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Effects of Nutrients on the Performance of the Biological Sulfur Recovery Unit for Sulfur Removal from Water

Ronny Purwadi [1], Dessi A. E. Br Ginting [1], Anbibie Anbibie [1], Wan Hanna Melini Wan Mohtar [2], Yusrin Ramli [3] and Antonius Indarto [1,4,*]

[1] Department of Chemical Engineering, Institut Teknologi Bandung, Jl. Ganesha 10, Bandung 40132, Indonesia
[2] Department of Civil Engineering, Universiti Kebangsaan Malaysia, Selangor 43600, Malaysia
[3] Graduate School of Science and Technology, Hirosaki University, 1-Bunkyocho, Hirosaki 036-8560, Japan
[4] Department of Bioenergy Engineering and Chemurgy, Institut Teknologi Bandung, Jl. Let. Jend. Purn. Dr. (HC) Mashudi No.1, Kabupaten Sumedang 45363, Indonesia
* Correspondence: antonius.indarto@itb.ac.id

Abstract: The Biological Sulfur Recovery Unit (BSRU) with Thiobacillus as biocatalysts is believed to be suitable for handling soluble sulfur in wastewater. The purpose of this study is to evaluate the effect of nutrient (SO_4^{2-}, PO_4^{3-}, and Fe^{2+}) concentration on BSRU performance, particularly on the conversion of sulfide to elemental sulfur. This study shows that the variation of SO_4^{2-} concentration does not significantly affect the conversion process, while a small increment of PO_4^{3-} (KH_2PO_4 1.7 g/L and K_2HPO_4 1.36 g/L) results in a higher yield of elemental sulfur production. Fe^{2+} also significantly affected the formation and conversion rate of elemental sulfur.

Keywords: elemental sulfur; nutrient; sulfur recovery unit; *Thiobacillus*; wastewater

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1. Introduction

Nowadays, combating sulfur content in wastewater has become an obligation due to the toxicity of sulfur [1,2]. Most sulfur content originates from industrial activities [3,4], such as wastewater from biogas, animal husbandry, animal skin tanning, and petroleum refineries. For instance, sulfate content in municipal wastewater was estimated at around 500 ppm [5], while in the mining and petroleum industry, the discharge outlet was up to 63,000 ppm and 10,000 ppm, respectively [6]. Surprisingly, the allowable sulfate content in water is as much as 250 ppm [6]. On the other hand, in tannery industries, the amount of sulfide is about 339 ppm [7] and a high amount of sulfide can lead to instant death for humans [8], although a small content of sulfide (0.5 ppm as hydrogen sulfide) just gives an unpleasant odor. Hence, reducing the sulfur content in wastewater is a must to avoid major health problems in society.

In some companies or industries that are concerned with the environment, sulfur-containing wastewater was further treated by chemical, physical [9], and/or biological processes. Among them, the Biological Sulfur Recovery Unit (BSRU) biological process is preferable since it is superior in cost-effectiveness and efficiency [10]. In addition, the sulfur from microorganism metabolism is environmentally safe and can provide benefits as an agricultural fertilizer and raw material in industries [11]. Consequently, the BSRU has become a promising method to tackle the sulfur problem in wastewater.

In the BSRU, sulfur can be recovered by converting soluble sulfur in liquid to elemental/solid sulfur. The BSRU is an alternative technology that converts soluble sulfur to elemental sulfur via a biological pathway [12,13]. It is suitable for treating highly sulfur-contaminated wastewater since it could result in high sulfur recovery, is environmentally friendly, and is easy to implement in industry [14]. The biological sulfur recovery process

results in more than 99.9% of sulfur removal and up to 96.5% of sulfur recovery [15,16]. The operating costs are reduced because there are no expenses for extra chemicals such as redox reagents and ligands [15]. The process does not require a high temperature or high pressure, so safety is enhanced, and capital expenditures are reduced. Consequently, the advantages acquired from this process are reduced operating costs, reduced capital expenditures, ease of operation, and safer operation.

The main key of BSRU is the living biocatalyst which is mostly microorganisms of the genus *Thiobacillus*. The microorganisms oxidize the S^{2-} ion that is present in the wastewater to S^0 to balance its internal redox reaction [12,17]. To perform the redox reaction, the nutrient has specific and important effects and it has been reported that some nutrient components are important in the biological conversion of H_2S [18–20]. A recent study shows that *Thiobacillus* grows on a medium containing sulfate, phosphorus, ferrous, and trace elements [21]. This recipe is widely used in cultivating *Thiobacillus* and includes the use of a mixed solution of metals, such as ethylenediamine tetraacetic acid (EDTA), zinc, calcium, manganese, iron, molybdenum, copper, and cobalt. Phosphate is utilized as a phosphor source to form adenylate phosphate (AMP, ADP, and ATP) as the supporting component in metabolism. Ammonium chloride is utilized as a nitrogen source, while magnesium and trace elements are required as a cofactor in enzyme systems.

Some researchers have reported that phosphate concentration in the growth medium contributes to the metabolism rate of microorganisms in the growth medium [20,22,23]. The phosphate concentration may also affect the oxidation of sulfide to elemental sulfur [24–26]. Nevertheless, few papers discuss other components, such as Fe^{2+} and the correlation between sulfate and phosphate concentration. Thus, this study aims to investigate the effect of SO_4^{2-}, PO_4^{3-}, and Fe^{2+} on the biological removal of hydrogen sulfide by oxidation into elemental sulfur. These nutrients are considered common components in wastewater [27–29], so the result can be easily interpreted on a commercial scale and increase the economic benefits since the needs of components can be minimized. Moreover, the nutrients have essential roles as coenzymes. The main novelty of this research is analyzing the influence of sulfate, phosphate, and ferrous rigorously on the formation of elemental sulfur, sulfide conversion, and the growth of *Thiobacillus*. In detail, sulfate is needed as a constituent of proteins, and phosphorus is used as a building block of nucleic acids, nucleotides, and phospholipids. In addition, iron plays a regulatory role and is present in ferredoxin and cytochrome [30]. Moreover, this research was also conducted at limited oxygen conditions to prevent sulfate formation; hence, elemental sulfur can be produced as the main product in this study which is evidenced by the low sulfate formation (4%) in this study.

