% for bioreactor A with 10% (v/v) *Acidithiobacillus thiooxidans*, 50.41% for bioreactor B with 20% (v/v) *Acidithiobacillus thiooxidans*, 40.82% for bioreactor C with 10% (v/v) *Pseudomonas putida*, and 42.04% for bioreactor D with 20% (v/v) *Pseudomonas putida.*

From the data in the table above could be seen that the decrease in the concentration of H2S occurs slowly on day 0 to day 15. It is because the stillage was by-product of the bioethanol producing process which contain alcohol or phenol. Alcohol or phenol has anticeptic ability, so it can affected bacterial growth in biofilms. This is seen on Figure 2.

From figure 2 a and b showed the correlation between H_2S concentration and bacteria population against time. H_2S concentration decreased for all bioreactors both on addition of *Acidithiobacillus thiooxidans* and *Pseudomonas putida* bacteria from day by day and it was accompanied by an increase bacteria population. But, the bacteria growth occurs slowly, so that it's greatly affected the degradation of H_2S in stillage.

If the bioreactor with the addition of *Acidithiobacillus thiooxidans* was compared with bioreactor with the addition of *Pseudomonas putida*, then the bioreactor that gives the best result is a bioreactors with the addition of *Acidithiobacillus thiooxidans*.

Bacteria from the genus *Thiobacillus* or *Acidithiobacillus* are more suitable for degrading sulfur or H_2S, because these bacteria needed a few nutrients to growth and they able to growth in acidic pH[5]. The main characteristics that the bacteria used to remove H_2S should have are the following : ability to convert H_2S to S^o, low nutrient requirement, low biomass accumulation, and high resistance to fluctuation in pH, temperature, moisture, polluting load, and O_2 [6].

C. Effect of Bacteria Population with BOD

Figure 3 shows the correlation between BOD of substrate and bacterial population in four bioreactors. Figure 3 a-d showed that in bioreactor A and bioreactor D, bacteria population increased slowly since the beginning of the process along with the decreasing of substrate concentration (BOD). This is happened because the substrate (stillage) contain alcohol or phenol, which has anticeptic ability for bacteria population. However, in bioreactor A and B, bacteria population were more than in bioreactor C and D. In bioreactor A and B with the addition of *Acidithiobacillus thioxidans*, which this bacteria are very suitable for operating conditions (acidic ph condition) and have ability to convert H_2S into So as energy sources rather than bioreactors C and D with the addition of *Pseudomonas putida*.

