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Screening of phosphate solubilizing bacteria from ex-coal mining soil in Central Bengkulu District, Indonesia

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Abstract The results of bacterial isolation obtained 402 bacterial isolates showed a clear zone, wherein 48 bacterial isolate had the potential ability to dissolve phosphate. From the 48 bacterial isolates, 10 which performed the highest phosphate solubilizing index (PSI) were selected. The PSBs selected PSBs were 9 gram-positive and 1 gram-negative. Biochemical test results showed that 7 isolates were closed to the genus *Bacillus*, 2 isolates were closed to *Mycobacterium*, and 1 isolate was closed to *Serratia*. Out of the 10 isolates, 2 best isolates were proceeded for quantitative performance test for solubilizing phosphate. The quantitative test revealed that BB3B27 and BB3B6 dissolved the highest phosphate on the 7th day, with 217.2 mg/L and 192.4 mg/L respectively. The 16S rRNA marker molecular showed that the selected BPF isolate BB3B27 had a 100% similarity in the nucleotide base sequence with the *Bacillus subtilis* strain BaBc-1.

Keywords: Coal mine, Phosphate solubilizing bacteria (PSB), Screening

Introduction

Coal mining activities are one that can bring detrimental impact in topsoil landscape. Many common phenomena that occur as a result of mining activities were loss of topsoil, drought, soil compaction, low water retention, toxic accumulation, acidic soil (pH), low soil fertility, high sulfate level, high salt level and nutrient-poor soils in macro nitrogen and phosphorus. This condition

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causes coal mining land to become less livable so that certain vegetation can not grow well and plants will experience stress (Rahayu *et al.*, 2021).

According to Whitmore *et al*. (2011), the plants cannot grow properly because of the limited penetration of roots into the soil to obtain nutrients and water. Water from rainfall and irrigation will be difficult to penetrate the soil surface due to pore closure. Seed germinations are also hampered in ex-mining land due to crust formation and increased soil strength when the soil became dry. A relatively large effort is needed to improve land with poor soil properties to support plant growth. To repair land with poor soil conditions, it can be overcome by planting pioneer plants such as sengon, lamtoro, or acacia which can better adapt to critical and nutrient-poor land. The presence of pioneer plants will improve the land and further facilitates another biota such as microbe (Novianti *et al.,* 2021), but still the roots of pioneer plants that grow in the area cannot dissolve the phosphorus (P) element from the ex-mining land. One effort to use ex-mining land to be more productive can be done by introducing phosphate solubilizing bacteria (PSB) as a Plant Growth Promoting Rhizobacteria (PGPR) which play a vital role in soil enrichment due to due to its phosphate solubilizing ability. These bacteria can secrete organic acids so that it decreases pH and breaks down bonds in some forms of phosphate compounds, thereby increases the availability of phosphate in the soil. Apart from solubilizing phosphate, these bacteria can solubilize potassium, increase nitrogen fixation, produce growth regulators such as auxins, cytokinins, and gibberellins while also protecting plants from pathogens because they secrete antibiotics and antifungals, and they are extraordinary biocontrol agents (Rawat *et al.,* 2020). Thus, efforts to utilize plants on infertile or less productive ex-mining land with the addition of phosphate solubilizing bacteria are needed to improve soil quality to obtain good re-vegetation outcomes.

Bacteria can act as phosphate solubilizers in the soil have been found, including *Pseudomonas putida* (Teng *et al.,* 2019), *Klebsiella variicola* (Nacoon *et al.,* 2020), *Bacillus megaterium, Rhizobium* sp. (Biswas *et al.,* 2018) and *Staphylococcus haemolyticus* (Hii *et al.,* 2020). These bacteria have the ability to solubilize phosphate elements that are bound to other elements (Fe, Al, Ca, and Mg) so that the P element becomes available to plant. Phosphate solubilizing microorganisms originating from ex-mining land have been studied, but there is no report from ex-coal mining. The objective of this study was to obtain potential phosphate solubilizing bacterial isolates by isolating, selecting, and characterizing phosphate solubilizing bacteria from ex-coal mining soil in Central Bengkulu Regency, knowing the potential phosphate solubilizing bacterial isolates based on 16S rRNA gene amplification and identifying the most potential phosphate solubilizing bacterial isolates.

Materials and methods

Sample site and collection

Soil samples were taken from the former coal mining land, Central Bengkulu Districts, Bengkulu Province, Indonesia which was carried out using a purposive sampling method. Samples come from the soil contained at each point of the sampling area. Soil samples were taken using a soil sample core or paralon pipe with a diameter of 3.3 cm at a depth of 0-15 cm from the surface. Soil samples taken were 3 paralon pipes for each soil location. There are 3 locations of land taken as samples, namely topsoil, dumping area, and reclaimed land. At the time of sampling, measurements of abiotic factors such as soil pH, humidity, temperature, and the coordinates of each sampling point were carried out.