2. Materials and Methods

2.1. Materials

Thiobacillus consortium was obtained from a commercial BSRU plant in Donggi, Indonesia and it was cultivated in Starkeya novella medium with some nutrient variations. The chemicals for the medium preparation consisted of K_2HPO_4, KH_2PO_4, NH_4Cl, $MgSO_4.7H_2O$, $Na_2S_2O_3.5H_2O$, Na_2-EDTA, $ZnSO_4.7H_2O$, $CaCl_2$, $MnCl_2.4H_2O$, $FeSO_4.7H_2O$, $(NH_4)_6Mo_7O_{24}.4H_2O$, $CuSO_4.5H_2O$, EDTA, KOH, $MgCl_2.6H_2O$, $NaHCO_3$, Na_2S, yeast extract, and distilled water.

2.2. Medium Preparation

The base medium used for the cultivation was the modified Starkeya Novella Medium No. 69 from DSMZ®. In brief, solutions A, B, C, and trace metal are presented in Table S1. To make the trace metal solution, a certain procedure should be followed. EDTA was dissolved first then the pH of the solution was adjusted to 7.0 using 2 N of KOH. Next, the remaining compound was added, and the pH was adjusted to 6.0. Each solution was sterilized separately by autoclaving at 121 °C. When cooled to room temperature, the solution was mixed with a proportional volume in a biological safety cabinet (BSC) using

a micropipette and sterile tips. The pH of the final medium was adjusted to pH 8 with sterile 0.5 N KOH.

On the other hand, the source of energy of *Thiobacillus* was the inoculation medium and the acclimatization medium. For the acclimatization medium, the composition of solution C in the base medium is substituted by the sodium sulfide and is described in Table S2. The composition of the rest solutions was the same as the inoculation medium. The fermentation medium is modified to make the sulfide conversion to elemental sulfur process more favorable than the synthesis of the cell. The recipe for the fermentation medium is briefly tabulated in Table S1. Solutions B, C, and D are phosphate, ferrous, and sulfate solutions, respectively. The compound of the solutions can be multiplied by 0, 1, 2, 3, and 4 to make the desired medium variation. The fermentation medium is made by mixing each solution from one per tenth of the initial volume.

2.3. Cultivation

The cultivation procedure was divided into three stages, which were inoculation, acclimatization, and fermentation. The cultivation process was conducted in the BSC to avoid contamination throughout the process. Inoculation was carried out in a 1 L Erlenmeyer flask with a 400 mL working volume, while acclimatization was carried out in an Erlenmeyer flask of certain size and working volume, as shown in Table S2. *Thiobacillus* was cultured in the medium using a 20% inoculum. Cultivation was carried out in a shaker incubator at 33 °C and 150 rpm and the pH of the initial medium was 8.0.

The microorganism consortium growth curve was analyzed before conducting the experiment. The growth curve was determined by using turbidity change over time. The growth curve assessment was to obtain the growth rate constant of *Thiobacillus* consortiums used in the experiment.

2.4. Preliminary Assessment

In the preliminary assessment, key nutrient concentrations were varied as presented in Table 1. The sulfate (SO_4^{2-}) concentration basis was selected based on the sulfate discharge concentration of the common Biological Sulfur Recovery Unit (BSRU) applied in industry. The concentration of each nutrient was adjusted from the fermentation medium composition (Table S2) as control variation and multiplied by 0, 2, and 3 to find out the effects of the nutrient. Then, 20 mL of acclimatization broth was mixed with 80 mL of sterile fermentation medium in a 300 mL Erlenmeyer flask. The pH was adjusted to 8.0 with sterile 0.5 N NaOH. The broth was cultivated for 48 h. The turbidity of the broth was analyzed at 0, 2, 3, 4, 6, and 8 h, while the pH was analyzed at 0, 2, 3, 4, 6, 8, 20, 24, and 48 h.

Table 1. Concentration variation of PO_4^{3-}, SO_4^{2-}, and Fe^{2+} for preliminary assessment.

Run	Nutrient (g/L)			
	PO_4^{3-}		SO_4^{2-}	Fe^{2+}
	KH_2PO_4	K_2HPO_4	Na_2SO_4	$FeSO_4 \cdot 7H_2O$
A1	0	0	0.09	0.05
A2	1.70	1.36	0.09	0.05
A3	3.40	2.72	0.09	0.05
A4	5.10	4.08	0.09	0.05
B1	1.70	1.36	0	0.05
B2	1.70	1.36	0.09	0.05
B3	1.70	1.36	0.18	0.05
B4	1.70	1.36	0.27	0.05
C1	1.70	1.36	0.09	0
C2	1.70	1.36	0.09	0.05

C3	1.70	1.36	0.09	0.10
C4	1.70	1.36	0.09	0.15

Note: A—phosphate nutrient is varied while sulfate and ferrous are constant; B—sulfate nutrient is varied while phosphate and ferrous nutrients are constant; C—ferrous nutrient is varied while phosphate and sulfate are constant.

