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ISOLATION AND CHARACTERIZATION OF SULFUR-OXIDIZING BACTERIA (SOB) FROM WASTE OF RUBBER FACTORY AND PAPER IN JAMBI

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Abstract. Sewage contains high concentration of sulfate and contaminates ground water supply. The production process in the factory produced sewage containing high amount of sulfur such as sulphide and sulphate. Sulfur bacteria, which are capable of oxidizing, reducing or partially oxidizing inorganic sulfur compounds such as *Thiomicrospira*, *Thiothrix*, *Desulforomonas*, *Achromatium*, and a group of *Thiobacillus*. Utilization of sulfur bacteria has done in attempt to find sewage bioremediation agents as well as to promote soil fertility. Isolation and characterization were carried out by growing the bacteria in the starkey selective medium, including pH reduction test, morphology, physiology and biochemical characterization. It has been observed that out of 2 isolates from different sources of samples (rubber and paper sewage), only one isolates related to sulfur-oxidizing bacterium (SOB) while the isolates AE01 related to true sulfur-oxidizing bacterium in genus *Thiobacillus*.

Keywords: bioremediation agent, sewage liquid, sulfur oxidizing bacteria (SOB), *Thiobacillus*

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1. Introduction

Factory waste contains high concentrations of sulfate which flows from sulfide-rich spots. Contamination of groundwater supply from factory waste can cause disruption to aquatic flora and fauna, corrosion of flow pipes and bridges, destruction of roads, etc. (Sajjad et al. 2016).

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The production process at rubber and paper factories produces waste containing large amounts of sulfur compounds such as sulfides and sulfates. Some additional compounds such as TMTD (tetramethyl triuram disulphide), sodium sulphide, ammonia (NH₃) and sulfuric acid are also used (Kantachote and Innuwat, 2004; Nurhayati & Mahmudin, 2012).

Sulfur bacteria consist of groups of heterogeneous organisms that have the ability to oxidize, reduce or partially oxidize inorganic sulfur compounds (Priyanka, Sivaji and Sridar, 2014). In addition to playing a role in increasing soil fertility through sulfur oxidation and solubilization of plant nutrients, sulfur bacteria can also be used as bioremediation agents for sulfur-containing wastes (Rojas-Avelizapa et al. 2013).

Sulfur bacteria can live in pH with a range of 1.0 - 10.5 (Priyanka, Sivaji and Sridar, 2014). Sulfur bacteria usually live in acidic environments such as soil containing a lot of sulfur (rhizosfir soil), factory waste and ex-mine land, and used tannery water (Vidyalakshmi and Sridar, 2006).

This study aims to isolate sulfur-oxidizing bacteria (SOB) from rubber and paper waste and characterize to determine the species of sulfur bacteria isolated from rubber and paper waste. The isolated SOB bacteria are used for alkaline soil reclamation, increasing sulfur oxidation in the soil as well as bioremediation agents for sulfur-containing wastes.

Sampling

The isolates of sulfur-oxidizing autotrophic bacteria were taken from 2 different sources which were rubber waste from rubber factories located in Batanghari Regency, Jambi Province and paper waste taken from paper mills located in Sabak, Tanjung Jabung Timur Regency, Jambi Province. The rubber waste samples taken were black while the rubber waste samples are golden clear. Rubber and paper waste samples were then measured for pH and electrical conductivity.

Media Preparation and Bacterial Isolation

The media used for isolating SOB bacteria is the Thiobacillus broth media which is a modification of the Starkey media, with a composition of 0.40 gr (NH₄)₂SO₄, 4.0 gr KH₂PO₄, 0.25 gr CaCl₂·2H₂O, 0.01 gr FeSO₄, 0.50 gr MgSO₄·7H₂O, 5.0 gr Na₂S₂O₃ and dissolved in 1000 ml of distilled water with pH 8.0. The media was sterilized and poured into 50 ml erlenmeyer as much as 20 ml each. The waste samples were then diluted through a serial dilution of 10⁻² and inoculated into 1 ml of Thiobacillus broth media. Erlenmeyer was incubated in an incubator at 30 ° C for 25 days. Bacterial growth is characterized by the presence of turbidity and biofilms found on the surface of the media and erlenmeyer walls.