Isolation of phosphate solubilizing bacteria

A total of 3 grams of compiled soil samples taken from the pipe paralon with each gram taken at each different point. The soil samples were put into 27 mL of 0.85% physiological saline solution, then a serial dilution was made up to 10^{-7}. A total of 0.1 mL of 10^{-3} to 10^{-7} dilution was spread over the surface of the Pikovskaya's (PVK) agar media. Then incubated at 37^{o}C for 72 hours. Phosphate solubilizing bacteria (PSB) were evaluated based on the presence of clear zones surrounding bacterial colonies.

Purification of phosphate solubilizing bacteria

Colonies forming clear zones were purified using quadrant scratch method then rejuvenated on Pikovskaya's agar slant (Lay, 1994). After that, it was incubated for 48 hours at 37^{o}C. Pure isolates obtained were observed for their morphological characters, such as colony shape, colony color, elevation, and edges. Isolated bacteria were kept on PVK agar slant at 4^{o}C for further study (Irianto, 2006).

Qualitative measurement of phosphate solubilization

Bacterial isolates were screened for their tricalcium phosphate (TCP) solubilizing activity on PVK agar media. Isolates were spot inoculated on the center of agar plate aseptically. All the plates were incubated at 28^{o}C for 7 days. A clear zone around a growing colony indicated phosphate solubilization

and was measured as phosphate solubilization index (PSI). PSI was calculated as the total diameter (colony + halo zone) to the colony diameter (Premono *et al*., 1996). The formula for calculating the phosphate solubilization index (PSI) as follows:

$$\text{PSI} = \frac{\text{Halozone diameter (cm)} - \text{Colony diameter (cm)}}{\text{Colony diameter (cm)}}$$

All the observations were recorded in triplicate. Strains developing clear zones around their colonies could easily be identified as PSBs.

Morphological and physiological characterization of PSB

Morphological identification of the selected isolates was carried out by the spotted method on Pikovskaya's agar media and incubated for 7 days at 37^{o}C. After incubation, colony morphology was observed, such as the shape, appearance, edges, elevation, and color. Afterwards, further identification was conducted through Gram staining and biochemical tests (Lay, 1994). Gram staining was then performed to determine the type and shape of bacterial cells. Physiological identification was done by biochemical tests. Biochemical tests were carried out in the form of catalase tests, motility tests, testing of sugars (lactose, sucrose, maltose, and glucose), urea tests, and citrate tests. Identification of bacterial isolates was referred on the 9th edition of Bergey's Manual of Determinative Bacteriology.

Determination of selected isolate growth curve

A total of 2 selected bacterial cultures were inoculated into 200 mL Nutrient Broth (NB) media, then incubated with a swaying incubator speed of 125 rpm at room temperature for 18 hours. Then 25 mL was taken and transferred into 250 mL Nutrient Broth (NB) media, then incubated in a swaying incubator. Every three hours, 2 mL of was taken to measure the absorbance value until it reached the death phase. The measurement of cell density was done using a Genesys 20 spectrophotometer at a wavelength of 600 nm to a value of Optical Density (OD) 0.6-0.8 with a range of bacterial cells reaching 107 CFU/mL to make a standard curve. The growth curve was determined by making a plot between the time and the OD value. Cell counts were calculated using the TPC (Total Plate Count) method on solid PVK media for each 10^{-3}, 10^{-5} and 10^{-7} dilutions of 1 mL of the same culture when testing cell density and incubated at room temperature for 24 hours (Fardiaz, 1992).

Quantitative measurement of phosphate solubilization

Quantitative test of isolates in solubilizing phosphate was carried out according to the method of Lynn *et al*. (2013) and Mursyida *et al*. (2015). One loop of bacterial culture was inoculated into 50 mL of liquid PVK media, then incubated in a shaking incubator for 48 hours. After incubation, 1 mL of culture was incubated into 100 mL of liquid PVK media and incubated in a shaker for 7 days at 37 ^{o}C. Every 24 hours, 1.5 mL of bacterial culture was centrifuged (Spectros Genesys 20) at 10,000 g for 10 minutes to separate bacterial cells from the supernatant. One mL of supernatant was taken and reacted with color-forming reagents (2.5 mL sodium molybdate (Na_2MoO_4) 2.5% and 1 mL hydrazine sulfate ($N_2H_6SO_4$) 0.3%), then heated for 10 minutes and cooled. After the blue color was formed, phosphate activity (P) was measured in a spectrophotometer at a wavelength (λ) of 830 nm for each day for 7 days. The standard curve was made using KH_2PO_4 with concentrations of 0, 20, 40, 60, 80, and 100 ppm.