2.5. Immobilized Cells

In order to improve the performance and flexibility of fermentation [31,32], the cell was immobilized by using a 6% Ca-alginate matrix. As a support, Ca-alginate is superior in biocompatibility, simplicity, availability, and low cost [33]. A mixture of 150 mL of sterile sodium alginate solution with 60 mL of acclimatization broth was prepared. The mixture was then added to the sterilized 1.5 L of 45 g/L $CaCl_2$ solution using a peristaltic pump and the mixture was stirred for 2.5 h to ensure the complete hardening of the Ca-alginate matrix. The immobilized cells were cultivated for 24 h using a final acclimatization medium at 20% immobilized cells. Lastly, 20 mL of the immobilized cells were added to the medium to make 150 mL of working volume. Additionally, to find out the effects of the nutrients, the variations were formulated by analyzing the preliminary assessment results (Section 2.5.) in the experimental result section. Then, the variations were reformulated following Table 2 to determine the effects of the nutrients on the sulfur conversion.

Table 2. Variations of nutrient concentrations.

Run	Nutrient (g/L)				Alginate Beads * (Immobilized Cells)
	KH_2PO_4	K_2HPO_4	$FeSO_4{\cdot}7H_2O$	Na_2S	
1	0	0	0.05	0.89	20 mL/150 mL
2	1.70	1.36	0.05	0.89	20 mL/150 mL
3	0	0	0.15	0.89	20 mL/150 mL
4	1.70	1.36	0.15	0.89	20 mL/150 mL
Blank	1.70	1.36	0.05	0.89	-

Note: * Fermentation was conducted in 150 mL of the working volume and 20 mL of alginate beads.

2.6. Sample Analysis

The analysis consisted of measurements of cells, sulfur, sulfide, sulfate concentration, and pH broth. Cells and sulfur concentration were measured using dry-weight analysis and measurements of particle density. Sulfide concentration was measured using the iodometry method while sulfate concentration was measured by its turbidity as barium sulfate. Dry-weight analysis, sulfide, and sulfate analysis were performed at the start and the end of the cultivation (t = 0 and t = 6 h) while turbidity was performed every hour for 6 h. The bacterial concentration in the alginate beads was measured by gravimetrics. One milliliter of beads was dissolved in a 9 mL solution containing 0.10 M of Na_2CO_3 and 0.04 M of citric acid by vigorous mixing for 5 min.

2.6.1. Dry-Weight Analysis

As much as 10 to 15 mL of cultivation broth was centrifuged using a 15 mL Falcon tube for 15 min at 4 °C and 6000 rpm. After centrifugation, the supernatant formed was then discarded from the Falcon tube. The precipitated biomass was dried at 50 °C in the oven for 1–2 days and then placed in a desiccator for 1–2 days. The dried residue was then weighed.

2.6.2. Measurement of Particle Density

The flasks were transferred from the shaker incubator and allowed to rest for 1 min to create a uniform sampling condition. Then, 2 mL of the broth was sampled using a micropipette and diluted with demineralized water to 10 mL of the final solution. The turbidity was measured with UV–Vis at 575 nm against demineralized water as a blank solution.

2.6.3. Determination of Sulfide Concentration

Methods for sulfide analysis were obtained from the Standard Methods for the Examination of Water and Wastewater [34]. The above method was scaled down to one-tenth of the sample volume and modified following former research [35]. The method was further modified based on the experiment to achieve the smallest error of mass balance in this study. The fermentation broth was centrifuged at 6000 rpm and 25 °C for 5 min to separate the precipitates. Then, 5 mL of the supernatant was transferred to another tube. Next, 1 mL of 2 N zinc acetate and 0.5 mL of 6 N NaOH solution were added to the tube to form zinc sulfide precipitates. The basic condition (addition of NaOH) was made to ensure the sulfide ion takes form as S^{2-} rather than HS^- which would, otherwise, not form a precipitate with zinc. The tube was centrifuged at 6000 rpm, 25 °C for 4 min to separate the precipitates from the solution. The precipitates were transferred to a flask, then 5 mL of standard iodine solution (0.025 N) was added. As much as 2 mL of 6 N HCl was slowly dropped into the solution to dissolve the remaining precipitates. The solution was back-titrated with 0.0250 N $Na_2S_2O_3$ with a starch solution as an indicator.

2.6.4. Determination of Sulfate Concentration

The turbidimetry method depends on the fact that barium sulfate is formed following barium chloride in addition to a sample tending to precipitate in colloidal form, and this tendency is enhanced in the presence of an acidic buffer (consisting of magnesium chloride, sodium sulfate, sodium acetate, and acetic acid [34]). This is a very rapid method and can be used for samples with sulfate concentrations of up to 10 mg/L. Thus, 5 mL of the sample was diluted into 100 mL to minimize the interference of other precipitates such as sulfur and phosphate salts. The solution was mixed with 15 mL of buffer and stirred at 150 rpm. Then, 0.1 g of barium chloride was added to the stirred solution. The turbidity was measured right after one minute of stirring at 420 nm.

3. Results and Discussion

3.1. Primary Evaluation of Cell Growth

The growth of *Thiobacillus* was evaluated by measuring cell mass concentration by determining the cellular dry weight and light absorption by suspended cells in sample culture media. Because of the autotrophic lifestyle, the biomass yield of *Thiobacillus* was very low [36]. Consequently, the produced elemental sulfur as a primary metabolite has more impact on the measured absorbance than the cells. Additionally, the measured absorbance of the culture media was significantly eventuated from elemental sulfur.

