The Potency of Purple Sulphur Bacteria for In-Situ Sulphide Bioremediation in Water

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Abstract. Sulphide is odorous water pollutants and is also toxic in the form of H2S. Generally, water with high sulphide content has a very low amount of dissolved oxygen. Purple sulphur bacteria can be used as in-situ waters bioremediation agents to overcome sulphide pollution, one of which comes from the decomposition of organic compounds. This study aimed to determine the potency of purple sulphur bacteria isolated from various water located in the Cibinong area, Bogor Regency in West Java Indonesia to remove sulphides. The Pfennig 2 media was used for culturing the isolate. In order to get a single colony of bacteria, isolation was carried out using multilevel dilution method under anaerobic conditions. From this study, seven isolates of purple sulphur bacteria could be isolated. All isolates were able to remove sulphide from the test medium with 6% and 12% of sulphide. The highest sulphide removal ability was found in CU1 isolate followed by L1 and L2. The growth of CU1 and L2 in Kali Item River water could be detected within 17 hours. Thus, CU1 and L2 isolate had the potency to be used as an in-situ bioremediation agent for sulphides in waters with low oxygen content because of its anaerobic nature suitable for such environment.

Keywords: water pollution, sulphide, anaerobic water, bioremediation, purple sulphur bacteria

1. Introduction
Sulphides in the form of H2S in waters are toxic compounds whose one of the sources is the result of decomposition of organic matter. A number of water bodies in Indonesia, especially in large cities, experience pollution of organic materials including H2S, which is characterized by blackened colours and smelly water that disturb the surrounding environment. The research conducted by the Research Center for Limnology LIPI in 2009 found that the increase in H2S levels in the waters of Lake Maninjau was one of the causes of mass fish mortality [1]. H2S pollution in Kali Item which caused a strong odor became a national issue when the Kemayoran Athletic Housing located near the river was used by athletes participating in the last 2018 Asian Games [2]. Purple sulphur bacteria is one of the bioremediation agents of sulphide because through its anoxygenic photosynthetic activity H2S is used as a source of electrons to fix CO2 so that at the end of
photosynthesis oxygen is not produced but nontoxic elemental sulphur[3,4]. In this kind of photosynthetic process sulphide, which is a form of reduced sulphur, acts as a source of energy and reducing energy [5]. Several studies have shown that anoxygenic photosynthetic bacteria in the form of a consortium with several other microorganisms can improve water quality in previously polluted waters [6,7].

This study aimed to determine the potency of purple sulphur bacteria isolated from various water located in the Cibinong area, Bogor Regency in West Java, Indonesia to remove sulphides including toxic H₂S. Kali Item River water is used as a test medium to determine the potential of selected purple sulphur bacteria in reducing sulphide concentrations in open water.

2. Methods

2.1. Isolation and Characterization of Isolate

The research was conducted in Cibinong, Indonesia. Sources of isolates were taken from the surface and bottom of the eel pond, catfish ponds and four small lakes (Situ Cibuntu, Situ Cilalay, Situ Cibinong and Situ Gedong) (Figure 1). Situ Cilalay is a small lake that has a spring in the center, does not receive water from the outside, and is pristine because it does not receive input from anthropogenic activities [8]. Situ Cibuntu and Situ Gedong are located near agricultural areas and receive intake from anthropogenic activities. Situ Cibinong is located at the edge of a highway and close to the people's market so that it often gets waste and wastewater intake, especially when it rains. The difference between the catfish and the eel pond was that eel ponds used a biofloc system while a catfish pond was a conventional pond without biofloc usage. Before taking water samples, the bottles for water storage were sterilized using autoclave at 121°C for 15 minutes. The sample was preserved by cooling in the refrigerator prior to use.