Screening of Sulfur Bacteria

The isolates previously grown on the Thiobacillus broth medium with initial pH 8.0 then measured the final pH using a pH meter. The isolate screening process was carried out by selecting isolates capable of reducing pH from 8.0 to .03.0. The selected isolates were then grown on solid Thiobacillus agar containing 0.40 gr $(\text{NH}_4)_2\text{SO}_4$, 4.0 gr KH_2PO_4 , 0.25 gr $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01 gr FeSO_4 , 0.50 gr $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0 gr $\text{Na}_2\text{S}_2\text{O}_3$, and 12.50 gr to be original, in 1000 ml of aquades with pH 8.0 to be further characterized.

Morphological and Biochemical Characterization

Characterization was carried out on isolates grown on Thiobacillus agar media, including Gram staining and several biochemical tests such as catalase test, oxidase test, motility test, starch hydrolysis, SIM (Sulfide Indole Motility) test and TSIA test (Triple Sugar Iron Agar) .

a. TSIA Test and H₂S Production

The suspected SOB isolates that have been screened, are grown on Triple Sugar Iron Agar (TSIA) media, then incubated at 300C for 24-48 hours. Then the color changes in the media were observed, the positive results of sugar fermentation were marked by changes in the color of the media from red to yellow. While the positive results of H₂S production are shown to change color from yellow to black.

b. Motility Test

The suspected SOB isolate that has been screened is grown on Sulfate Indol Motility (SIM) media, by inserting an ose needle containing bacterial isolates on the media. Then observed changes in the media, positive results are shown with certain patterns on the media as long as the puncture results.

c. Indol Test

The suspected SOB isolates that have been screened are grown on Sulfate Indol Motility (SIM) media, then incubated at 300C for 24-48 hours. Positive results are indicated by pink colored rings at the top of the media after being pressed with Kovac's reagent.

d. Starch Hydrolysis Test

Bacteria that have been isolated and seen growth in the culture are poured lugol solution and left for several minutes. Then observed the color changes that occur around the colony. Positive results are indicated by the formation of clear zones around the colony after iodine drops.

e. Catalase Test

The suspected SOB isolates that have been screened are reviewed on sterile glass objects. Then drop 3% H₂O₂, observe whether there is a bubble. Positive results are indicated by the presence of gas bubbles.

f. Oxidase Test

SOB suspected isolates that have been screened, taken using an ose and applied to a piece of filter paper. Then drop 1-2 drops of oxidase reagent in bacterial culture. Then observe changes in color in bacterial culture. Bluish purple formation shows positive results.

Identification of Bacteria

After morphological and biochemical characterization, identification of bacterial species was carried out using the book Bergey's Manual of Determinative Bacteria (Holt et al. 1994) and the Bergey's Manual of Systematic Bacteria Vol 2A (Brenner et al., 1923).

Results and Discussion

Screening of Sulfur Bacteria from Rubber Waste

A total of 2 isolates of sulfur oxidizing bacteria (SOB) were successfully obtained from liquid waste from the rubber industry in Jambi Province. Both isolates were isolates of AE01 and AE02, respectively. The results of SOB bacterial culture showed growth in the medium of Thiobacillus broth which was characterized by the formation of mucous layers (biofilms) on the surface of the media after incubation for \pm 25 days (Rosa, et.al, 2017).

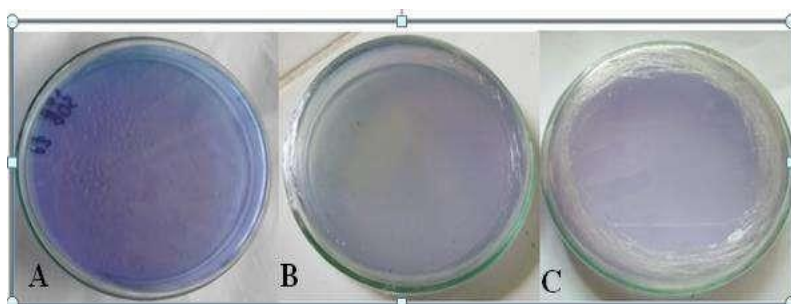


Figure 1. Culture of sulfur oxidizing bacterial isolates (SOB) in starkey media with Bromocresol purple A). Medium before bacterial isolation B). Growth of AE01 isolate C. AE02 isolate

