

# Isolation and Potency Test of Sulfate Reducing Bacteria (SRB) as Bioremediation Agent for Ex-Coal Mining Soil

*by* Hary Widjajanti

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**Submission date:** 20-Sep-2022 03:10PM (UTC+0700)

**Submission ID:** 1904380260

**File name:** ency\_Test\_of\_Sulfate\_\_SRIWIJAYA\_JOURNAL\_OF\_ENV\_31\_MARET\_2020.pdf (571.19K)

**Word count:** 3487

**Character count:** 18148

## Isolation and Potency Test of Sulfate Reducing Bacteria (SRB) as Bioremediation Agent for Ex-Coal Mining Soil

Aria Yanka Paula Putri<sup>1</sup>, Hary Widjanti<sup>2</sup>, Harminuke Eka Handayani<sup>2</sup>

<sup>1</sup>Environmental Management, Postgraduate Program, Sriwijaya University, Indonesia.  
Email: ariayankapaulaputri@gmail.com

### Article history

| Received         | Revised in revised form | Accepted      | Available online |
|------------------|-------------------------|---------------|------------------|
| 19 December 2019 | 30 March 2020           | 31 March 2020 | 31 March 2020    |

**Abstract:** Mining activities can have negative impact on the environment which lead to water and soil pollution. The physical, chemical and biological nature of the soil are changed which characterized by low organic content, low soil pH, low water holding capacity, inadequate nutrient and acid generating material. Coal mining land is acidic due to reaction between sulfur oxidation and rainwater which generate sulfate compounds. Bioremediation technique is a wise effort to overcome environmental pollution by utilize Sulfate Reducing Bacteria (SRB). SRB could decrease sulphate by express of pH increasing which indicates of a SRB population development and sulfate content decrease. This study aims to obtain SRB isolate as potential bioremediation agent for ex-coal mining soil. The treatment of experiment is SRB isolate which obtained from ex-coal mining soil at PT. Bukit Asam Tanjung Enim, South Sumatera. The result of this study obtained ten SRB isolates at the isolation stage. The selection stage obtained five SRB isolates with the criteria of solid black in medium marked equal with the similar number of colonies which has potency as a bioremediation agent for ex-coal mining soil. SRB isolates P<sub>10</sub>T<sub>1</sub>R<sub>2</sub>, P<sub>9</sub>T<sub>2</sub>R<sub>2</sub>, P<sub>6</sub>T<sub>1</sub>R<sub>3</sub>, P<sub>5</sub>T<sub>1</sub>R<sub>1</sub> and P<sub>2</sub>T<sub>1</sub>R<sub>2</sub> respectively were evaluated to increase pH and reduce sulfate on ex-coal mining by potential tested. The P<sub>6</sub>T<sub>1</sub>R<sub>3</sub> SRB isolates were able to increase into pH 6 by percentage increasing 40,3% and decreasing sulfate concentration from 4340,9 ppm to 3596,1 ppm percentage decreasing 17,15%. The SRB isolate can be used as bioremediation agent with indicator of pH increasing and sulfate declining during application on ex-coal mining soil.