![RESEARCH LOCATIONS](image)

**Figure 1.** Sampling site locations at Cibinong Bogor, West Java Province

The isolation of purple sulphur bacteria was initiated by bacterial enrichment using Pfennig 2 liquid media [9] with modification of 0.25% NaCl salinity. As much as 1 mL of sample was put into a sterile tube containing 9 mL of Pfennig 2 media. Then 2 ml of sterile paraffin was added to each tube for anaerobic environment, after which the tube was closed tightly. Incubation was carried out at room temperature in a light enough place (200-1000 lux) for 14 days (Figure 2).

Isolates grown at the enrichment stage were purified using a multilevel dilution method [10] in liquid Pfennig 2 media. The dilution factor was ranged from 10⁻⁶ – 10⁻¹⁵. Dilution was carried out by inserting 0.5 ml of sample suspension into a tube containing 4.5 ml of liquid Pfennig 2 media solution, and then 2 mL of sterile liquid paraffin was added for anaerobic conditions. The diluted culture was incubated for 7 days at room temperature where it was sufficiently light (200-1000 lux). Culture purity was studied through staining bacterial preparations and microscopic observation at 1000x magnification. The purest cultures were then used as stock cultures for the next testing phase.
Identification was held to study the type of bacteria. Each isolate was observed including the colour form of bacterial culture, cell form and Gram test [11]. The spectral pattern test using a spectrophotometer aimed to determine the presence of bacteriochlorophylls and carotenoids which were characteristic of purple sulphur bacteria. Spectral pattern tests were carried out in the wavelength range of 300-950 nm [12]. The 3-4 days of isolates grown in liquid Pfennig 2 liquid media were taken 1 ml and then put in 4 ml of 60% sucrose solution in a test tube. The samples were stirred until homogeneous after which the absorbance values were measured in the range of waves 200-1000 nm using a spectrophotometer [9]. Isolate that have bacteriochlorophyll peak in its spectral absorbance then was used for the sulphide removal activity test and growth pattern of selected isolates.

2.2. Sulphide Removal Activity Test
The sulphide removal activity test in purple sulphur bacteria was carried out in two stages, namely the initial screening stage and the advanced test phase. Both of testing used a liquid Pfennig 2 medium with a treatment of sulphide concentration (as Na₂S.10H₂O). In the initial screening stage, the sulphide concentration was 6%, while in the advanced test the sulphide concentration used was 6% and 12%. The activity test was carried out by inserting 1 ml of 3-day-old test culture into 9 ml of liquid media Pfennig 2 with the addition of 2 ml paraffin to conduct an anaerobic condition. At the initial screening stage, the culture was incubated at room temperature for 72 hours with light radiation from 200-1000 lux. The sulphide content was observed qualitatively by plate drops technic every 24 hours using the methylene blue method. Sulphide was positive when the colour formed was blue, but there is no sulphide if the colour was yellow or clear thin red. The formed colour becomes an assessment of sulphide removal activity for strong (+++: red and yellow clear colours), medium (++: greenish yellow), or low (+: bluish) activity. The results obtained from the initial screening took the best three isolates for further testing. At the advanced test stage, the measurement of sulphide content was carried out quantitatively using the methylene blue method [13] at the initial and end of the incubation period (40 hours). At the end of test bacterial population was measured as optical density (OD) value using Hach spectrophotometer with a wavelength of 600 nm and then the sample culture was filtered using a sterile cellulose nitrate membrane (pore size 0.45 um). The 5 ml filtrate was pipetted and then 0.3 ml of amin H₂SO₄ reagent, 0.1 ml of FeCl₃ reagent, and 0.5 ml (NH₄)₂HPO₄ reagent were added respectively. The absorbance was measured at a wavelength of 664 nm and the sulphide concentration was calculated.

The percentage of sulphide removal was calculated by the following equation:

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\text{% SulphideRemoval} = \frac{(S_0 - S_1)}{S_0} \times 100\%
\]
2.3. Growth Patterns Study
Observation of the growth patterns of selected isolate was aimed to obtain data regarding the growth phases of purple sulphur bacteria. The test of the growth pattern was carried out in the following way: as much as 9 ml of liquid Pfennig 2 media were inserted into the cuvet tube. Then 1 ml of isolate was added with paraffin, the tube was tightly closed and incubate under 200-1000 lux at room temperature. Bacterial growth was observed every 2 hours through the measurement of bacterial optical density (OD) using the Hach spectrophotometer at a wavelength of 600 nm [14].