As elemental sulfur is the primary metabolite, its concentration in the medium is eligible to be used to represent cell concentration. The suspended matter concentration from the fermentation stage is shown in Figure 1a. The growth characteristics of *Thiobacillus* followed several phases, namely, (1) the lag phase, (2) the exponential phase, (3) the stationary phase, and (4) the death phase. The lag phase in the inoculation took place in the first hour of the inoculation and continued by an exponential growth phase that took place for the next two hours. After 3 h, the stationary and death phases occurred. The death phase of *Thiobacillus* in the growth curve was not clearly shown as the dead cells were also measured in the gravimetric and spectrophotometric methods. Therefore, total cell mass concentration may stay constant, but the number of dead cells may increase [30].

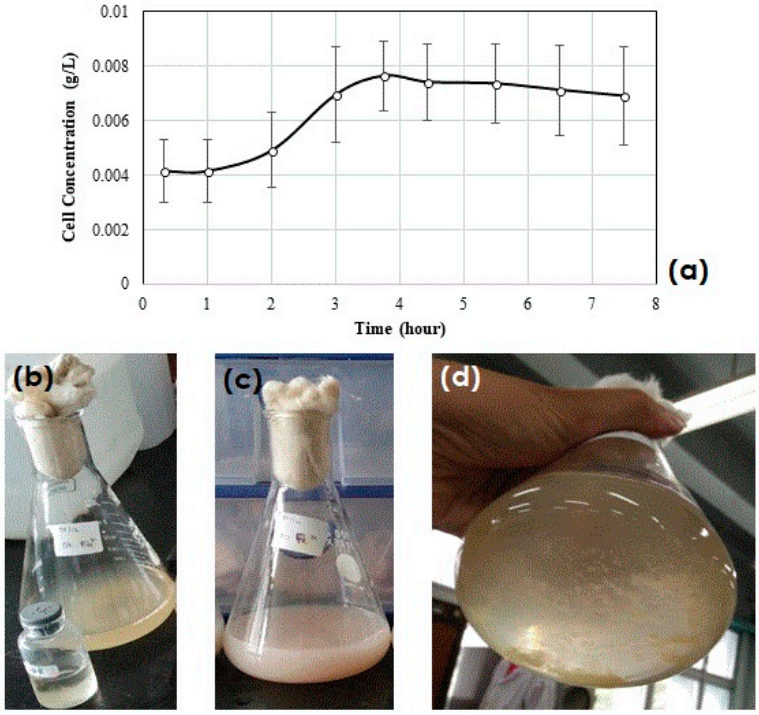

Figure 1. Experimental results of the sulfur recovery: (**a**) Thiobacillus growth curve, (**b**) initial condition, (**c**) cell formation peak of fermentation (4–6 h), (**d**) solid sulfur precipitation at the end of fermentation (>20 h).

This experiment discovered the specific rate of growth in the exponential growth phase. The specific growth rate of the inoculum was around 1.15–1.20 h^{-1}. The doubling time (τ_d) of the inoculum was 0.57–0.60 h. These growth parameters were used for the sampling schedule in the fermentation. Compared to other studies, the specific growth rate from microbes in biological sulfur recovery may vary from 0.04 to 0.20 h [37,38] and the doubling time is between 2.85 and 17.2 h [38–40]. The rapid growth of *Thiobacillus* in this study can be caused by the availability of sulfur components as energy sources [41]. Regarding the results, *Thiobacillus* cultures in this research have compatibility with the medium and can give economical benefits since the growth duration is relatively shorter.

3.2. Influence of Nutrients on the Cell Growth

3.2.1. Evaluation of Turbidity in the Medium

The nutrient effects were assessed by using phosphate, ferrous, and sulfate ions at four levels (variations) of concentration. The sulfur compound is insoluble in water; thus, precipitation of the produced sulfur occurs in the solution. The precipitation of sulfur caused the reduction of the measured sulfur as the absorbance of the solution. When the rate of sulfur precipitation is higher than the sulfur production rate, the absorbance might decrease. The transition between the increment and decrement of absorbance would be seen as the peak of the sulfur component in the solution. The time needed to reach the peak was used to represent the rate of the process of sulfide to elemental sulfur conversion. Bacteria grow on sulfide as an energy source, producing sulfur as the bacteria grow. The broth turbidity increased as sulfide was converted to elemental sulfur.

As shown in Figure 2a, the maximum absorbance was reached after 1.5 h for variations A2, A3, and A4. Nonetheless, the maximum absorbance for the no phosphate variation (A1) was reached after 4 h; also, the maximum absorbance in A1 variation is the lowest (0.35), as depicted in Figure 2a. It is clear that phosphate is essential in bacteria growth, although at lower concentrations, the time required to reach the highest absorbance is longer. Obviously, at higher phosphate concentrations, the bacteria grow faster. However,

there is practically no difference in the time needed for reaching the maximum absorbance in phosphate variations A2, A3, and A4 which are recorded to be as high as 0.38, 0.38, and 0.40, respectively.

As presented in Figure 2b, the ferrous effect has the same patterns as phosphate variation. In short, the ferrous component can accelerate the growth rate of bacteria simultaneously and a significant gap of maximum absorbance occurred in each variation. Nonetheless, variations B1 and B2 have a similar duration (about 3 h) to reach the maximum absorbance, and variations B3 and B4 have a similar duration (about 1.5 h). By some considerations such as the roles of the ferrous component in cell growth and metabolism, variation B2 was chosen as a low ferrous variation, while variation B4 was chosen as a high ferrous variation.

On the other hand, Figure 2c does not show a significant change in maximum absorbance and the time needed to reach maximum turbidity since each medium turbidity is relatively unchanged, but in higher sulfate variations, it shows a slight drop in maximum absorbance. In detail, the maximum absorbance in variation C1 is about 0.37, but those of variations C2, C3, and C4 are 0.35. Moreover, the time required to reach the highest absorbance for each variation is similar, being about 1.5 h. Briefly, the additional sulfate as a nutrient was not needed in the immobilized cell experiment in this study.

