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Cover Page Footnote

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Role of Indigenous Nitrogen-fixing Bacteria in Promoting Plant Growth on Post Tin Mining Soil

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Abstract

Post tin mining soil is generally marginal with low pH, has poor nutrient content, and is thus unfavorable for plant growth, particularly for *Sorghum bicolor*, which is a nutrient-demanding plant. Indigenous bacteria are usually used in bioaugmentation to ameliorate environmental degradation due to their ability to adapt well. This research aimed to isolate indigenous nitrogen-fixing bacteria and evaluate its potential for promoting the growth of *S. bicolor* on post tin mining soil. Nitrogen-fixing bacteria were isolated from post tin mining soil by using specific media and identified by Bergey's manual. Twenty five isolates were obtained, and eight of them (*Azospirillum* sp., *Azospirillum lipoferum*, *Azotobacter chroococcum*, *A. paspalii*, and *Rhizobium* sp.) were identified as nitrogen-fixing bacteria. A greenhouse experiment was conducted using factorial completely randomized design with three replications. The first factors were fertilizers, i.e., NPK; *A. lipoferum* CBT4 + NPK; *A. lipoferum* CBT4; and without fertilizer (control). The second factors were soil types, i.e., A (fertile soil from Cibinong), B (soil from Bangka Botanical Garden), C (soil from post tin mines two years after mining), and D (soil from active tin mining). Result showed that *Azospirillum lipoferum* CBT4 isolated from C (soil from post tin mines two years after mining) exhibited the highest IAA, Ca-P solubilizing ability, and PME-ase activity. This species survived up to a population of 10^7 CFU/gram soil in the three types of post tin mining soils and could be a potential plant-growth promoting rhizobacteria (PGPR) species for effectively improving the growth of *S. bicolor* plant on post tin-mining soil.

Keywords: nitrogen fixing bacteria; PGPR, sorghum, tin mining soil, Bangka Island

Introduction

Nitrogen-fixing bacteria (NFB) are symbiotic and nonsymbiotic microorganisms. Symbiotic bacteria (*Rhizobium*) live freely and symbiotically infects legume roots, forming root nodules. Nonsymbiotic bacteria (*Azotobacter* and *Azospirillum*) live freely in various types of soil and rhizosphere. These bacteria can be associated with various types of plants that grow in different types of environments. The existence of these bacteria in soil is influenced by soil fertility, pH, contents of carbon (C), nitrogen (N), phosphorus (P), potassium (K), and micro nutrients [1], and soil aeration [2]. Several types of NFB can adapt to different habitats with varied temperature, acidity, and extreme oxygen pressure [3]. Some bacteria can live in any environment, such as in marginal ex-tin mining soil contaminated with heavy metals.

Tin mining activity damages the environment and leads to a decline in soil quality because of the high content of heavy metals, loss of macro and micro nutrients from top soil, disruption of humidity, temperature, pH, and exudates produced by plant roots, and reduced microbial activity in the rhizosphere [4]. Microbes play an important role in the mineralization of macro and micro elements for plant growth [5] as well as in metabolism and development of plants [6]; microbes also act as indigenous component in bioaugmentation. Microbes have great potential to speed up the rate of degradation of contaminated soil. Some bacteria, such as *Rhizobium*, *Azotobacter*, and *Azospirillum* cannot only to tie up nitrogen but also dissolve phosphates bonded to Al, Fe, and Ca [7] and in inorganic form [8]. N and P are essential elements in the soil to improve biogeochemical cycles and microbial activity in the rhizosphere of plants that grow in post-mining soil [9]. Bacteria can be a catalyst in nitrogenase cycle to improve the fertility of post mining soil due to its ability to reduce N_2 gas into ammonium in the atmosphere [4] and produce plant growth hormones, such as indole acetic acid (IAA), gibberellins, and cytokinins [10]. Therefore, that nitrogen fixing bacteria (*Rhizobium*, *Azotobacter*, and *Azospirillum*) are considered an important component of biological organic

fertilizers [11], especially for the basic ingredients of biofertilizer, to stimulate the growth of plants through revegetation of degraded lands, such as soil of post tin-mining, particularly in the Island of Bangka.