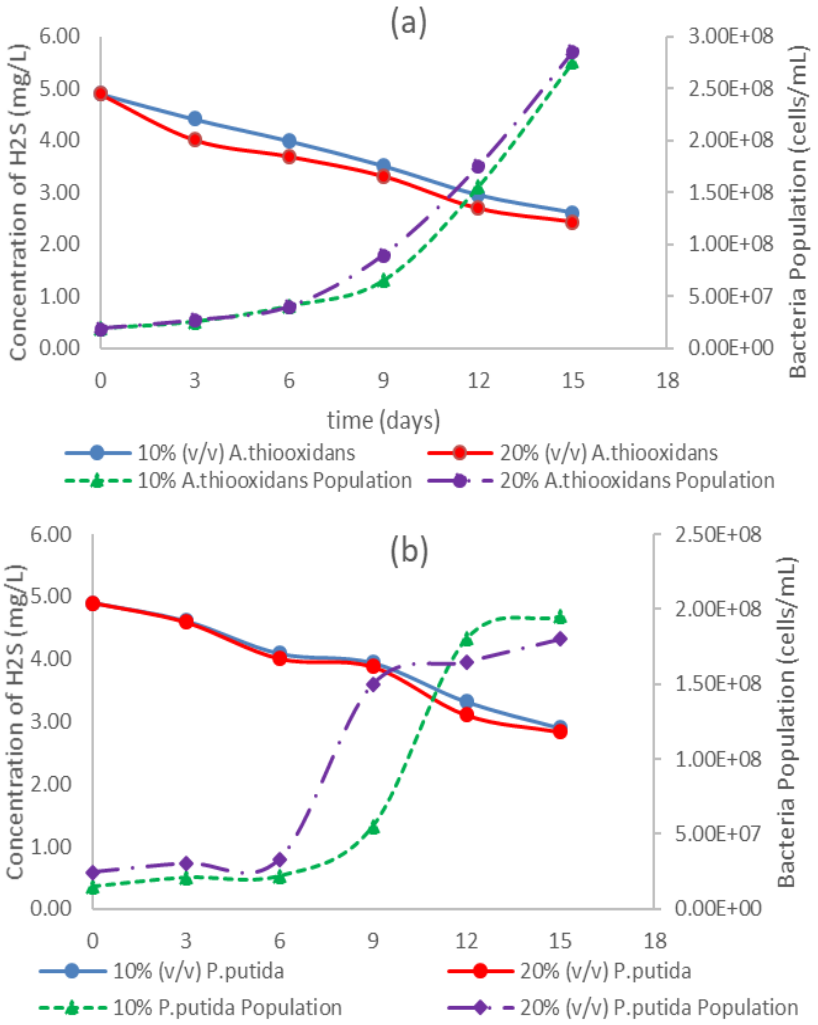
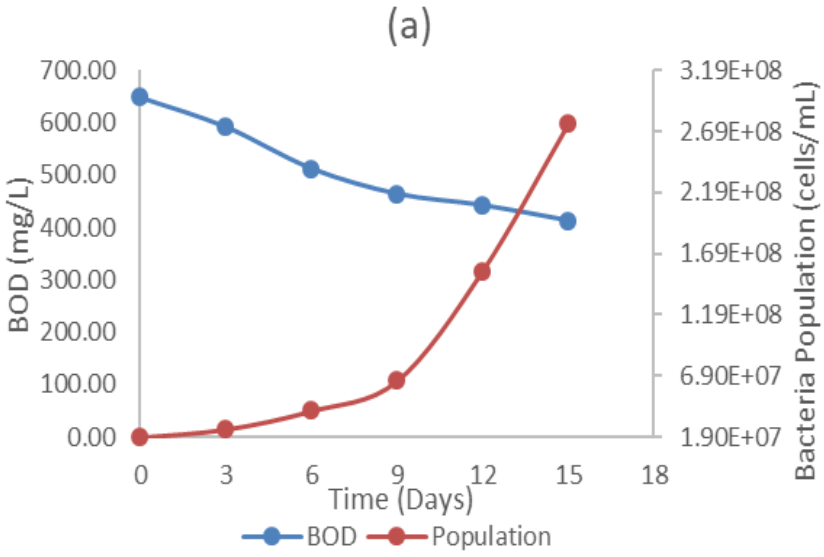


Figure 2. The Correlation of H_2S Concentration and Bacteria Population to Time (days) with addition of (a) *Acidithiobacillus thiooxidans* and (b) *Pseudomonas putida*