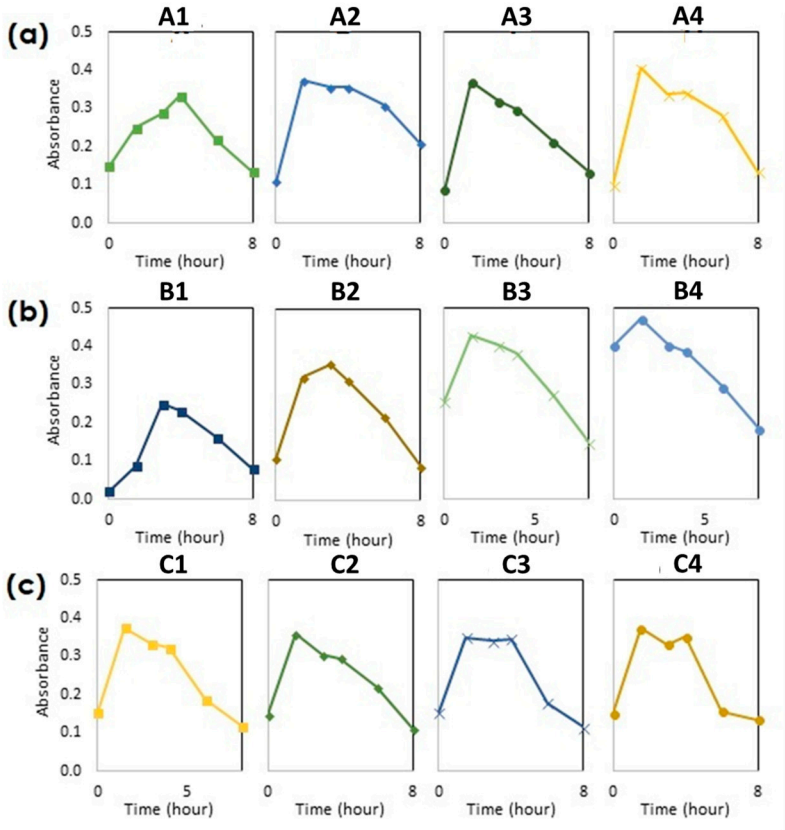

Figure 2. Turbidity changes during fermentation time for different nutrients: (**a**) phosphate, (**b**) ferrous ion, and (**c**) sulfate.

3.2.2. Evaluation of Liquid Acidity

The graphics for the pH trend at phosphate nutrient variation can be seen in Figure 3a. It is confirmed that sulfide biological oxidation by *Thiobacillus* was divided into several stages. In the first stage, sulfide was biologically oxidized to sulfur. The pH increased for the first 3 h when the conversion rate of sulfide to elemental sulfur was much higher than the sulfate formation rate. Precisely after the first peak, the pH tremendously declined as sulfate formation took over the elemental sulfur formation. Besides, as the prominent

growth substrate (sulfide) was used up, *Thiobacillus* made its way to utilize thiosulfate. The utilization of thiosulfate generated sulfate compounds which also greatly dropped the pH. The time preceding the first peak of the pH is employed as the time range for observing and assessing the sulfide-to-elemental conversion process. After 24 h of fermentation, fermentation broths were notably clearer and some precipitates at the bottom of the flasks were observed in Figure 1d. The precipitate's color was yellow to reddish-brown. It is depicted that the precipitates consist of sulfur and biomass.

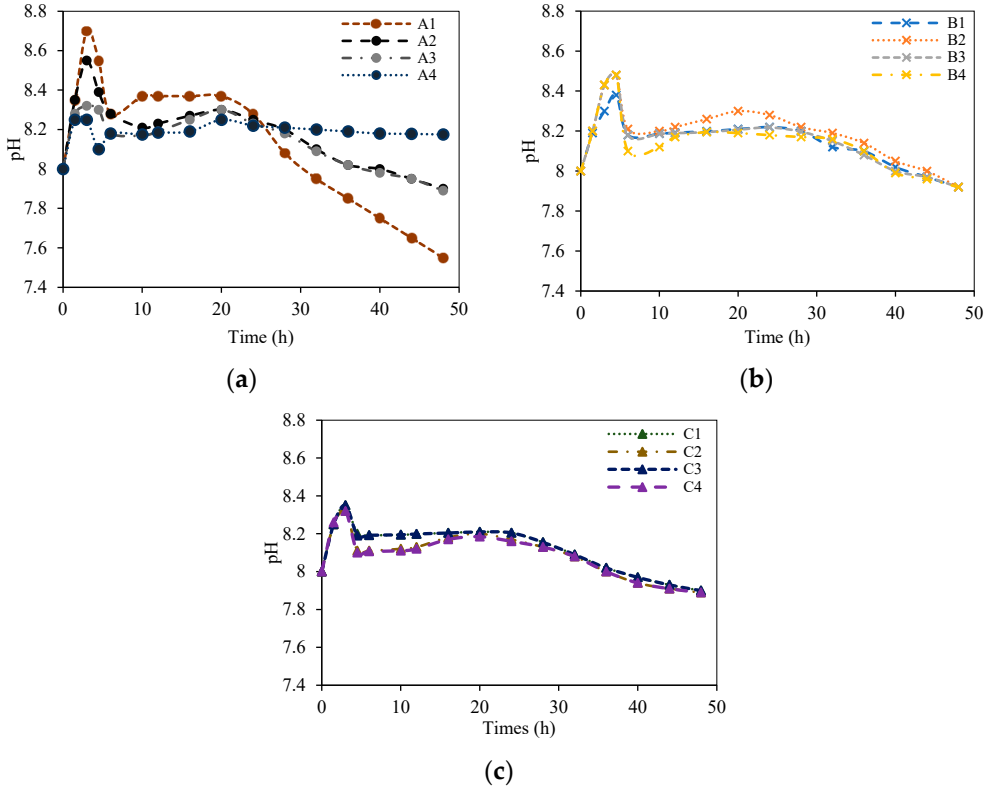

Figure 3. Trend of pH during fermentation time for different nutrients: (**a**) phosphate, (**b**) ferrous ion, and (**c**) sulfate.

