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Isolation and Identification of Endophytic Bacteria Related to Plant Nutrient Level in Coal Mining Site from East Kalimantan Indonesia

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ABSTRACT
East Kalimantan has a very wide area for coal mining. However, not all coal mining companies carry out land reclamation before coal mine was abandoned. This marginal land is very potential to be utilized as productive land, although efforts must be done to minimize the main limiting factor in coal mining site, namely low soil fertility by utilizing endophytic bacteria found in ex-coal mining sites. This research aimed to isolate and identify endophytic microorganisms in coal mining sites with potential to play a role in coal mining reclamation related to plant nutrient level. The method used in this research was exploration method, by determining sample points in former coal mining sites with high representation value. Samples were taken from 5 points in 3 different former mining sites located in East Penajam Paser Regency, East Borneo, with a total of 15 sample locations. Bacterial isolation was performed followed by characterization and identification using gram staining and calculation of comparable coefficients. To determine the percentage of comparable coefficients (S_s) of positive and negative similarities of characteristics from each bacterial species, the morphological and physiological test results of the *Bacillus* bacteria were used. The isolation results showed that there were 11 (eleven) isolates which could be identified into 11 types of bacteria which were potential as a nutrient level role based on the results of the characterization ex-coal mining sites; *Bacillus pantothenticus, B. mycoides, B. firmus, B. brevis, B. stearothermophilus, B. anthracis, B. laterosporus, B. sphaericus, B. sphaericus, B. alvei*, and *B. firmus*. All isolates came from Bacillus genus with similarity percentage at 65-70%.

Keywords: *Coal mining area, Identification of endophytic microorganisms, East Borneo.*

1. INTRODUCTION

The East Borneo region has a mining business of around 4.4 million hectares (http://green.kompasiana.com/). This area has the potential to be utilized as a productive agricultural area considering the extent of ex-mining land that has not been used optimally because not many mining companies pay attention to the reclamation of ex-mining land, especially ex-coal mining, causing a lot of land was left abandoned. In some locations, coal mining in East Borneo is carried out using open mining techniques, causing environmental damage. This technique is carried out by clearing the land and then taking and moving the soil in the top soil area which results in damage to physical, chemical and biological properties of soil, causing low nutrient levels in this region [1].

Kesumaningwati *et al.* [2] stated that in general former coal mining sites had low soil fertility with a texture of clay with limiting factors including high sulfate levels, high salt levels, acid soils with Al toxicity and low level of K. Another report showed that ex-coal mining site in East Borneo had ultisol soil type which has low soil fertility with various limiting factors such as low organic matter, high cation exchange capacity so that it is only suitable to be used as plantation land, including acidic soils with very saturated of Al [3]. Similar results were revealed by Mashud and Manaroinsong [4] which showed that ex-coal mine site had low fertility rate due to various limiting factors. Therefore, treatment is needed to overcome these limiting factors before the former coal mining land can be used as agricultural land. If these limiting factors are not overcome then plants that planted on this land will experience stress.

Various soil microorganisms have an extraordinary role in maintaining the level of soil fertility, including mycorrhizae which have an important role in supporting the rehabilitation of mining site [5]. The main function of mycorrhizae in mine site rehabilitation is to facilitate the absorption of nutrients by plants, so that it can have an impact on increasing plant growth and production [6]. In addition, mycorrhizae can also help expand the function of plant root systems in obtaining nutrients [7]. In terms of nutrient absorption, mycorrhiza can increase absorption of relatively immobilized soil nutrients such as sulfur (S), copper (Cu), zinc (Zn), boron (B) [8], and also various essential nutrients such as N and P. Mycorrhizae can also help maintain the stabilization of heavy metal elements such as Hg, Pb, Cd, As so that they do not directly harm plants [9].

Phosphate solubilizing bacteria can be used as biological agents for soil reclamation due to the low levels of P in coal mining site, given its role to increase the availability of P nutrients in the soil, where phosphate is one of the essential nutrients for plants so that an enhance in P availability will also can improve plant growth [10]. These include hydrocarbon solubilizing bacteria that have an important role in degrading abundant hydrocarbon compounds in coal mining site. This bacterium degrades hydrocarbon compounds by cutting the hydrocarbon chains shorter with the help of various enzymes so that the hydrocarbon compounds are not toxic to plants and can increase soil nutrients derived from the results of breakdown of hydrocarbon compounds [11,12]. In addition, nitrogen fixing bacteria can also utilized because of their ability to provide nitrogen nutrients in the soil [13], because they have nitrogenase enzymes that can tether free nitrogen in the air and convert it to ammonium [14]. With the availability of nitrogen for plants, it can increase plant growth because nitrogen is an essential element needed to compile structural and functional proteins including those used for the formation of growth hormones namely auxin, gibberellin, and cytokines [15].