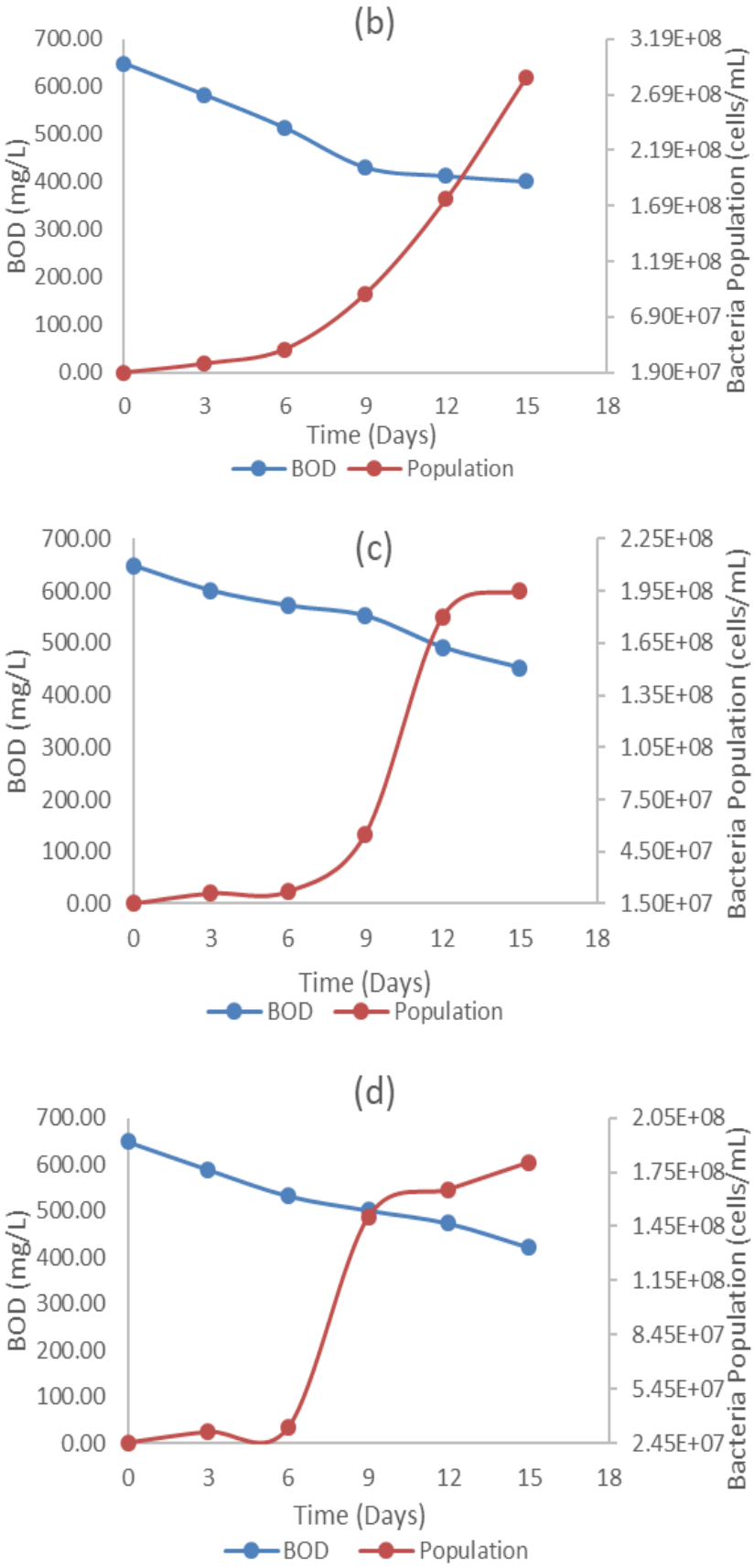


Figure 3. Correlation between BOD and bacteria population in bioreactors with addition of (a) 10% (v/v) *Acidithiobacillus thiooxidans*; (b) 20% (v/v) *Acidithiobacillus thiooxidans*; (c) 10% (v/v) *Pseudomonas putida*; and (d) 20% (v/v) *Pseudomonas putida*

IV. CONCLUSION

From the research that has been done, it could be concluded as follows :

1. Stillages containing sulfur above the specified threshold required further treatment before being released into the environment.

2. Stillage was contained alcohol which has anticeptic ability for bacteria, so it can be slowing the bacteria growth on biofilm
3. Type and concentration bacteria affects the rate of H_2S degradation. Best result of H_2S degradation occurred in 20% (v/v) of Acidithiobacillus thiooxidans addition with H_2S removal efficiency of 50.41% for 15 days operation.

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