**Keywords:** Isolation, test potential, sulphate reducing bacteria, bioremediation

**Abstrak:** Aktivitas penambangan memiliki dampak negatif terhadap lingkungan sekitar dan menimbulkan pencemaran air dan tanah. Sifat fisik kimia juga lingkungan biologi berubah ditandai dengan kandungan organik yang rendah, pH tanah yang rendah, kapasitas memegang air yang rendah, pasokan unsur hara tanaman yang tidak memadai dan bahan pembangkit asam. Lahan yang berasal dari penambangan batubara bersifat asam karena oksidasi sulfur yang bereaksi dengan air hujan menghasilkan asam sulfur sehingga kondisi tanah asam. Kajian bioteknologi merupakan upaya yang bijaksana dalam mengatasi pencemaran lingkungan dengan menggunakan Bakteri Pereduksi Sulfat (BPS) sebagai agen bioremediasi. BPS dapat mengkontaminasi sulfat dengan indikator peningkatan pH yang menandakan adanya peningkatan populasi BPS dan penurunan kadar sulfat. Penelitian ini bertujuan untuk memperoleh isolat BPS yang berpotensi sebagai agen bioremediasi tanah pascatambang batubara. Penelitian merupakan eksperimen dengan isolat sebagai perlakuan yang diperoleh dari tanah pascatambang batubara di PT. Bukit Asam, Tanjung Enim, South Sumatera. Hasil penelitian ini diperoleh sepuluh isolat BPS yang diperoleh dari tahap isolasi bakteri. Pada tahap seleksi bakteri diperoleh lima isolat BPS yang memiliki kriteria warna hitam pekat pada media dengan jumlah koloni yang sama sehingga memiliki potensi sebagai agen bioremediasi tanah pascatambang. Isolat BPS P<sub>10</sub>T<sub>1</sub>R<sub>2</sub>, P<sub>9</sub>T<sub>2</sub>R<sub>2</sub>, P<sub>6</sub>T<sub>1</sub>R<sub>3</sub>, P<sub>5</sub>T<sub>1</sub>R<sub>1</sub> dan P<sub>2</sub>T<sub>1</sub>R<sub>2</sub> dilakukan uji potensi pada tanah pascatambang batubara untuk menaikkan pH dan menurunkan sulfat. Isolat BPS P<sub>6</sub>T<sub>1</sub>R<sub>3</sub> mampu menaikkan pH tanah menjadi 6 dengan persentase kenaikan sebesar 40,3% dan menurunkan konsentrasi sulfat 4340,9 ppm menjadi 3596,1 ppm dengan persentase penurunan sebesar 17,15%. Isolat BPS P<sub>6</sub>T<sub>1</sub>R<sub>3</sub> dapat digunakan sebagai agen bioremediasi dengan indikator kenaikan pH dan penurunan sulfat yang diaplikasikan pada tanah pascatambang batubara.

**Kata kunci :** Isolasi, uji potensi, bakteri pereduksi sulfat, bioremediasi

### 1. Introduction

The mining activity have a negative impact on the environment around coal mining areas, including

land subsidence, damage the aquatic environment and soil pollution. Then the physical, chemical and biological nature of the soil become change by

characterizer of low organic content, low soil pH, low water holding capacity, inadequate nutrient and acid generating material [7]. The land that comes from coal mining is acidic due to the oxidation of sulfur reacts with rainwater to produce the sulfur acid [5].

The study of bioremediation is a wise and appropriate efforts to resolve environmental pollution. Alternative management can be done by [4] utilizing biological agent in the form of Sulfate Reducing Bacteria (SRB). SRB is able to decrease the content of sulfate and increase soil pH which indicated an increasing population of SRB [8]. SRB has tolerance on the various concentrations of sulfate (acidic) [13].

SRB isolate type of *Desulfovibrio* sp. is obtained from sulfate soil. *Desulfovibrio* sp. are motile, gram-negative bacteria, vibrio [12], can be grown on a medium of sulphate-lactate anaerobic conditions that sufficient to sustain the development of the cell [1], able to survive in toxic conditions contains hydrogen sulphide in the sediments with depth 2-3cm and the ground [6].

A habitat of coal mining area is suitable for the growth of the SRB [15], SRB sourced from ex-coal

mining soils which are rich in sulphide minerals produce sulfate compounds caustic acidic pH with high acidity [2]. The SRB can be found in soil layers of the ex-coal mining due to the soil organic matter which contaminated land [3], as a carbon source for SRB growth [9].

The research of the biological agents that using the SRB is an attempt treatment by preventative measures as precautionary of the environmental pollution, need to be done for the bioremediation of the ex-coal mining soil that isolated from ground source ex-coal mining soil itself which is safe for the environment.

## 2. Methods

The research was carried out in the Laboratory of Integrated Research Graduate Program of Sriwijaya University. The soil samples are obtained from ex-coal mining soil site and point coordinates S 03° 43' 46" E 103° 50' 07, 834 m, IUP Banko Barat Pit 3 West Tanjung Enim Mining Unit, PT. Bukit Asam Tbk, South Sumatra (Figure 1). This study was conducted from April to November 2019.