Given the beneficial nature of soil microorganisms, it increase the reason for applying multisymbiotic studies of soil microorganisms to be involved in minimizing limiting factors in an effort to make a reclamation model [16, 17, 18, 19] for ex-coal mining site by empowering plants from mining sites that have potential as bioremediator through multisymbiotic relationship between host plants and endophytic soils microorganisms based on the concept of tripartite symbiosis between plants, soil bacteria, and mycorrhizae [20]. This research was a preliminary aimed to isolate, characterize, and identify endophytic microorganisms which in the next stage play a role in the reclamation model of ex-coal mining site utilizing the multisymbiotic interaction patterns of endophytic microorganisms by empowering plants that have the potential as bioremediators from coal mining locations.

2. METHODS

This exploratory study took location in former coal mining sites in the East Penajam Paser Regency, East Borneo, Indonesia. There are three locations of former coal mining site. At each location, 5 (five) locations will be taken as samples so that in total there were 15 locations of soil samples. From these soil, bacteria were isolated. Ten mL of sterile distilled water was prepared to put 1 gram of soil sample, continued by homogenized process. After this process, 1 mL was taken and put in a test tube containing Czapex Broth without sucrose, continued by incubated process in 7 days at 300°C. During incubated for 7 days, 1 mL of each samples was inoculated using a pour plate method in petri dishes containing KNA media and incubated at 30°C. A test tube containing KNA media was prepared to identify the growing bacteria inoculated, after that the pure isolates are obtained, and than the results of morphological gram staining and physiological tests of bacterial colonies were performed.

Furthermore, observations of macroscopic colony, cellular microscopic (Gram staining), and physiological characteristics were carried out using Microbact Identification Kits (MicrobactTM GNB 12 A and 12 B) and identification of bacteria using the book Bergey's Manual of Determinative Bacteriology By Holt [21].

The procedure for identifying bacterial isolates is as follows.

1. Rejuvenate pure culture of bacteria that will be identified with elements ranging from 18 - 24 hours on oblique NA
2. Gram staining to find out the type of Gram and form of bacteria to be identified
3. Conduct an oxidase test from an oblique NA bacterial sample to determine the type of kit to be used. Positive oxidase is indicated by the presence of purple on the oxidase strip paper, while the negative oxidase does not have a purple color
4. Make a bacterial suspension in a physiological salt solution of 0.85% NaCl of 10 mL
5. Inserting a bacterial suspension into each well as much as 250 µL
6. Closing the parts of the well that are circled in black with emersion oil if the oxidase test is positive and the parts circled in black and black are dotted for the negative oxidase test
7. After all suspensions have entered, close the well seal and incubate in the incubator at 37 °C for 24 hours
8. After 24 hours, the results read in the wells are recorded and matched with the MicrobactTM

Gram Negative Identification System determination key. Can be seen in the reaction table

9. Addition of reagents for wells that require further detection

a. Well of 8 - indole (2 drops of kovacs reagent, reaction is read in 2 minutes)

b. Well of 10 - VP (1 drop of VPI and VPII, reaction is read in 15 - 30 minutes)

c. Well of 12 - TDA (1 TDA test, reaction can be observed directly)

d. Nitrate reduction test (o-nitrophenyl-β-d-galactophyranoside (ONPG))

Well 7 - ONPG after reading the ONPG reaction, 1 drop of nitrate A and nitrate B was added. The determination will indicate a reduction in nitrate to nitrite (NO_2), when there is a formation of red color within a few minutes after the addition of the reagent.

The results of testing positive Gram bacteria were done by calculating the comparable coefficients. To determine the percentage of comparable coefficients (S_s) that indicated positive and negative similarities of the properties of each bacterial species of the genus *Bacillus*, the morphological and physiological test results of the *Bacillus* bacteria were used [22].

To determine the identification of bacteria isolated from real bacteria using the similarity coefficient formula:

$$Similarity\ coefficient = \frac{A}{A+B+C} \times 100\% \qquad (1)$$

with:

A: Positive traits for both strains
B: Positive trait for strain one, negative trait for strain two
C: Negative trait for strain one, positive trait for strain two

Data including the results of isolation and identification of endophytic microorganisms were analyzed descriptively.