Genomic DNA isolation of bacteria, PCR amplification, and sequencing of 16S rRNA gene

Bacterial DNA genome was extracted using the protocol of the Presto^{TM} gDNA Bacteria Mini Kit (Geneaid). After DNA extraction, Polymerase Chain Reaction (PCR) was performed in a vertiti thermal cycler. The specific prokaryotic primers, 63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387R (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al*., 1998) were used for the amplification of the 16S rRNA gene fragment. PCR amplifications were carried out in 10 µl PCR reaction mixture consisted of 0.5 µl primer 1387R, 0.5 µl primer 63F, 5 µL GoTag, 3.7 µL ddHO, and 0.3 µl DNA template. The amplification cycle consisted of an initial denaturation step of 5 min at 94^{o}C, followed by 35 cycles of 30 sec at 92^{o}C (denaturation), 30 sec at 58^{o}C (annealing), 1,.5 sec at 72^{o}C (elongation), and a final extension step for 5 min at 72^{o}C with cooling (storage) for 10 min at 4^{o}C. PCR products were electrophoresed using 1.5% agarose stained with Ethidium bromide (EtBr 0.5 lg/mL) for 45 min, and visualized using under UV light at 1% 80 Volt. The amplified 16S rRNA gene was seen with the formation of a ± 1300 pb band. The results of amplification were then sequenced to determine the sequence of nucleotide bases through PT. Indonesian Science Genetics (Sambrook and Russel, 2001). All the bacterial isolates were classified in BLAST analysis of their respective 16S rRNA gene partial sequences. Various database including NCBI and Ribosomal Database Classifier (RDC) were used to determine the

exact taxonomical calssification of individual organism. For determination of closet type strains NCBI Blast was used.

Phylogenetic analysis

Sequence data were compared visually and sequences were aligned using the Clustal W software and distances were calculated according to Kimura's two-parameter method. Phylogenetic trees were produced using the neighbor-joining (NJ) method. Bootstrap analysis was based on 1000 resamplings. The MEGA (Molecular Evolutionary Genetics analysis) 6.0 package was used for all phylogenetic analysis. The final sequence was submitted to GenBank.

Results

Sample site and collection

Soil sampling was conducted using purposive sampling method with 3 different points based on the height of the location and the astronomical location, namely top soil surface with a height of 285 m and astronomical location 03°45'50.4 "LS, 02°30'42.6" BT, land dumping area with a height of 281 m and astronomical location 03°45'32.8 "LS, 02°30'31.4"BT, and reclaimed land with a height of 282 m and astronomical location 03°44'59.9 "LS, 02°30'31.0" BT. From each location point, physical environmental factors such as soil pH, soil humidity, and air temperature were measured (Table 1).

Table 1. Data on physical factors from the study location

Parameters of Environmental Physical Factors						
No	**Location**	**pH**	**Soil Moisture (%)**	**Air Temperature (°C)**	**Altitude Coordinate**	**Point Location**
1	Top Soil	6.16	2.26	35 °C	285 m	03°45'50.4" LS 02°30'42.6" BT
2	Dumping Area Soil	3	8	39 °C	281 m	03°45'32.8" LS 02°30'31,4" BT
3	Reclaimed land	4.46	5.8	37 °C	282 m	03°44'59.9" LS 02°30'31.0" BT

Isolation and purification of phosphate solubilizing bacteria

The total number of bacteria growing on Pikovskaya's media was carried out using dilutions 10^{-3}, 10^{-5}, and 10^{-7}. They were isolated using the scatter cup method. The obtained results of bacterial colony growth calculated using counter colonies. The results of counting is presented in Table 2. A total of 402 phosphate solubilizing bacteria colonies were isolated from the former coal-mining soil. There were 48 isolates of phosphate solubilizing bacteria were selected which based on their phosphate solubilizing performances and different morphological observations from each colony.

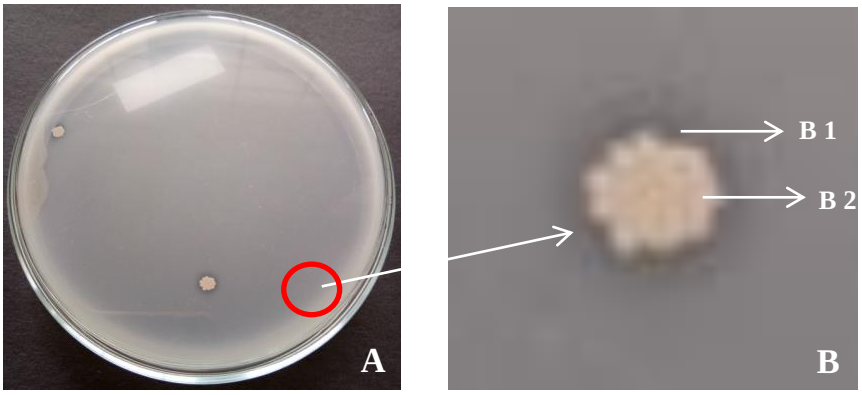

Figure. 1. The appearance of the phosphate solubilizing bacteria colony was characterized by a clear zone (A) original image and (B) enlarged image (B1) clear zone (B2) bacterial colony, which grew on the Pikovskaya incubation media for 72 hours at a temperature of 37 ^{o}C