As shown in Figure 3a, at various PO_4^{3-} concentrations, the variation in pH values was relatively high. This has to do with the buffer capacity that the phosphate compound delivers to the inoculation solution. The lesser the amount of phosphate compound added to the solution, the lower the buffer capacity. The solution with the least phosphate ended up with the widest pH deviation (the highest was 8.7 and the lowest was 7.6) from the initial pH (pH = 8.0), while a solution with the greatest phosphate concentration ended up with the least pH deviation from the initial pH. Although the pH reached 8.7 for a while, the cells could still grow, since the pH range of *Thiobacillus* to oxidize sulfide is between 1 and 9 [42]. Therefore, it can be concluded with a higher phosphate concentration, the solution pH could be maintained better.

In contrast to the profile obtained from PO_4^{3-}, varying Fe^{2+} and SO_4^{2-} concentrations in the medium did not significantly affect the pH of the solution. As evidence, the pH peak in ferrous and sulfate variations was around 8.3–8.5; however, it gradually declined to around 8.0 at the end of fermentation time. The trend of the pH value in both Fe^{2+} and SO_4^{2-} variation solutions showed the typical characteristic with two peaks and without pH deviation in the variation, as illustrated in Figure 3b,c. Figure 3b explains that the ferrous ion does not contribute significantly to the pH change, but, interestingly, the variation without ferrous ion addition shows better pH stability in the early step. Because

ferrous ion can react with sulfide to form FeS [43], which is an alkaline compound, the pH is able to increase in ferrous addition variations.

Additionally, the increasing and decreasing pH value of the medium in this study may not have a significant impact since the pH was more than 6 and less than 9. As mentioned earlier, *Thiobacillus* can oxidize at a wide range of pH (1–9), but to ensure that the chemical process does not compete with the biological process, the pH should be more than 6 [36]. Hence, the pH change in this study is acceptable since *Thiobacillus* can keep their roles.

3.3. Sulfur and Sulfate Formation by Immobilized Cells

Evaluation of the sulfur and sulfate formation was conducted by turbidity measurements and gravimetric analysis for 6 h, as mentioned in Section 2.6. As shown in Figure 4, the measured sulfide concentration of the pure sulfide solution with the same sulfide concentration in the broth was 253 mg/L in the high phosphate and high ferrous condition. This study shows that the overall conversion sulfide accounted for 84%, of which 14%, 4%, and 66% were converted to sulfur, sulfate, and other compounds (including bacteria growth), respectively.

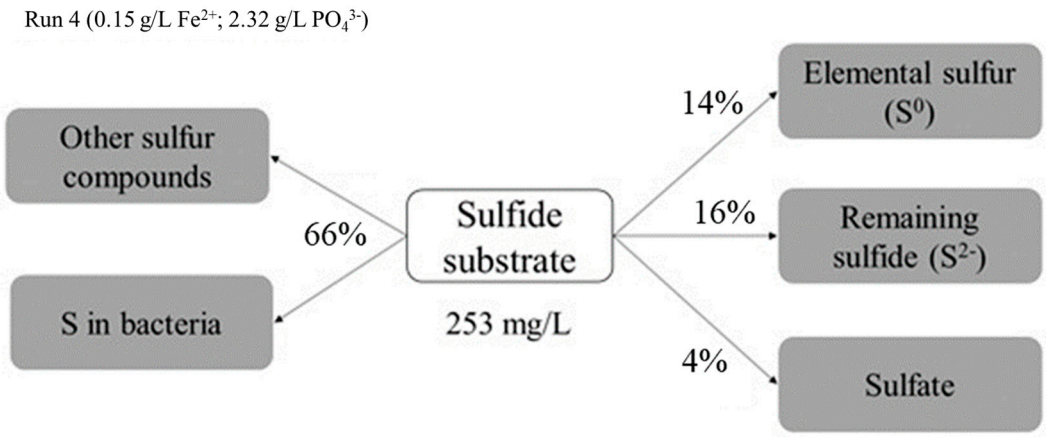

Figure 4. Schematic of the sulfur balance.

Moreover, the phosphate-containing variations have a similar initial sulfide concentration, which is lower than the no-phosphate variation's initial concentration, as shown in Table 3. Despite the similar final sulfide concentration for each variation, it is obvious that the high-ferrous variations show a slightly lower average final sulfide concentration than in low-ferrous variations. Table 3 also describes the sulfide concentration in the initial and final conditions together with the converted amount. It also shows that the initial sulfide concentration for each variation is different. As noted in Table 3, the highest initial concentration is acquired by Run 2, followed by Run 1. Most sulfide was converted in the aforementioned variations. The blank naturally contains the most converted sulfide because it has the lowest initial concentration. According to the observations, the no-phosphate variants converted more sulfide than other variants. The higher initial sulfide concentration was the main contributing factor.

Table 3. Sulfide, sulfate, and sulfur concentration in the immobilized cells experiment.