3. RESULTS AND DISCUSSION

All isolate showed the gram-positive bacteria (Table 1) due to the coloring results using four reagents used in this staining. All cells will form a CV-I (Crystal violet-Iodine) complex which will bind to the Mg-RNA component of the cell wall, forming an Mg-RNA-CV-I complex which is insoluble in alcohol. These will give a violet crystal base color. Fat will be dissolved when 95% alcohol as a decolorization compound added. While, the Mg-RNA-CV-I complex could be seemed clearly when lugol as a strengthening solution added.

In gram positive bacteria, the primary dyes are difficult to wash and cells turn purplish in blue. As we known that gram positive bacteria have a small fat content. When the alcohol is used to wash, the fat will dissolve and a small pore form which is then covered by alcohol dehydrated protein, so that the pore is closed. While large pores will be formed in gram negative bacteria due to a lot of fat, so when washing with alcohol, the large pores that cannot be covered by dehydrated pores, as a result alcohol cleans all Mg-RNA-CV-I complexes and cells lose color. By using safranin as the fourth reagent in coloration will replace the base color that has been lost due to alcohol leaching. The gram-positive bacteria remain purple, while the gram-negative bacteria will be red [23,24].

Table 1. Identification of endophytic bacteria in the fifteen location of former coal mining sitesure

No	Bacterial Isolate Code	Shape	Identification Results	
			Species Name	Probability (%)
1	6 II B3 (gram positive)	Bacil	*Bacillus mycoides; B. pantothenticus*	60%
2	IE 5 (gram positive)	Bacil	*Bacillus mycoides*	60%
3	2 IA3 (gram positive)	Bacil	*Bacillus firmus; B. pantothenticus; B. brevis; B. stearothermophilus*	65%
4	8 III B4 (gram positive)	Bacil	*Bacillus anthracis; B. mycoides; B. pantothenticus; B. laterosporus; B. sphaericus; B. stearothermophilus*	65%
5	II A4 (gram positive)	Bacil	*Bacillus sphaericus*	70%
6	7 III B3 (gram positive)	Bacil	*Bacillus mycoides*	70%
7	4 III E3 (gram positive)	Bacil	*Bacillus pantothenticus*	70%
8	1 IA4 (gram positive)	Bacil	*Bacillus alvei*	65%
9	3 IB4 (gram positive)	Bacil	*Bacillus firmus; B. pantothenticus; B. brevis; B. sphaericus*	65%
10	5 IB3 (gram positive)	Bacil	*Bacillus anthracis; B. mycoides; B. sphaericus; B. stearothermophilus*	65%
11	I D3 (gram positive)	Bacil	*Bacillus stearothermophilus*	70%

Table 2. Biochemical tests (physiology) of isolate bacteria from soil former coal mining site

Bacterial Samples	Oksidase	Motility	Nitrate	Lysine	Ornithine	H_2S	Glucose	Manitol	Xylose	ONPG	Indole	Urease	V-P	Citrate	TDA	Gelatin	Malonate	Inositol	Sorbitol	Rhamnose	Sucrose	Lactose	Arabinose	Adonitol	Raffinose	Salicin	Arginine	Katalase	Indosphora
6IIB3	+	-	-	-	-	-	-	-	-	+	-	-	+		-	+	-	-	-	-	+	-	-	-	-	+	+	+	+
IE5	+	-	+	-	-	-	-	-	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+
2IA3	+	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	
8IIIB4	+	-	+	-	-	-	-	-	-	+	-	-	+	-	-	+	-	+	+	-	+	+	-	-	-	-	-	+	+
IIA4	+	-	-	+	-	+	-	-	-		-	+	-	-	-		-	-	-	-	-	-	-	-	-	-	+	+	+
7IIIB3	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+
4IIIE3	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	+
1IA4	+	-	-	-	-	-	+	+	-	+	+	-	+	+	-	+	-	-	-	-	+	-	-	-	-	-	-	+	+
3IB4	+	+	+	-	-	-	-	+	-	+	-	-	-	+	-	+	-	-	-	-	+	-	-	-	-	-	+	+	+
5IB3	+	-	+	-	-	-	-	-	-		-	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	+	+
ID3	+	+	+	-	-	-	-	+	-	+	-	+	+	-	-	+	-	-	+	-	+	-	-	-	-	-	-	+	+

Table 1 shows that from each plate colony there were found more than one isolate bacteria, so that the type of bacteria found were *Bacillus pantothenticus; B. mycoides; B. firmus; B. brevis; B. stearothermophilus; B. anthracis; B. laterosporus; B. sphaericus; B. sphaericus; B. alvei;* dan *B. firmus.* However, in general all derived from the genus Bacillus with a percentage between 65% to 70%. After identifying, followed by determining the characterization of these indigenous bacteria by using the results of biochemical activity tests carried out by comparing the biochemical activities of each different bacteria due to different enzymatic process in bacterial [25].