2.4. Viability Test of Selected Isolates
The viability study was aimed to determine the ability of selected isolates to grow in polluted aquatic environments. Water from two site locations of Kali Item River in Kemayoran, Central Jakarta was used as a test medium (Figure 2). The river water used as a test medium was not enriched with any material nor sterilized. The viability test was carried out by inserting 1 mL of 3 days-old test culture into 150 mL of Kali Item River water, then 5 mL of sterile liquid paraffin was added to conduct anaerobic condition. Incubation was carried out in room temperature with a lighting strength of 200-1000 lux for 24 hours. Bacterial growth was observed through the formation of red in the test culture.

3. Results and Discussions

3.1. Isolate Characteristics
The enrichment results with the incubation time of 14 days showed that purple sulfur bacteria were found in almost all sampling locations except for the bottom of catfish and eel ponds and surface water of Situ Cilalay. The presence of purple sulphur bacteria can be seen from the color change of the medium.
that was originally clear to become brownish red and pink (Table 1). According to Dworkin et al [9] the formation of purple, red, and pink in culture indicates the growth of purple bacteria.

Table 1. Appearance of cultured sample sulphide after enriched by Pfennig 2 liquid medium

| Sample          | Catfish pond | Eel pond | Situ Cibuntu | Situ Cilalay | Situ Cibinong | Situ Gedong |
|-----------------|--------------|----------|--------------|--------------|---------------|-------------|
| Surface water   | (+)          | (+)      | (+)          | (-)          | (+)           | (+)         |
| Bottom water    | (-)          | (-)      | (+)          | (+)          | (+)           | (+)         |

The isolates that could be purified from enriched culture were 7 isolates, which were 2 isolates from catfish ponds (L1 and L2), 2 isolates from Situ Cibuntu (CU1 and CU2), 1 isolate from Situ Gedong (GO), 1 isolate from eel pond (SD), and 1 isolate from Situ Cibinong (PS) (Table 2). The culture from Situ Cilalay was not successfully purified because in its development there was a shift in the bacterial community which was marked by changes in the color of the culture which was originally red to green. The pure isolates L1, L2, CU1, CU2, and GO were obtained at 10⁻⁶ dilution rates, whereas SD and PS isolates were obtained from 10⁻¹⁵ dilution rates. Higher dilutions in Situ Cibinong and eel ponds was caused by bacterial cells still mixed in dilutions up to 10⁻¹⁴ which indicates that there are more diverse sulfur bacteria in both locations.

The Gram stain test was found that all of seven isolates belonged to Gram negative (Table 2). This result was in accordance with a study conducted by Kumar et al [15], which said that most purple sulfur bacteria belong to Gram negative. The microscopic observation found that there were 4 basil-shaped isolates (L1, L2, CU1, and CU2), and 3 isolates in the form of cocobasil / oval (SD, PS and GO) (Table 2). According to Shabebet all [16] some purple sulfur bacteria have different cell forms such as cocobasil / oval, basil, and spiral with distinctive color. The morphology form of purple sulfur bacteria differs from each species and makes it characteristic for identification [17].