Concentration	Units	Blank	Run 1	Run 2	Run 3	Run 4
			Sulfide			
Initial	mg/L	114	180	212	135	135
Final	mg/L	49	45	47	51	41
Conversion	mg/L	65	135	165	84	94

	%	57	75	78	62	69
Sulfate						
Initial	mg/L	27	105	111	116	116
Final	mg/L	232	52	256	158	145
Formation	mg/L	206	n/a	145	42	30
	%	767	n/a	132	36	27
Sulfur						
Final/generated	mg/L	0.5	3.8	17.0	2.7	34.8

On the other hand, Table 3 also presents the sulfate concentration in the initial and final conditions together with the generated amount. Sulfate is expected to be generated, as bacteria converted sulfide into several products, which are elemental sulfur, thiosulfate, sulfite, and sulfate [17]. Contradictory to the research by Vishniac and Santer (1957), this experiment shows that phosphate has a negative effect on the oxidation of sulfide to sulfate [44]. This phenomenon can be explained by the proposed thiosulfate oxidation reaction steps [45].

Step 1: $S_2O_3^{2-} + 0.25\ O_2 + 0.5\ H_2O \rightarrow 0.5\ S_4O_6^{2-} + OH^-$ (enzymatic)
Step 2: $2\ S_4O_6^{2-} \rightarrow S_3O_6^{2-} + S_5O_6^{2-}$ (nonbiological dismutation)
Step 3: $S_5O_6^{2-} \rightarrow S_4O_6^{2-} + S^0$ (nonbiological decomposition)
Step 4: $S_3O_6^{2-} + H_2O \rightarrow S_2O_3^{2-} + SO_4^{2-} + H^+$ (nonbiological hydrolysis)
Step 5: $S_3O_6^{2-} + 2\ O_2 + 2\ H_2O \rightarrow 3\ SO_4^{2-} + 4\ H^+$ (enzymatic)

As demonstrated by the reaction steps, the oxidation of thiosulfate to tetrathionate is a biological reaction, so the higher the phosphate concentration, the faster the rate. Nevertheless, the last step which produces sulfate requires oxygen. Considering that the fermentation took place in limited oxygen conditions, step 5 of the reaction might be slower. Therefore, the second to fourth steps would be more favorable, resulting in more elemental sulfur than sulfate. In contrast, it is unable to account for Run 3's higher sulfate production than the phosphate-containing variation. It is too complex to deduce an acceptable explanation from the way the medium's constituent parts interact. Since less sulfate was produced, it is only reasonable to conclude that phosphate-containing media are better for the conversion of sulfide to sulfur.

Run 1 depicts a result that is incongruent with other variations. Run 1 which had lower ferrous and no phosphate concentrations lost sulfate rather than generating it. This result is in agreement with the control result, as the control has a similar composition to Run 1. Thereby, it can be assumed that there was another interference in the sulfate analysis as it is hardly possible for *Thiobacillus* to convert sulfate into a more reduced compound. The medium contained a considerably high concentration of calcium ions, 0.02 M, which can form precipitates with sulfate. Hypothetically, calcium was released from the beads during the fermentation due to the decrease in the sulfate ions concentration, hinting there were more calcium sulfate or other sulfate salts precipitating during the fermentation. Consequently, Run 1 is less preferable than the other variations.

In industry, a high elemental-sulfur-to-sulfate molar ratio is more desirable as it is able to produce more products (elemental sulfur) and fewer wastes (sulfate salts). In addition, there will be less additional post-treatment to separate sulfate and sulfur. Therefore, the elemental-sulfur-to-sulfate molar ratio was calculated and is tabulated in Table 4. It is clear that at a high phosphate variation, the molar ratio is higher than in the no-phosphate variation. Run 4 resulted in the highest molar ratio, which is 3.5.

Table 4. Experimental results for the immobilized cells experiment.

Parameter	Units	Run 1	Run 2	Run 3	Run 4
Sulfur formation rate	-	0.78	7.35	0.65	8.75
Final sulfide	mg/L	45	47	51	41

Sulfide conversion	%	82	80	81	84
Sulfur-to-sulfate molar ratio	-	n/a	1.2	0.06	3.5

Furthermore, as depicted in Figure 5, the absorbance tends to increase in all solutions, though at different increment rates, with the blank solution having the lowest rate of sulfur production. From Figure 5, the linear gradient from the sulfur graph is made to obtain the rate of sulfur production, as tabulated in Table 4. Interestingly, Table 4 describes that Run 2 and Run 4 have high sulfur formation rates, being 7.35 and 8.75, respectively. In order to determine the phosphate effect, solutions Run 1 and Run 3 were compared to Run 2 and Run 4. In Table 4, it is shown that Run 2 and Run 4 generally have approximately 10 times higher sulfur production rates than Run 1 and Run 3, indicating a positive influence of phosphate on sulfur production by *Thiobacillus*. Phosphate also has a prolonged effect on the sulfur production time. By comparing Run 1 and Run 3 with Run 2 and Run 4 in Figure 5, a solution with high phosphate concentration exhibits a longer time of increasing absorbance before decreasing, compared to a solution with low phosphate concentration.