One characteristic of SOB bacteria was its ability to reduce pH. The pH value greatly affects the metabolic activity of SOB bacteria in oxidizing the substrate where it grows. The two isolates obtained from rubber wastewater were also tested for metabolic activity. The results of the tests conducted indicate that there had been a decrease in pH on the medium of Thiobacillus broth. This can be seen in the decreasing pH of media which was originally 8.0 to 2.63 (AE01) and

2.61 (AE02). Dara (2000) states that SOB bacteria are always found to live at low pH and are found mostly in soil and water containing sulfate.

According to Donati *et al.* (1996), pH reduction on selective media of Thiobacillus broth is caused by the production of sulfuric acid which causes the pH of the previously alkaline media to turn sour due to the growth of SOB bacteria. Nurseha and Djajakirana (2004) also add that SOB bacteria generally get energy from the oxidation of one or more reduction of sulfur compounds. Some examples of SOB bacteria such as *Thiobacillus ferrooxidans* add energy by oxidizing ferro to ferric.

SOB bacteria carry out oxidation processes directly or indirectly. Regusa and Medgwick (1990) concluded that microorganisms successfully dissolve heavy metals and sulfides directly through dependence on metabolism or indirectly with products of their metabolism. pH reduction tests carried out on SOB bacteria have also been reported by several previous studies (Donati *et al.* 1996; Vidyalakshmi and Sridar, 2006; Priyanka, Sivaji and Sridar, 2014 and Sajjad *et al.* 2016). Ferrous sulphate contained in the media will be rapidly oxidized by acidophilic microbes when there is oxygen and/or ferric iron in the environment (Sajjad *et al.* 2016).

These microbes are acidic (acidophilic), using source C from inorganic materials (lithotrophs or muscrof) and using energy sources from oxygen (Wentzel, 2004). The T.ferrooxidans species, known as chemolytotrophy and synthesizing its cells from carbon obtained enzymatically from CO₂, turned out to be able to use organic carbon in a limited way (Bacelar-Nicolau and Johnson, 1999). The presence of BOS will drive AMD to 500,000-1,000,000 times compared to geochemical reactions (Mills, 2004) so that in this case the microbial group is very detrimental to the environment in which they live.

Table 3. Characteristics of Morphology of SOB bacterial isolates originating from rubber factory waste

No	Code Isolate	Morphology						Optical characteristic
		Colour	Form	Size	Elevation	Surfaces	Margin	
1	AE01	grey	filamentous	Medium	Flate	smooth-shiny	filamentous	Transparance
2	AE02	white	round	small	Flate	smooth	Full	Partly transparance

Characteristics of morphology, physiological properties and biochemical activity of bacteria

The two bacterial isolates suspected of SOB were then characterized based on morphological characteristics, physiological properties and biochemical activities. This is an important part of bacterial identification (Berge's Manual of Systematic Bacteriology).

The two isolates showed different characteristics (Table 3). Differences in these characteristics include colony color, shape, colony size, elevation, surface appearance and margin. However, the optical characteristic test performed on the two isolates showed the same results, They were Translucent. Pelczar and Chan (1988) state that each genus of bacteria will generally form its own characteristics but some of the characteristics that are also shown to be possible will be the same.

The process of identification of bacteria was carried out after going through several kinds of biochemical tests such as the Gram staining test (Figure 2), biochemical tests (Table 4) which include: catalase test, SIM test to measure indole production and motility, TSIA test to measure H₂S production, gas production, and the ability of bacteria to ferment three types of sugar, pH reduction test and amylum hydrolysis test (starch hydrolysis).