Table 2. The amount of phosphate solubilizing bacteria that grew on Pikovskya's media incubated at 37^{o}C for 72 hours

No	Depth location	Sample point	Sample Code	Total Number of Bacterial Colonies			Number of Bacterial Colonies Forming Clear Zones		
				10^{-3}	10^{-5}	10^{-7}	10^{-3}	10^{-5}	10^{-7}
		1	BB1A	300	73	36	300	73	6
1	A	2	BB2A	TBUD	TBUD	7	TBUD	TBUD	2
		3	BB3A	TBUD	TBUD	7	TBUD	TBUD	2
		1	BB1B	17	5	2	4	3	1
2	B	2	BB2B	160	0	4	0	0	4
		3	BB3B	0	3	6	0	2	3
Total isolat							304	80	18
							402		

Description: TBUD = Too Much To Calculate, BB = Coal, 1,2,3 = sampling point, A = surface depth range 0-1 cm, B = surface depth range 14-15 cm.

The growth of phosphate solubilizing bacteria was characterized by the presence of clear zones on Pikovskaya media around the colonies (Figure 1). The formation of clear zones was caused by the dissolution of phosphate by organic acids produced by microbial colonies. The source of phosphate used in the media was $Ca_3(PO_4)_2$. The effectiveness of phosphate dissolution was influenced by the specific characteristics of each phosphate solubilizing microbial and environmental or media differences.

Qualitative measurement of phosphate solubilization

From the 48 selected isolates, based on the value of the phosphate solubilizing index, the 10 best isolates were selected from the largest clear zone size. The phosphate solubilizing index (PSI) values of the 10 selected phosphate solubilizing bacteria (PSB) isolates are displayed in (Table 3).

Table 3. The phosphate solubilization index (PSI) value of 10 selected phosphate solubilizing bacteria (PSB) isolates from the former coal mining land in Central Bengkulu Districts

No	Isolate Code	Phosphate Dissolution Index (cm)
1	BB1B1	0,25
2	BB3B5	0,45
3	BB3B6	0,66
4	BB1B15	0,22
5	**BB3B27**	**0,83**
6	BB2A34	0,25
7	BB2A35	0,4
8	BB3A39	0,45
9	BB1A45	0,26
10	BB1A46	0,32

Description: BB = Coal, 1,2,3 = sampling point, A = surface depth range 0-1 cm, B = surface depth range 14-15 cm, 1,5,6,15,27,34,35, 39.45, and 46 = number of isolates.

Morphological and physiological characterization of PSB

The obtained pure isolates were observed for morphological characters, namely colony shape, color, elevation, and edges. The selected isolates shared characteristic as well as distinc properties as shown in Table 4. Gram staining showed that from 10 isolates of selected phosphate solubilizing bacteria, 9 of them were gram-positive and 1 isolate was gram-negative (Table 5). The results of observations using a microscope with a magnification of 1000x, obtained the form of bacteria in 10 selected bacterial isolates, namely bacilli, with arrangement of long bacilli, streptobacillic, and short bacilli (Figure 2).

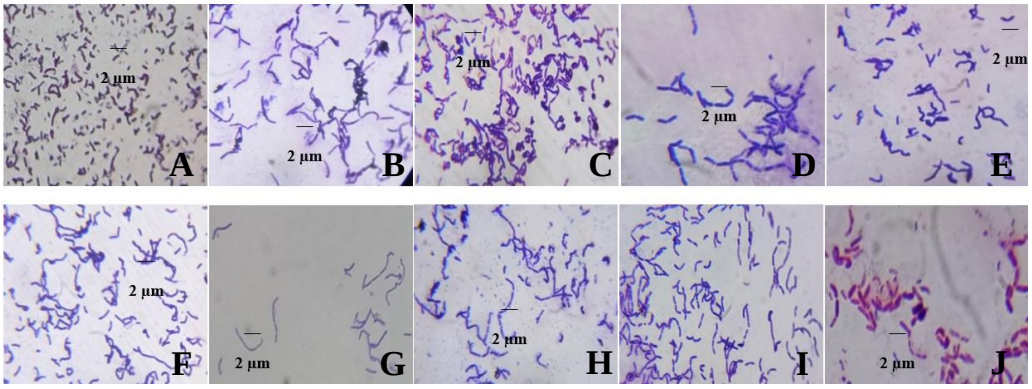

Figure 2. Gram staining results were observed at 1000x, BB = coal magnifications, (A) = BB3B27, (B) = BB3B6, (C) = BB3B5, (D) = BB3A39, (E) = BB2A35, (F) = BB1A46 (G) = BB1B1, (H) = BB1A45, (I) = BB2A34, and (J) = BB1B15

Physiological identification of biochemical tests showed isolates BB3B27, BB3B5, BB3A39, BB1A46, BB1B1, BB1A45, BB2A34 were close to the genus *Bacillus*. BB3B6 and BB2A35 isolates have proximity to the genus *Mycobacterium* and BB1B15 isolates to the genus *Serratia* (Table 5).