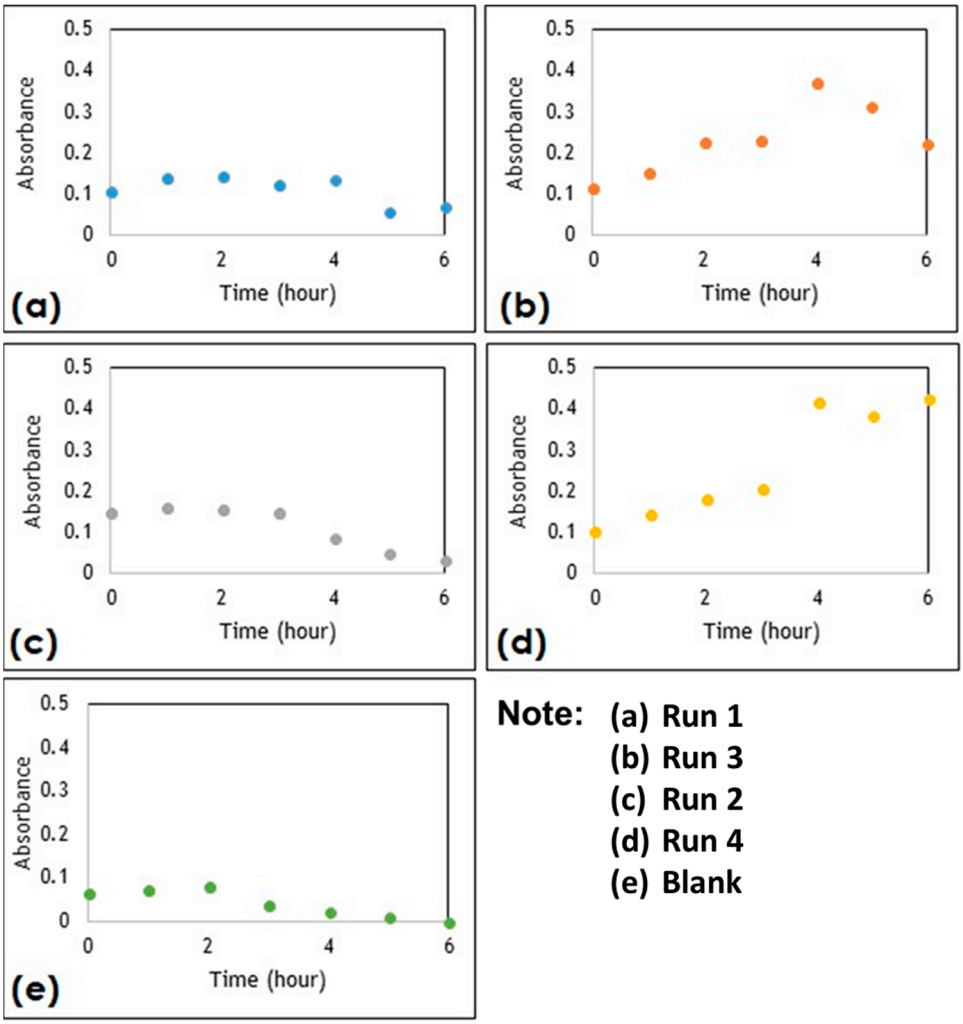

Figure 5. Turbidity changes for each variation and the blank in calcium alginate beads.

On the other hand, the ferrous effect in the solution is assessed by comparing Run 2 and Run 4. Conforming to Table 4, Run 4 has a slightly higher sulfur formation rate compared to Run 2. This is because ferrous ions acted as a catalyst by forming ferric ions that can be used by *Thiobacillus* in oxidizing sulfide ions to elemental sulfur [46]. In gravimetric analysis, the weighed solids were sulfur precipitates because *Thiobacillus* cells were

already entrapped in alginate beads and no longer interfered with the gravimetric analysis. The positive results of dry-weight analysis proved that the produced sulfur is extracellular sulfur or non-membrane-associated sulfur. As shown in Tables 3 and 4, the most satisfactory result of elemental sulfur production is found in the Run 4 solution. The sulfur concentration in the Run 4 solution was up to 35 milligrams/mL followed by the Run 2 solution being 18 mg/mL.

Table 5 presents the comparison of this study to other studies. The performance of *Thiobacillus* in Run 4 is still acceptable since another study using *Thiobacillus* generally has lower sulfur formation (lower by >10%) and sulfide conversion. However, the studies which are conducted with sludge show better performance, as evidenced by the sulfide conversion being able to be more than 90% and sulfur formation being up to 90%. This might happen since the sludge has not only sulfide-oxidizing bacteria, but also sulfate-reducing bacteria [47]. Therefore, after oxidizing the sulfide to sulfate, the sulfate can be converted to sulfur.

Table 5. Sulfur removal study performance from several studies.

Consortium	Formed Sulfur (%)	Formed Sulfate (%)	Sulfide Conversion (%)	Ref.
Sludge *	58.9–90.5	-	96.41	[48]
Active sludge	50.0–90.0	50–90	90.0	[49]
Thiomirospia	-	-	68.4–94.7	[50]
Thiobacillus	0.0–3.7	9.0–60.5	58.0–86.0	[8]
Arcobacter	60	17–19	~100	[29]
Thiobacillus	14.0	4.0	84.0	This work

Note: * The sludge mainly consists of *Sulfurovum* and *Sulfurimonas.*

4. Conclusions

The influence of nutrients on the performance of the sulfur conversion process, i.e., the sulfur yield, the rate of sulfur production, the final sulfide concentration, and the elemental sulfur to sulfate molar ratio, was investigated. Referring to the above discussions, the addition of phosphate and ferrous nutrients was proved to result in the fastest sulfur production rate. Moreover, it also produced the highest sulfur precipitates in the final solution, the lowest final sulfide concentration, and the highest elemental sulfur-to-sulfate molar ratio. Therefore, Run 4, i.e., a high phosphorus and ferrous nutrient concentration variation, was selected as the best variation among other variations. In this experiment, the sulfur yield for the fermentation was ca. 0.14 g/g. The sulfide conversion of the fermentation could be increased up to 84% for 6 h of fermentation with an elemental sulfur-to-sulfate molar ratio of 3.5. Eventually, this study discovered another option for the BSRU process and contributed to the sustainability of the sulfur recovery system.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w15030530/s1, Table S1: Medium preparation compositions; Table S2: Solution C composition for the acclimatization medium.

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