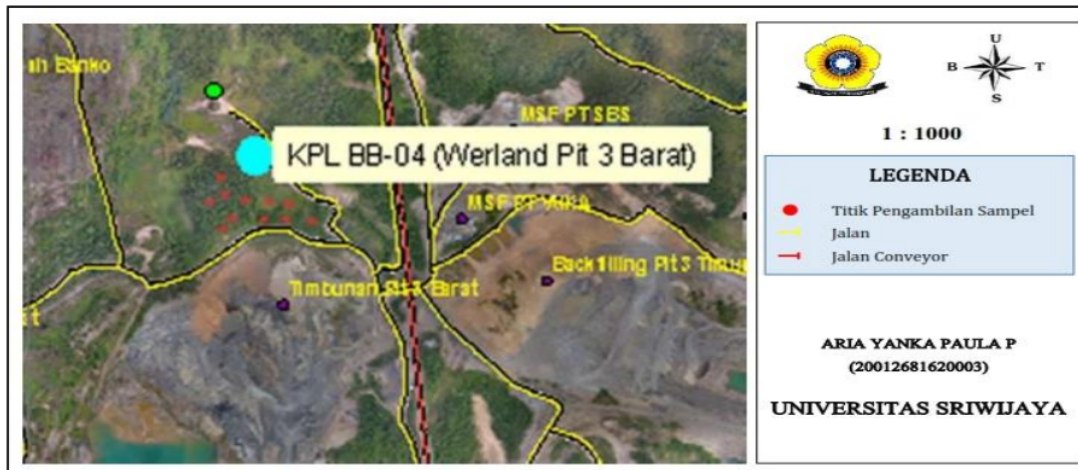


Figure 1. The Map of Soil Sampling Locations

The apparatus are bioreactor, Uv-Vis spectrophotometer and GPS. The materials needed in the research are alcohol 70%, aquades, physiological salt (NaCl), one liter postage of medium [15] which consisted of sodium lactate (8mL), magnesium sulphate (1g), ammonium chloride (0,5g), potassium dihydrogen phosphate (1g), iron phosphate (0,1g), ascorbic acid (0,5g), glucose (0,1g), calcium chloride (0,1g), sodium sulphate (0,5g) and extract of yeasts (0,1g), aluminium foil, methylene blue, gas pack and soil.

The sampling technique use simple random sampling. Each of the dilution tubes were taken 1 mL and inserted into test tube containing 15 mL of the postgate medium. Tubes closed tightly and labeled, incubated anaerobically in desiccator with gas pack and methylene blue solution for 7-14 days at room temperature. Observations of bacterial growth are marked by change of medium into black color (sulfide formation) indicating the activity of SRB. The suspension of SRB isolates (15 ml) insert to glass bottle containing 1L of the postgate medium.

The calculation of total bacteria used total plate count method. The potential test of SRB isolate showed pH increasing and sulfate concentration declining in bioreactors. It is contained of 500 g ex-coal mining soil which added 50 ml SRB isolate suspension and

500 ml distilled water, then stirring until saturated form a paste/mud to make the media in anaerobic conditions that SRB isolate could be grown on the medium. The stages of research are presented in the Figure 2.