Table 4. Characteristics of 10 selected isolates through morphological observation

No	Isolate Code	Morphological observation				
		Colony Surface	Appearance of Colonies	Elevation	Edge of the colony	Colony Color
1	BB3B27	Slippery	Round	Convex	Entire	Cream white
2	BB3B6	Contoured	Irregular	Raised	Lobate	Yellowish white
3	BB3B5	Contoured	Irregular	Raised	Undulate	Cream white
4	BB3A39	Contoured	Irregular	Raised	Lobate	Cream white
5	BB2A35	Contoured	Irregular	Raised	Lobate	Cream white
6	BB1A46	Contoured	Irregular	Raised	Lobate	Cream white
7	BB1B1	Contoured	Irregular	Convex	Lobate	Yellowish white
8	BB1A45	Contoured	Irregular	Raised	Lobate	Cream white
9	BB2A34	Contoured	Irregular	Raised	Lobate	Cream white
10	BB1B15	Contoured	Irregular	Raised	Undulate	Cream white

Table 5. Gram staining and biochemical tests results of 10 selected PSBs isolates from ex-coal mining land in Central Bengkulu Regency

No	Isolate Code	Gram Staining		Biochemical Test								Species Name
		Gram	Cell Form	K	U	M	S	G 1	2	3	4	
1	BB3B27	+	Basil	+	-	+	+	+	+	+	-	*Bacillus* sp 27
2	BB3B6	+	Long Basil	-	-	-	-	+	+	+	+	*Mycobacterium* sp 6
3	BB3B5	+	Streptobasil	+	-	+	+	+	-	-	+	*Bacillus* sp 5
4	BB3A39	+	Streptobasil	+	-	+	-	+	-	+	-	*Bacillus* sp 39
5	BB2A35	+	Short Basil	+	+	-	-	+	-	+	-	*Mycobacterium* sp 35
6	BB1A46	+	Basil	-	-	+	+	+	-	+	+	*Bacillus* sp 46
7	BB1B1	+	Streptobasil	+	-	+	+	+	-	+	-	*Bacillus* sp 1
8	BB1A45	+	Streptobasil	+	-	+	+	+	-	+	-	*Bacillus* sp 45
9	BB2A34	+	Streptobasil	+	-	+	-	+	-	+	-	*Bacillus* sp 34
10	BB1B15	-	Streptobasil	-	-	+	-	+	+	+	-	*Serratia* sp 15

Description: BB = Coal, 1,2,3 = sampling point, A = surface depth range 0-1 cm, B = surface range depth of 14-15 cm, 1,5,6,15,27,34,35,39,45, and 46 = number of isolates. K = catalase test, U = urease test, M = motility test, S = citrate test , G1 = Glucose test, G2 = maltose test, G3 = sucrose test, G4 = lactose test, (+) = gram positive bacteria, and (-) = gram negative bacteria.

Determination of selected isolate growth curve

Bacterial growth can be observed through increasing cell numbers over time. BB3B6 isolate experienced a lag phase of up to the 6th hour with an average bacterial count of $7.0x10^5$ CFU/mL, after which it was followed by a log (exponential) phase up to the 9th hour with an average bacterial count of $8.9x10^5$ CFU/mL. Then it entered the stationary phase until the 42nd hour with an average number of bacteria $10.8x10^5$ CFU/mL. BB3B27 isolate experienced a lag phase up to the 6th hour with an average bacterial count of $6.1x10^5$ CFU/mL, after which it was followed by a log (exponential) phase up to the 9th hour with an average bacterial count of $8.5x10^5$ CFU/mL. Then entered the stationary phase until the 45^{th} hour with an average number of bacteria $9.9x10^5$ CFU/mL (Figure 3).