The spectral pattern of bacteria showed that all isolates were purple bacterial species indicated by the presence of bacteriochlorophylls. Bacteriochlorophylls possessed by seven isolates were classified as bacteriochlorophyll a. This was indicated by the presence of maximum wavelengths at 375, 595, 805,855 and 870 (Table 3). Pfennig all in Dworkins et al [9], states that a bacteriochlorophyll a has a maximum wavelength range at 375, 590-600, and 800-900 nm. Carotenoid pigment in all of the seven isolates were identified at the wavelengths of 440, 460 and 490 indicated that the pigments belonged to spirilloxanthin. There are 5 types of caroteoids as bacterial light-capturing pigments, namely lycopene, rhodopin, anhydrobodovibrin, rhodovibrin and spirilloxanthin, which are characteristic of photosynthetic bacteria [18]. Spirilloxanthin has a maximum wavelength absorption of 440-490 nm [19]. Isolate GO, CU2, and L2 had seven wavelength peaks where as CU1, L1, SD and PS isolates as many as 6 wavelength peaks. These differences thought to be influenced by culture conditions and
bacteriochlorophyll location in their cells. The difference in wavelength absorption is caused by the type of bond and the molecular location of the bacteriochlorophyll in the pigment protein complex of photosynthetic organelles [14].

Table 2. The morphology and characteristics of the isolates

| Sample location  | Isolate | Gram Test | Cell Shape | Color of bacterial liquid culture |
|------------------|---------|-----------|------------|----------------------------------|
| Catfish pond     | L 1     | Negative  | Rod        | Pink                             |
|                  | L 2     | Negative  | Rod        | Pink                             |
| Situ Cibuntu     | CU 1    | Negative  | Rod        | Red brown                        |
|                  | CU 2    | Negative  | Rod        | Red brown                        |
| Situ Gedong      | GO      | Negative  | Spherical  | Red brown                        |
| Situ Cibinong    | PS      | Negative  | Oval/Coccibacillus | Brown                  |
| Eel pond         | SD      | Negative  | Oval/Coccibacillus | Brown                  |

Table 3. The result of spectral absorbance observation

| Isolate | Wavelength Peak | Number | Bacteriochlorophyll Type |
|---------|-----------------|--------|--------------------------|
| GO      | 375 440 460 490 | 7      | a                        |
| CU1     | 375 440 460 490 | 6      | a                        |
| CU2     | 375 440 460 490 | 7      | a                        |
| L1      | 375 440 460 490 | 6      | a                        |
| L2      | 375 440 460 490 | 7      | a                        |
| SD      | 375 440 460 490 | 6      | a                        |
| PS      | 375 440 460 490 | 6      | a                        |

3.2. Sulphide Removal Ability

The initial screening test was found that the seven isolates could reduce the sulphide concentration in the medium so that it could be concluded that the seven isolates were purple sulfur bacteria. In the span of 48 hours the highest decrease in sulphide concentration was found in isolates L1, L2, and CU1, followed by CU2 and GO with moderate levels of decline, and PS and SD which at least reduced sulphide concentrations. Based on the results of the test isolates L1, L2 and CU1 were selected for further testing.

The results of the advanced testing of the three isolates were presented in Table 4. From the table it was known that the increase in sulphide concentration in the test media resulted in an increase in the bacterial population and an increase in the ability of sulphide removal in all of test isolates. This is in accordance with the opinion of Schlegel et al [14] that the characteristic of purple sulfur bacteria is its ability to oxidize sulphide compounds through photosynthesis in cells and the presence of sulphides can accelerate bacterial growth. L1 isolates had the strongest sulphide removal specific value, followed by L2 isolates and CU1 isolates respectively. However, overall CU1 isolates produced the highest percentage of sulphide reduction followed by L2 and L1 isolates respectively.

The high percentage of sulphide concentration decrease in CU1 isolate was caused by the higher reproductive capacity of these isolates than other isolates. This can be seen from the OD value of the isolates (Table 4). Observations on the growth pattern showed that CU1 isolates reached the stationary phase at 32nd hour with an OD value of 0.602. While L1 isolates reached the stationary phase at the 48th hour with an OD value of 0.579 and L2 isolates at 72 hours with an OD value of 0.608 (Figure 3).
So with higher bacterial cell counts than L1 and L2, CU1 isolates become more effective in removing sulphides.