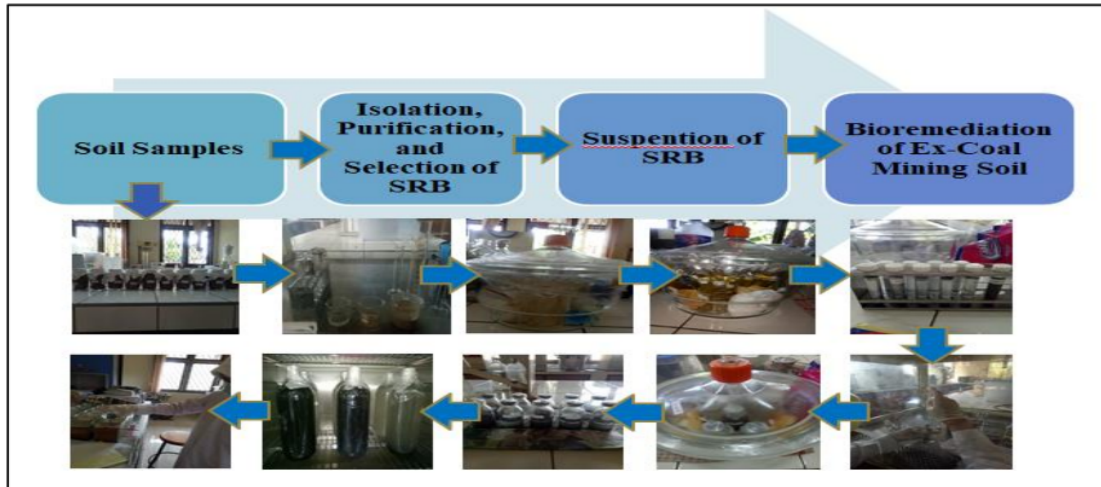


Figure 2. The Stages of Research

Bioreactors were incubated for 14 days at room temperature, and sulfate concentrations measured at 0 day, 7 d<sub>4</sub> and 14 days by using turbidimetry method. The study was designed using a completely randomized design with 5 treatments SRB isolate and control (without SRB isolate) and 4 p<sub>4</sub> plicates. The data of sulfate concentration are analyzed by analysis of variance 5% and LSD test 5%.

### 3. Results and Discussion

#### 3.1 Isolation and Selection Sulfate Reducing Bacteria (SRB)

Sulfate Reducing Bacteria (SRB) pure isolates were isolated from ten samples soil at the

mining area of PT. Bukit Asam Tanjung Enim, obtained 10 SRB isolates grown on postage medium. Postgate medium is a selective medium for SRB growth. Then ten of SRB isolates were assessed based on the black density which imply sulfide formation as indicator the presence of SRB activity. The ten SRB isolates were selected based on dark black criteria, the five SRB isolates were obtained (Figure 3) with the characteristics of physical, chemical and biology properties, color of medium (black), the smell of suspension, temperature, pH, sulfate concentration and total microbes as presented in Table 1.



Figure 3. SRB isolates: (a) P<sub>9</sub>T<sub>2</sub>R<sub>2</sub>, (b) P<sub>10</sub>T<sub>1</sub>R<sub>2</sub>, (c) P<sub>5</sub>T<sub>1</sub>R<sub>1</sub>, (d) P<sub>6</sub>T<sub>1</sub>R<sub>3</sub>, (e) P<sub>2</sub>T<sub>1</sub>R<sub>2</sub>



The characteristics of SRB isolate based on Table 1 show that the SRB isolate indicated deep black suspension on medium and smells of rotten eggs which indicate of presence of hydrogen sulphide gas. SRB isolates have optimum temperature range at 25-40°C, the measurement result show that 32°C is optimum temperature range for SRB growth. The sulfate concentration could affect pH value because the increasing of sulfate concentration generate strong acid conditions then decline pH value as result. The strong acid condition also has effect on

bacterial growth. SRB isolates P<sub>9</sub>T<sub>2</sub>R<sub>2</sub> have 1,13x10<sup>7</sup>cfu/ml bacterial colonies as the smallest colonies. Small total bacterial colonies will affect ability to decrease sulfate concentration.

Low total bacteria have a low sulfate reduction ability which also reduce ability to increase pH. The low pH conditions will affect the structure of enzymes and proteins in bacterial cells because of the intercellular physiological activity in pH 6. The low pH will inhibit the formation of ATP then SRB isolate growing is not optimal due to longer growth time [10].

Table 1. The Characteristics of SRB Isolates Suspension

| SRB Isolates                                  | Medium of Color (Black) | The Smell Of Rotten Eggs | Temperature (°C) | pH  | Concentration Sulfate (ppm) | Total Mikrobess (CFU/ml) |
|---|-------------------------|--------------------------|------------------|-----|-----------------------------|--------------------------|
| P <sub>10</sub> T <sub>1</sub> R <sub>2</sub> | Deep black              | Very smelly              | 32               | 4,7 | 834                         | 4,89 x 10 <sup>7</sup>   |
| P <sub>9</sub> T <sub>2</sub> R <sub>2</sub>  | Deep black              | Very smelly              | 32               | 4,5 | 1350                        | 1,13 x 10 <sup>7</sup>   |
| P <sub>6</sub> T <sub>1</sub> R <sub>3</sub>  | Deep black              | Very smelly              | 32               | 4,6 | 586                         | 1,40 x 10 <sup>7</sup>   |
| P <sub>5</sub> T <sub>1</sub> R <sub>1</sub>  | Deep black              | Very smelly              | 32               | 4,7 | 962                         | 2,08 x 10 <sup>7</sup>   |
| P <sub>2</sub> T <sub>1</sub> R <sub>2</sub>  | Deep black              | Very smelly              | 32               | 4,6 | 654                         | 1,88 x 10 <sup>7</sup>   |