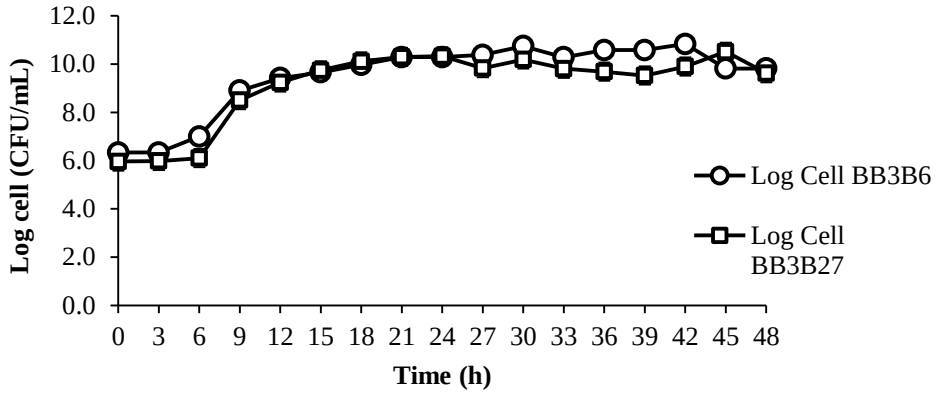

Figure 3. Growth curve of two BB3B27 and BB3B6 isolates on Nutrient Broth medium for 48 hours incubation at 27 ^{o}C

Qualitative measurement of phosphate solubilization

The quantitative test results of phosphate dissolution from two selected isolates showed that each isolate has a different optimum time (day) in solubilizing phosphate. The quantitative measurement of phosphate solubilization results showed that BB3B6 isolate dissolved the highest phosphate on day 7, which was 192.4 mg/L while isolate BB3B27 dissolved the highest phosphate on day 7, which was 217.2 mg/L. The number of cells and the concentration of phosphate dissolution of the two selected isolates are shown in Figure 4.

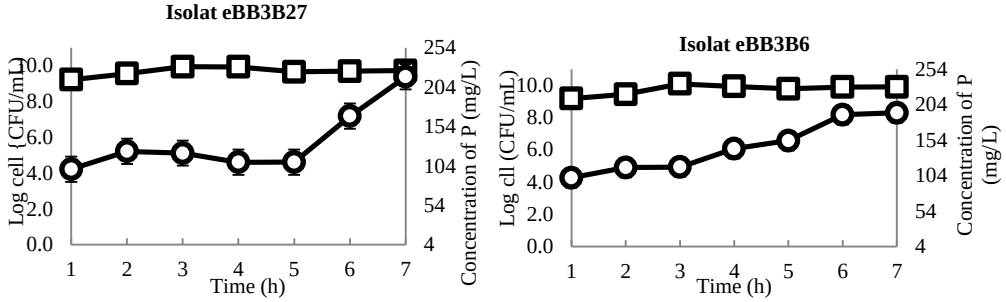

Figure 4. Number of cells and phosphate dissolution concentration (A) isolate BB3B27, (B) isolate BB3B6, (-o-) = log cell number, and (- □ -) = phosphate concentration

16S rRNA gene amplification

Amplification of the 16S rRNA gene from selected isolates using 63f and 1387r primers produced one amplicon measuring around 1300 bp (Figure 5). Phylogenetic tree analysis showed that BB3B27 isolates were closely related to *Bacillus subtilis* with 100% similarity (Figure 6).

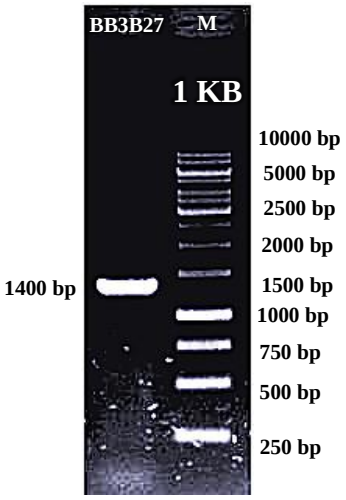

Figure 5. Results of BB3B27 isolate electrophoresis amplification based on 16S gene rRNA. M = 1 kb marker; BB3B27 = PCR product

The results of the reading and comparison with the genebank data were then taken from the comparative 16S rDNA gene sequence data which had a similarity between 90% - 100%. Based on the comparison of DNA base sequences of isolates with genebank data, the species names of the closest bacterial isolates were obtained as in Table 6.

Table 6. Results of 16S rRNA gene sequence analysis from BB3B27 isolates based on NCBI database

No	Comparison Line Description	E Value	Identity	Access number
1	*Bacillus subtilis* strain BaBc-1	0.0	100.00%	MK254686.1
2	*Bacillus subtilis* subsp. subtilis strain VITAPRRKSMG-2	0.0	100.00%	MH118517.1
3	*Bacillus vallismortis* strain APBSMLB224	0.0	100.00%	MG706003.1
4	*Geobacillus sp.* strain APBSDSB9	0.0	100.00%	MG705555.1
5	*Bacillus tequilensis* strain CN17-8	0.0	100.00%	MH762888.1
6	*Bacillus licheniformis* strain X-3	0.0	100.00%	MH628666.1