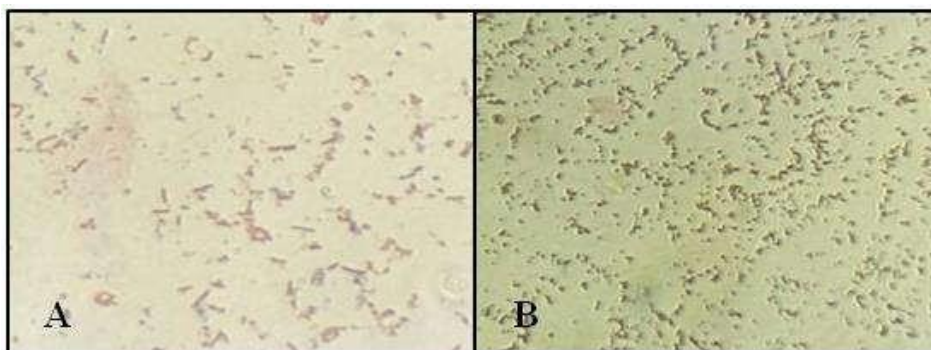


Figure 2. The staining results of the two sulfur oxidizing bacterial isolates (SOB). A) Isolate AE01, B). Isolate AE02

Based on the characteristics of the isolates in Table 4, it was assumed that AE01 isolate was the *Thiobacillus ferrooxidans*. This is because the characteristics possessed by the bacteria *Thiobacillus ferrooxidans* were in accordance with the characteristics possessed by AE01. The characteristics include the same life ability in the range of pH 1.3-4.5; classified as bacil Gram negative bacteria; and the isolates obtained were classified as facultative aerobes. Robertson & Kuenen (2005) states that bacteria with these characteristics generally use the oxidation results from Fe²⁺ and reduce sulfur as their energy source, as well as ammonium and nitrate as their nitrogen source. This bacterium also needs oxygen for life. This is also in accordance with the

test results that have been carried out. Nevertheless, further investigation at the molecular level using rRNA is needed to obtain accurate data on the species obtained.

Microorganisms will use energy produced from the oxidation / reduction process of metals or other harmful compounds for growth. Thiobacillus is able to reduce pH to the range 5.6-6.2 to 2.6- 2.8, and utilize sulfur and thiosulphate as its energy source (Vidyalakshmi & Sridar, 2006). According to Sajjad et al. (2016), most sulfur bacteria (sulfur-oxidizing bacteria) are a group of bacteria belonging to the genus Thiobacillus, Thiomicrospira, Thiothrix, Desulfotomaculum and Achromatium. However, sulfur oxidation is not only limited by true bacterisulfur but can also be carried out by other heterotrophic bacteria isolated from soil or aquatic environments which are capable of carrying out the same oxidation mechanism (Das et al. 1996).

Table 4. Characteristics of Physiological Properties and Biochemical Activities Bacterial isolates of SOB bacteria originating from rubber factory wastes

No	Physiological Test	AE01	AE02
1	Form	Bacil	Coccus
2	Gram stain	negative	negative
3	Catalase	-	-
4	Motility	+	+
5	Gas production	+	-
6	H ₂ S production	-	-
7	TSIA	<i>Alkaline slant</i>	<i>Acid slant</i>
		<i>Alkaline butt</i>	<i>Alkaline butt</i>
8	<i>Bromocresol purple</i>	+	-
9	pH reduction	good	good

The use of SOB bacteria has been widely used. Handayani (1997) reports that, SOB microorganisms that have the potential to oxidize iron and sulfur include Thiobacillus ferrooxidans, Thiobacillus thiooxidans, Leptospirillum ferrooxidans and genus from Sulfolobus

such as *S. acidocaldarius*. *Thiobacillus Ferrooxidans* is also reported as one of the safest and most environmentally friendly types of alternative bacteria for coal desulfurization. *Thiobacillus ferrooxidans* has the ability to oxidize iron and sulfur.

Conclusion

Based on the research that has been done, it can be concluded that:

- a. It has been found 2 isolates of SOB bacteria derived from liquid waste from rubber plantations. However, the researchers did not succeed in finding SOB bacterial isolates originating from paper industry wastewater.
- b. From the identification carried out, the two bacterial isolates were thought to belong to SOB bacteria which were able to oxidize sulfur as a carbon source. The isolate with the AE01 isolate code is thought to be a bacterium with the species *Thiobacillus ferrooxidans*.

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