### 3.2 Potential test *Sulfate Reducing Bacteria (SRB) as a bioremediation agent for ex-coal mining soil*

The four SRB isolates were tested which show pH value increasing and sulfate reduction potentially in ex-coal mining soil. The pH and sulfate concentration measurements were carried out of 3 times starting on 0<sup>th</sup>, 7<sup>th</sup> and 14<sup>th</sup> day. The total treatments are four SRB isolates and control (without isolate SRB treatment). The results of study show that pH increasing (Figure 4) and sulfate concentration declining (Figure 5) by activity of isolate SRB in ex-coal mining soil.

The result of pH measurement of the control treatment showed relatively has no changes in pH value. The SRB isolate as treatment was able to increase pH to 6,5. The SRB isolates P<sub>6</sub>T<sub>1</sub>R<sub>3</sub> was able to increase pH 3,7 to 6,2 by 40,3% percentage increasing during application on ex-coal mining soils. The increasing of pH value with SRB isolates

treatment is affected by the SRB isolate activity that decreases sulfate to sulphide. The process of sulfate reduction by SRB isolates are sulphide reacted with dissolved metal ions forming metal sulphide which is not dissolved. The SRB isolates have the ability to transfer electrons or hydrogen to sulfate as an electron acceptor. In the reduction of sulfate reaction produces hydrogen sulfate can decrease pH because of the sulfate is a strong acid which then decreases to form as sulphides is a weak acid so that to increase pH. In the reduction of sulfate reaction, is produced hydrogen sulfate that decrease pH, because initially sulfate is a strong acid then decreases as sulphides form result which is a weak acid that increasing pH. More sulfate concentration decrease then pH increase as result. SRB isolate can increase into pH 6 in accordance with environmental quality standards for coal mining in environmental regulation No. 113 of 2003 which contained of mining waste quality standards.

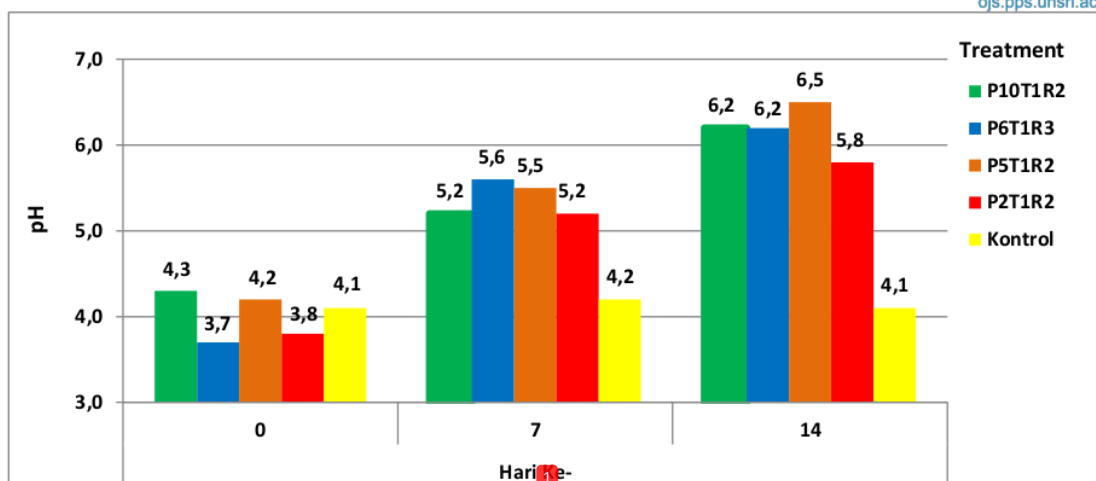


Figure 4. The increase of pH with Sulfate Reduction Bacteria (SRB) as bioremediation agent for ex-coal mining soil