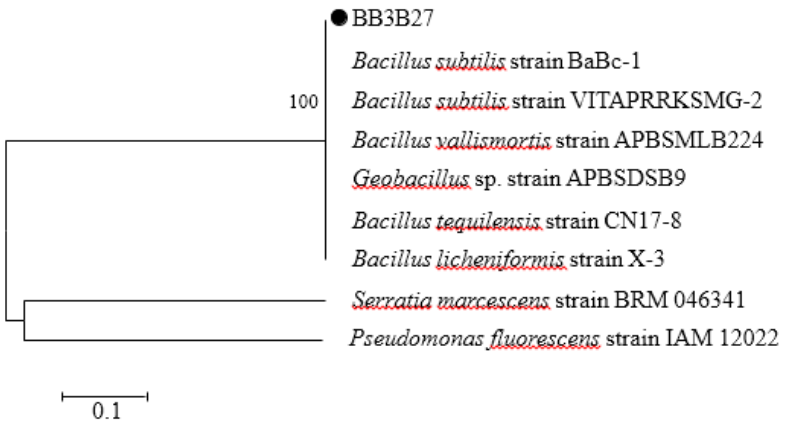

Figure 6. Construction of phylogenetic trees from selected isolates BB3B27 based on 16S rRNA gene sequences constructed with Neighbor Joining with 1000 resampling

Discussion

The three locations have soil pH from very acidic to acidic. The results of this measurement consistent with previous work of Sukarman and Gani (2020), who found that the pH of the soil in the former coal mining land in South Kalimantan was 4-6. Dick *et al*. (2006) also found that the pH of the soil on ex-mining land was 4-5. The acidity of soils collected from mining sites may result from coal, which mostly contains sulfate. The reaction of coal with rainwater could produce Fe, Al, and several other oxidized metals that cause high acidity (Agus *et al.*, 2016).

Soil depth determines the growth of bacterial colonies (Table 2). PSB and TBUD colonies at ground level between 0-1 cm were found more than 14-15 cm deep with similar colonies. According to Wei *et al*. (2017), each location has a very diverse growth rate of phosphate solubilizing microorganisms. The above results suggested that PSB inoculation may not inhibit indigenous microbial growth activities and diversity, and the change of physicochemical environment (e.g., temperature, pH, organic matter content, etc.) in different stages is more crucial that could lead to the bacterial community composition succession. The third location (reclaimed land) has more bacteria because reclaimed soil contains organic material (pioneer plant litter such as sengon) which functions as a source of bacterial nutrition.

BB3B27 isolate had the highest phosphate dissolution index value of 0.83 cm which was isolated from the third location (reclamation soil) at a dilution of 10-7 at a depth of 14-15 cm (Table 3). BB3B6 isolate has the second-highest phosphate dissolution index value of 0.66 cm which was isolated from the

location (reclamation soil) at 10-5 dilutions at a depth of 14-15 cm. The ability of phosphate solubilizing bacteria in the two isolates with the highest IP were tested quantitatively.

The mechanism of phosphate dissolution occurs chemically and biologically. Chemically, PSB produces organic acids such as citric acid, gluconic acid, oxalic acid, and tartaric acid which are able to dissolve inorganic phosphates such as Fe-P, Al-P, and Ca-P in the soil in 4 ways, namely, (a) chelation of cations bound to phosphates, (b) lowering the pH, (c) complexation with metal ions bound to phosphates, and (d) challenging P for adsorption sites Kishore *et al.* (2015). Joner *et al*. (2000) stated that phosphate dissolution was carried out using enzyme phosphatase which was secreted by plant roots and soil microbes. When the mineralization process of organic matter does not have enough phosphate, the enzyme phosphatase produced by microbes will convert P-organic to inorganic P to increase phosphate levels.

Phosphate solubilize tests produce varying dissolution index values. The variation in the phosphate solubilize index value is caused by differences in the ability of bacteria to secrete extracellular organic acids. The main mechanism for solubilizing phosphate is insoluble through the production and secretion of organic acids (Archana, 2007). The clear zone formed around the colony is not always caused by the size of the colony. This was shown by Mursyida (2015) who found that the diameter of a large bacterial colony does not always indicate a large clear zone as well.

The results of the observation showed that BB1B15 isolates belonged to the gram-negative bacteria that produced a red color. The Gram-negative bacteria have thin cell walls and double cell membranes, so that during the bleaching process with 96% alcohol, the cell wall is not strong enough to bind the crystal violet base color. The Gram-negative bacteria can also absorb safranin dyes which cause red bacteria. According to Fardiaz (1989), gram-negative bacteria contain more lipids which allow the enlargement of pores easily. Enlargement of these pores causes dissolution of crystal violet when washing with alcohol.