Table 2 indicates the biochemical activities of bacteria isolated from the former coal mining site. Physiological characteristics include the nature and ability of bacteria to grow on media (Lysine, Ornithine, Arginine, ONPG, indole, TDA, Gelatin, Malonate-amino acids) H_2S, citrate, Urease, VP, (Glucose, Mannitol, Xylose, ONPG, Inositol, Sorbitol, Rhamnose, Sucrose, Lactose, Arabinose, Adonitol, Raffinose, Salicin - carbohydrates). The table shows that all bacteria have positive responses to the oxidase and catalase tests, while other tests varied which showed the nature of different bacteria.

The bacterial species that were successfully isolated and identified in the former coal mining site are eleven bacterial species, however, of the eleven species of bacteria this does not mean that one isolate is only identified as one bacterial species because there are several isolates consisting of more than one bacterium, namely isolate 6 II B3, 2 I A3, 8 III B4, 3 I B4, 5 I B3, while the other isolates are isolates with a single bacterial species.

As we known that that biodegradation of various compounds that exist in ex-coal mining site by microbial communities depends on the adaptive response of bacterial community to the presence of compounds in the mining site, which is hydrocarbon as a dominant compounds [26]. This means that when indigenous bacteria are used in the bioremediation process of this former coal mining site, then in its management it relies on the process of adaptation of the bacteria used to the environmental conditions that exist in the former coal mining site as well as how these bacteria have a mechanism to tolerate conditions that are generally not beneficial [27].

Bioremediation is a biological mechanism for converting waste compounds into other forms that can be used by organisms. Bioremediation involves the process of degradation, eradication, immobilization, or detoxification of various hazardous substances and chemical wastes by utilizing the activities of microorganisms community [28]. Bacteria can be considered able to increase their role in improving the condition of plant nutrient availability [29]. The fungus, yeast, algae and protozoa turned out to be unsuitable due to their size and inability to grow in existing conditions, especially in reservoirs for remediation if using a reactor. Fernandes *et al.* [31] stated that bacterial communities on mining site and soil near mining site can be utilized as a bioremediation strategy for soil reclamation. Bacteria that can be used for bioremediation in mining site area are bacteria that have the potential to trigger plant growth and bacteria that can survive in stress conditions such as *Pseudomonas* sp. and *Acinetobacter* sp. [24].

This indicates that the success of microbes in degrading hydrocarbon compounds (for example hydrocarbons that exist in ex-coal mining site) that are highly based on their activities to change existing carbon complex to become another small molecule [31]. For example, if it is in reactor conditions, for example in relatively high temperatures, pressure, and media of salinity which allows the bacteria to be able to conduct their activities well including to grow and to develop. Therefore, thermophilic isolates, even anaerobic thermophilic bacteria have good potential for use and can be isolated and cultured. Ansari *et al.* [32] reported that thermophilic bacteria were able to degrade soybean oil, olive oil, glycerol and crude oil.

4. CONCLUSION

The isolation results showed that there were 11 (eleven) colony plates based on the characterization results, 11 types of bacteria were identified, i.e. *Bacillus mycoides*; *B. pantothenticus, B. firmus; B. brevis; B. stearothermophilus; B. anthracis; B. laterosporus; B. sphaericus; B. sphaericus; B. alvei*; and *B. firmus*. However, in general all derived from the genus Bacillus with a percentage similarity between 65% to 70%. In addition, the results show that several isolates do not only contain one isolate. The isolates that have been identified need to be tested for their ability to dissolve phosphates, to degrade hydrocarbon compounds before being tested at the field level. Bacteria community plays an important role in nutrient level in the soil.

AUTHORS CONTRIBUTION

All authors conceived and designed this study. All authors contributed to the process of revising the manuscript, and at the end all authors have approved the final version of this manuscript.

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