Table 2. The average of pH on 0<sup>th</sup>, 7<sup>th</sup> and 14<sup>th</sup> day during the SRB isolates as bioremediation agent for ex-coal mining soil

| Treatment                                     | Average of pH |                  |                   |
|---|---------------|------------------|-------------------|
|   | Day- 0        | Day- 7           | Day- 14           |
| P <sub>10</sub> T <sub>1</sub> R <sub>2</sub> | 4,3           | 5,2 <sup>b</sup> | 6,2 <sup>bc</sup> |
| P <sub>6</sub> T <sub>1</sub> R <sub>3</sub>  | 3,7           | 5,6 <sup>b</sup> | 6,2 <sup>bc</sup> |
| P <sub>5</sub> T <sub>1</sub> R <sub>1</sub>  | 4,2           | 5,5 <sup>b</sup> | 6,5 <sup>c</sup>  |
| P <sub>2</sub> T <sub>1</sub> R <sub>2</sub>  | 3,8           | 5,2 <sup>b</sup> | 5,8 <sup>b</sup>  |
| Control                                       | 4,1           | 4,2 <sup>a</sup> | 4,1 <sup>a</sup>  |
| BNT (5%)                                      | 0,575         | 0,761            | 0,433             |

Description : The numbers followed by the same letter are not significantly different at the 5% level according to the LSD test

The average of pH in the Table 2 shows that there is a difference between the SRB isolates and controls on ex-coal mining soils applying. There was a significant difference in the average pH between treatment with isolate and controls which indicated by the F value > F critic. Based on the LSD test results shown in Table 2, it is also known that there is

a significantly difference between SRB isolates and control on the average of pH at the 7<sup>th</sup> and 14<sup>th</sup> day measurements. The increasing of pH due to SRB isolates decreasing sulfate to sulfide that previously in the form of strong acids (sulfate) then change into weak acids form (sulfide) which increase the pH.

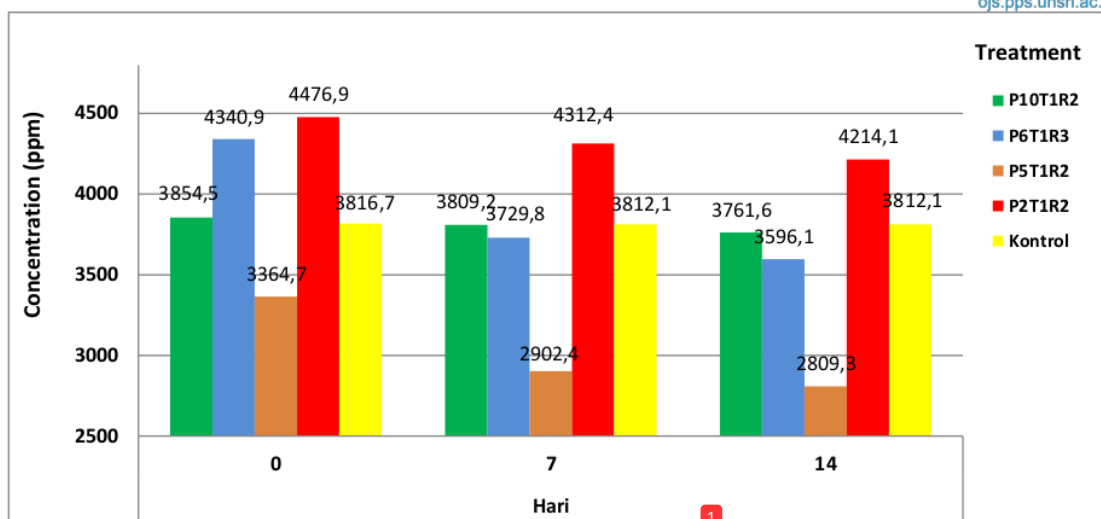
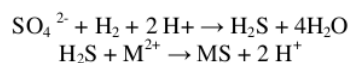


Figure 2. The Decrease of sulfate concentration with Sulfate Reduction Bacteria (SRB) as bioremediation agent for ex-coal mining soil

The sulfate concentrations of control treatment showed relatively no changes in sulfate concentrations decreasing (Figure 4). Whereas the SRB isolates treatment showed change sulfate reduction. The SRB isolates P<sub>6</sub>T<sub>1</sub>R<sub>3</sub> can decrease sulfate concentrations from 4340,9 ppm to 3596,1 ppm by 17,15% percentage reduction during applications in ex-coal mining soils.