The growth curve of BB3B6 and BB3B27 isolates showed the similarity of time to reach the lag phase which is from the 0^{th} hour to the 6^{th} hour. The lag phase or adaptation phase is the phase that bacteria need to adapt to their new environment. In this phase, bacteria are growing in new media which is completely differed from their natural media. NB is a new media or carbon source from Isolates BB3B6 and BB3B27, where previously they ~~used~~ were in Pikovskaya media.

BB3B6 and BB3B27 isolates also had the same time to reach the logarithmic phase which ended at 9^{th} hour. The logarithmic phase or

exponential phase is the time to be needed by bacteria to divide or grow in a relatively short time. This lag phase is experienced by BB3B6 and BB3B27 isolates where both only require the 6th to 9th hours to reach the phase.

Furthermore, BB3B6 and BB3B27 isolates entered the stationary phase. The stationary phase is the phase where bacteria balance the amount between dead and living bacteria. Balancing is done to compensate for decreasing in nutrient levels in NB media due to increase in the number of bacteria. This phase is also called the equivalent or balanced phase. BB3B6 isolates reached the stationary phase at 42^{nd} hour, while the BB3B27 at 45^{th} hour (Figure 3). According to Pelczar and Chan (1986), time differences can be influenced by several factors, such as carbon sources, energy sources, pH, oxygen, temperature, incubation period, or the nature of the organism.

Quantitative test results on both isolates showed that the amount of phosphate concentration began to increase with the rise number of bacterial cells that occurred in the stationary phase. According to Chen *et al*. (2006), the dissolution of phosphate in culture media can be influenced by several factors such as the composition of the bacterial medium, the presence of phosphate solubilizing bacterial strains, and changes in the pH of the culture medium. The quantitative test results on BB3B6 was 192.4 mg/L and BB3B27 was 217.2 mg/L.

It is found that phylogenetic trees were divided into 2 large groups, where BB3B27 is not in the same branch as comparable phosphate solubilizing bacteria such as *Serratia* and *Pseudomonas*. The BB3B27 bacteria are on the same line as *Bacillus subtilis* strain BaBc-1, *Bacillus subtilis* subsp. *subtilis* strain VITAPRRKSMG-2, *Bacillus vallismortis* strain APBSMLB224, *Geobacillus* sp. strains of APBSDSB9, *Bacillus tequilensis* strain CN17-8, and *Bacillus licheniformis* strain X-3. It indicates that BB3B27 bears 100% similarity with nucleotide bases. In the second branch, *Serratia* and *Pseudomonas* bacteria are in the same branch, while BB3B27 was in a different branch. It indicated that BB3B27 does not have the same sequence of nucleotides bases with *Serratia* and *Pseudomonas*.

Bacillus is rod-shaped bacteria that can be found in soil and water including seawater (Pelczar *et al*., 1976). *Bacillus subtilis* is a member of the genus *Bacillus* which is a gram-positive, positive catalase test, and isolated from the soil. This bacterium is known to be able to produce various kinds of extracellular enzymes such as protease, lipase, amylase, nuclease, and phosphatase (Slepecky and Henpill, 1992). *Bacillus vallismortis* is a gram-positive bacterium that was successfully isolated from desert land (Robberts *et al*., 1996). *Geobacillus* sp. is a gram-positive bacterium isolated from soil (Ren *et al*., 2010). *Bacillus tequilensis* is a gram-positive bacterium isolated from

tombs and these bacteria form spores (Gatson *et al*., 2006). *Bacillus licheniformis* is a saprophytic, Gram-positive organism, endospore formation that occurs in plants and soil (Veith *et al*., 2004).

Bacillus subtilis has a 100% similarity with *Bacillus vallismortis*, *Geobacillus* sp., *Bacillus tequilensis*, and *Bacillus licheniformis* because it is a bacterium that can be found on the ground.

It is concluded that isolation of bacteria taken from ex coal-mining soil in Central Bengkulu Regency resulted in 402 bacterial isolates, of which 48 had the high phosphate solubility. Of the 48 bacterial isolates, 10 bacterial isolates were selected with the highest dissolution index of 0.83 cm (BB3B27 isolates). Of the 10 BPF isolates selected, 9 of them were gram-positive and 1 of them was gram-negative isolate. The biochemical test results showed that 7 isolates were closed to the *Bacillus*, 2 isolates had proximity to the *Mycobacterium*, and 1 isolate was closed to the *Serratia*. Of the 10 isolates, 2 of the best isolates were selected to be tested for quantitative bacterial abilities. The quantitative test results showed that the highest phosphate dissolution was carried out by BB3B27 and BB3B6 which occurred on the 7th day of 217.2 mg/L and 192.4 mg/L respectively. The molecular test of 16S rRNA showed that the nucleotide base sequence of isolate BB3B27 had a 100% similarity with *Bacillus subtilis* strain BaBc-1.

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