### Table 4. Effectiveness value of sulphide removal with the addition of Na$_2$S 6% and 12%.

| Isolate | Removed Sulfide (mg/L) | OD | Sulphide removal (%) | Specific Sulfide Removal | Removed Sulfide (mg/L) | OD | Sulphide removal (%) | Specific Sulfide Removal |
|---------|------------------------|----|----------------------|--------------------------|------------------------|----|----------------------|--------------------------|
| L1      | 22.91                  | 0.500 | 92 %                | 45.82                    | 36.71                  | 0.613 | 87 %                | 59.88                    |
| L2      | 24.75                  | 0.569 | 99 %                | 43.49                    | 37.67                  | 0.689 | 91 %                | 54.67                    |
| CU1     | 24.68                  | 0.585 | 99 %                | 42.18                    | 39.35                  | 0.730 | 97 %                | 53.90                    |

#### Figure 4. Growth curve of Isolate L1, L2 and CU1.

### 3.3. Viability of Selected Isolates in Polluted Water

The Kali Item River, which is located near Wisma Atlit Kemayoran, Central Jakarta, has long been known for its black and foul-smelling appearance that disturbs the surrounding environment. The water current is almost invisible. With these conditions it can be estimated that Kali Item is anaerobic waters with high sulphide content. The results of measurements of sulphide content in water samples at two sampling locations showed an average concentration of 2.575 mg / L in locations 1 and 1.431 mg / L at location 2.

The results of the viability test of CU1 and L2 isolates on Kali Item River water showed that both isolates could grow in water from both river locations. This was indicated by the formation of red color after incubation for 17 hours (Figure 4) whose intensity increases until the 4th day. The red color in water from location 1 was more concentrated than location 2. This was because the sulphide content at location 1 was higher than location 2. On day 5, a greenish layer began to appear at the bottom of the container. On the 7th day red in the water column began to fade while the green layer at the bottom of the container thickened. This change showed a shift in the pattern of microorganism populations along with changes in the chemical content of water. The reduction of toxic sulphide concentrations by the activity of purple sulfur bacteria resulted in the triggering of growth of susceptible sulphide organisms and conversely a decrease in sulphide concentration resulted in a decrease in the population of purple sulfur bacteria. A similar phenomenon was also found in the control medium, but the process occurred more slowly and the formation of red was not as exact as the sample with purple sulfur bacterial isolates. The ability of CU1 and L2 isolates to grow in Kali Item River medium provided an opportunity for the use of both isolates as in-situ bioremediation agents in open water. In-situ bioremediation has been
widely carried out and provides significant results at low costs. Gao et al [6] used a consortium of more than 100 types of microorganisms including anoxicogenic photosynthetic bacteria to improve the water quality of the Chengnan River, China by direct application of the consortium. Within 18 days after the stocking, there was an improvement in water quality. Bioremediation can be combined with biological or non-biological systems to get maximum results as done by Sheng et al [7]. Sheng et al [7] combined aeration technology, bacterial bioremediation, membrane bioreactors, artificial biofilms and floating wetlands to improve the quality of Dihe River, China, in-situ. The bioremediation agent used is Anoxigenic Photosynthetic Bacteria and Bacillus subtilis which were directly added to the river. Within two months water quality improvements began to appear and there was a change in aquatic community structure in the river.

![Figure 5. Color of selected purple sulphur bacteria cultures in Kali Item River water after 17 hours incubation](image)

4. Conclusion
In this study, seven purple sulphur bacterial isolates from natural waters and aquaculture were obtained. All of the isolates were Gram negative and had colour which varied from pink, red brown and brown colour. The isolates had spectral absorbance that indicate the presence of a bacteriochlorophyll-a type which is the characteristic of photosynthetic bacteria. As sulphur bacteria, the seven isolates were able to reduce sulphide concentration with the highest activity owned by CU1 isolates from Cibuntu Situ. CU1 and L2 isolates have the potential to be used as bioremediation agents for sulphide pollution in waters because of their ability to reduce sulphide concentrations through photosynthesis. The application of isolates can be carried out in-situ for water types similar to Kali Item River.

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