The treatment of SRB isolate has ability to decrease sulfate concentration on ex-coal mining soil because SRB isolates utilize sulfates which found in ex-coal mining soils as energy sources in the form of carbon acceptors (C). This carbon source as act an electron donor in metabolism of the bacterial and the constituent cells of the bacterial body. The sulfate reduction reaction characterized by the sulfate reduction by SRB isolates is shown in the following reaction:



The SRB isolates utilize the energy from the reduction reaction sulfate into sulfide. This reduction reaction produces hydrogen sulfide (H<sub>2</sub>S) which is useful in the deposition of metals (metal sulfides). In the ex-coal mining area H<sub>2</sub>S gas will bind to the metals contained in the land and settle in form of reductive metal sulfide [14]. The formation of stable metal sulfide is difficult to dissolve that has beneficial for the environment. This compound precipitate toxic metals because metal solubility is very low and expected that concentration is harmless and meets environmental quality standard requirements when carried to the waters [11].

Table 3. The average of sulfate concentration on 0, 7 and 14 days during the SRB isolate as bioremediation agent for ex-coal mining soil

| Treatment                                     | The average of Sulfate concentration (ppm) |           |           |
|---|--|-----------|-----------|
|   | Day-0                                      | Day-7     | Day-14    |
| P <sub>10</sub> T <sub>1</sub> R <sub>2</sub> | 3854,6 bc                                  | 3809,2 bc | 3562,9 b  |
| P <sub>6</sub> T <sub>1</sub> R <sub>3</sub>  | 4340,9 d                                   | 3729,8 b  | 3596,1 bc |
| P <sub>5</sub> T <sub>1</sub> R <sub>1</sub>  | 3364,6 a                                   | 2902,4 a  | 2809,4 a  |
| P <sub>2</sub> T <sub>1</sub> R <sub>2</sub>  | 4476,9 d                                   | 4312,4 d  | 4214,2 c  |
| Control                                       | 3816,7ab                                   | 3812,1 bc | 3812,1 bc |
| BNT (5%)                                      | 462,9                                      | 453,7     | 468,2     |

Description : The numbers followed by the same letter are not significantly different at the 5% level according to the LSD test

The average of sulfate concentration shown in Table 3, it is known that there is a difference between SRB isolates and control treatment on ex-coal mining soil. The significant difference in the average of sulfate concentration between treatment with SRB isolates and control is indicated by the calculating of F value > F critic. Based on the LSD test result shown in Table 3, it is also known that there is a significantly difference between SRB isolate treatments and control.

SRB isolates have a role in reducing environmental pollution by reducing sulfate concentration which can increase pH then reduce dissolved metals by deposition of metals to overcome environmental pollution. Soil bioremediation by SRB isolates will help the rehabilitation activities of ex-coal mining soil.

#### 4. Conclusion

1. This study can be concluded that four SRB isolates namely P<sub>10</sub>T<sub>1</sub>R<sub>2</sub>, P<sub>6</sub>T<sub>1</sub>R<sub>3</sub>, P<sub>5</sub>T<sub>1</sub>R<sub>1</sub> and P<sub>2</sub>T<sub>1</sub>R<sub>2</sub> respectively were has potentiality to increase pH and decrease sulfate on ex-coal mining soil.
2. SRB isolates P<sub>6</sub>T<sub>1</sub>R<sub>3</sub> were able to increase into pH 6 by percentage increase 40,3% and decrease sulfate concentrations from 4340,9 ppm to 4596,1 ppm by 17,15% percentage reduction during applications in ex-coal mining soil as a bioremediation agent for ex-coal mining soils.

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