Plant growth will be successful if it can utilize nitrogen-fixing and phosphate-solubilizing indigenous bacteria of tin mining soil. Bacteria can be inoculated to improve the growth of plants, such as *Sorghum bicolor*. This versatile plant can be used as a source of food, animal feed, and industrial raw materials. The plant can also grow on marginal land, even the growth of plant roots does not show symptoms of poisoning on the soil of post-mining [12]. This research was conducted to contribute to the limited information about the potential of indigenous bacteria as plant-growth promoting rhizobacteria (PGPR) from soil post tin mining soil in Bangka Island. The aim of the research was to obtain nitrogen-fixing indigenous bacteria of tin-mining soil that can dissolve phosphor, produce PMEase enzyme, and IAA hormone and has the potential as PGPR for improving the growth of *Sorghum* in marginal soils (ex tin mining).

Materials and Methods

Location and Soil Sampling. Soil samples were obtained from Bangka Island, Bangka Belitung Province, Indonesia at three locations, namely, 1) reclaimed post tin-mining in Bangka Botanical Garden, Pangkalpinang (S: 0.2° 0,9' 05,3'' and E: 106° 0,9' 28,9''), coded B (the dominance of vegetation: *Pinus merkusii* Jungh & De Vr, and *Malaleuca leucadendron* L); 2) two-year-old abandoned mining soil in the village of Batu Belubang, Subddistrict of Pangkalan Baru, Central Bangka (S: 02° 11' 409'' and E: 106° 11' 265''), coded C (the dominance of vegetation: pioneer plant *Melastoma malabathricum* L. and *Acacia mangium* Willd.); and 3) active mining soil in the village of Semabung Lama, Pangkal Pinang (S: 2° 9' 36'' and the E: 106° 9' 4''), coded D (no vegetation). About 1 kg of soil sample was collected taken by creating a plot size of 50 m × 50 m at 25 sampling points with a depth of 20 cm. Soil samples were transported to laboratories for physical and chemical analyses following Rowell's method [13]. Based on the result, the soil was classified as non-fertile soil.

Isolation and Identification of Bacteria. NFB and phosphate-solubilizing bacteria were screened with a plate assay method using selective media, i.e., YEMA, mannitol Ashby [14], and Caceres [15]. About 10 g of soil was placed into an Erlenmeyer flask containing 90 mL of sterile distilled water and mixed on a rotary shaker at 120 rpm for 30 minutes. The solution was serially diluted from 10^{-2} until 10^{-7}. About 0.2 mL of the soil extract from the serial dilution (10^{-3}, 10^{-5}, and 10^{-7}) was placed in a sterile Petri dish, which was then poured with selective agar media (50 °C), namely, YEMA (10 g of mannitol, 0.5 g of K2HPO4, 0.2 g of MgSO4.7H2O, 0.1 g of NaCl, 1.0 g of yeast, 20 g of agar, 1 L of aquadest + Congo red 2.5 mL/L in 1% solution) for *Rhizobium*, mannitol Ashby (20 g of manitol, 0.2 g of K2HPO4, 0.2 g of MgSO4.7H2O, 0.2 g of NaCl, 0.1 g K2SO4, 5 g of CaCO3, 15 g of agar, and 1 L of aquadest) for *Azotobacter*, and Caceres medium (0.5 g of K2HPO4, 0.2 g of MgSO4.7H2O, 0.1 g of NaCl, 0.5 g of yeast extract, 0.02 g of CaCl2, 0.01 g of FeCl3.6H2O, 5.09 g of D-L malic acid, 4.8 g of KOH, 20 g of agar, and 15 mL of 0.25% Congo red per liter) for *Azospirillum*. All media were adjusted to pH 7.0, inoculated with the sample, and incubated at room temperature for 7 days. The obtained isolates were purified and stored on LB slant. After 3 days of incubation, the isolates were identified based on morphological characteristics, such as cell shape (cocci, rod, or short rod), Gram positive/ negative status, and cell movement (motile, spore formation, single, paired, or chain). Biochemical characteristics of the isolates were determined based on the guidelines of Bergey's Systematic Bacteriology [16]. The bacterial cultures were maintained on LB slants and stored at room temperature for further use in screening and characterization of PGPR activity to promote plant growth.

Functional Characterization of Isolates: Nitrogen Fixation. Selection of NFB was conducted by Dobereiner method [17]. The bacteria from the selective medium were grown into a test tube containing a semi-solid medium of NFB (nitrogen-free bromthymol blue) without N (0.5% DL-malic acid, 0.4% KOH, 0.05 % K_2HPO_4, 0.01% $MgSO_4.7H_2O$, 0.005% $MnSO_4.H_2O$, 0.002% NaCl, 0.001 % $CaCl_2$, 0.005% $FeSO_4.7H_2O$, 0.0002% $Na_2MoO_4.2H_2O$, 0.175% Bacto agar, and 2 mL of 0.5% bromothymol blue). The tube was then incubated at room temperature for 5–7 days. NFB were identified as colonies that formed a circular fog like a ring below the surface of the medium in the tube.

Functional Characterization of Isolates: Indole Acetic Acid Production. Isolates were inoculated into a flask containing 50 mL of *King B* broth with 200 ppm tryptophan as a precursor for biosynthesis of auxin to analyze physiological IAA. The flasks were incubated at room temperature for 24, 48, and 72 hours. About 2 mL of the culture suspension was obtained after each incubation period and centrifuged for 5 minutes. The supernatant was collected, placed into a test tube, and added with 4 mL of Salkowski reagent. IAA production was indicated by the color pink on the extraction of bacteria [18]. Further quantitative analysis of IAA production was conducted with a spectrophotometer at λ = 540 nm by interpolation on the calibration curve of IAA.

Functional Characterization of Isolates: P Solubilization. The ability of bacteria to dissolve P was determined according to the method [19]. The isolates were grown on Pikovskaya's solid media (10 g of glucose, 0.5 g of (NH4)2SO4, 0.3 g of NaCl, 0.3 g of KCl,

0.5 g of $MgSO_4.7H_2O$, 0.03 g of $FeSO_4.7H_2O$, 0.03 g of $MnSO_4.4H_2O$, 10.0 g of $Ca_3(PO_4)_2$, and 20.0 g of Bacto agar. The pH was adjusted to 7.0 by using $Ca_3(PO_4)_2$ as a P source. After 5 days of incubation at room temperature, the isolates that can dissolve P were the colonies surrounded by a clear zone (*halo zone*). Solubilization index (SI = [diameter of colony + diameter of halo zone]/colony diameter) was calculated using the method [20]. Orthophosphate in the liquid media of Pikovskaya was determined after 3, 6, and 9 days of incubation in accordance with the method [21].

Functional characterization of isolates: Phosphomonoesterase enzyme activity. Phosphomonoesterase or phosphatase activity (PMEase) acids and bases were determined following the method [22]. The substrate solution used for PMEase activity included p-nitrophenyl phosphate disodium (pNPP 0.115 M) and p-nitrophenol (pNP). Absorbance was recorded (yellow) using a spectrophotometer with a wavelength of 400 nm. Control was prepared using the same procedure for the sample, but the substrate solution was added with 0.5 M $CaCl_2$ and 0.5 M NaOH. The activity of PMEase was determined using standard method with 1–10 ppm p-nitrophenol, and that in the blank was determined using water distillation. PME-ase activity unit was defined as mol/h of p-nitrophenol. Analysis of PMEase activity was conducted after incubation for 3, 6, and 9 days.

Bioassay in Greenhouse. Sorghum seeds were cleaned with alcohol and soaked in sterile water for 1 hour in Backer glass. The seeds ware arranged in sterile Petri dish lined with filter paper and incubated at room temperature until sorghum seeds germinated. The seeds were soaked in 25 mL of the bacterial inoculant suspension containing 10^9 cells/mL for 1 hour. The inoculum used was the strain with the highest plant growth promoting activity (*Azospirillum lipoferum* CBT4). Two seeds were planted to experimental pots (300 g) containing sterile soil (soil types A, B, C, and D). The experiment adopted factorial completely randomized design with three replications. The first factors were fertilizers, i.e., NPK, *A. lipoferum* CBT4 (10^9) + NPK, *A. lipoferum* CBT4 (10^9), and without fertilizer (control). The second factors were soil types, i.e., A (fertile soil from Cibinong), B (soil from post-mining reclaimed into Bangka Botanical Garden), C (soil from post tin mines 2 years after mining), and D (soil from active tin mining). At 45 days after planting, the growth of sorghum was evaluated by measuring plant height, and dry weight. The number of bacterial populations from each pot was counted using plate count method [23]. The results will indicate the effectiveness of bacteria as a candidate for post mining land reclamation.

Results and Discussion

Isolation and Identification of Bacteria. Twenty-five isolates were obtained from the selective medium (YEMA with Congo red, Caceres, and Mannitol Ashby). Thirteen isolates (BBG1-BBG13) were isolated from soil B, six isolates (CBT1- CBT6) were isolated from soil C, six isolates (DBT1- DBT6) were isolated from soil D, and only 11 isolates were known (Table 1).

Based on the results of isolation (Table 1), the soil of the reclaimed tin mining area, namely, Bangka Botanical Garden (B), had more isolates, particularly in the rhizosphere, than the 2-year-old abandoned tin mining soil (C) and active tin mining soil (D). The same results were reported as such, the diversity and population of bacteria in the rhizosphere were higher than those in areas without vegetation (open land) [24-27]. The diversity and determinants of bacterial communities in the soil or rhizosphere were dependent on soil type, soil depth, plant type, number of plants that grow in that habitat [28][29], availability of nutrients, pH, water content, soil texture, and artificial interferences, such as artificial farming, pesticides, and pollution [30].

Based on the analysis of the bacterial population in the tin mining soil, soil type A was found to be fertile with bacterial content 10^7 CFU gram soil, whereas soil types B, C, and D were less fertile with bacterial population density of less than 10^7 CFU/gram of soil (Table 2). Fertile soil should have a bacterial content of at least 10^7 CFU/gram of soil [31]. The low content of bacterial populations on tin mining soil, may be caused by low nutrient content . Heydarnezhad *et al*. [32] reported that the land of post tin mining has low nutrient status and stabilization structures. The availability of organic materials such as macro and micro elements in the soil is a limiting factor to the growth of bacteria. Bacterial populations are also affected by the type and number of plants that grow in that area because the plant root would emit beneficial nutrients to promote bacterial growth [33].

Twenty-five indigenous isolates were obtained as follows: 13 isolates from soil type B, 6 isolates isolated from soil type C, and 6 isolates isolated from soil type D. Based on the identification guidelines of Bergey's Systematic Bacteriology [16], only 11 of the isolates were identified as *Azospirillum* sp. (BBG3), *Azotobacter paspalii* (BBG4), *Bacillus weihenstephanensis* (BBG6), *Klebsiella* sp. (BBG7), *Azospirillum* sp. (BBG8), *Azospirillum* sp. (BBG9), *Azotobacter chroococcum* (BBG13), *Rhizobium* sp. (CBT2), *Azospirillum lipoferum* (CBT4), *Azospirillum* sp. (CBT5), and *Enterobacter cloacae* (DBT6).

Table 1. Location of Sampling and Results of Isolation and Identification

Location	Source of material	Selective medium	Isolate code	Identification result
B	Soil	Mannitol ashby	BBG1	Unidentified
S: 0.2º0,9'	Soil	Mannitol ashby	BBG2	Unidentified
05,3"	Soil	Caseres	BBG3	*Azospirillum* sp.
and	Rhizosphere of *Pinus merkusii* Jungh.& De Vr	Mannitol ashby	BBG4	*Azotobacter paspalii*
E:106º0,9'	Soil	YEMA congo red	BBG5	Unidentified
28,9"	Rhizosphere of *Citrus* sp.	Natrium Agar	BBG6	*Bacillus weihenstephanensis*
	Rhizosphere of *Pinus merkusii* Jungh.& De Vr	Natrium Agar	BBG7	*Klebsiella* sp.
	Rhizosphere of *Vigna unguiculata* L	Caseres	BBG8	*Azospirillum* sp.
	Watery soil	Caseres	BBG9	*Azospirillum* sp.
	Soil	YEMA congo red	BBG10	Unidentified
	Rhizosphere of *Malaleuca leucadendron* L	YEMA congo red	BBG11	Unidentified
	Rhizosphere of *Citrus* sp.	YEMA congo red	BBG12	Unidentified
	Rhizosphere of *Malaleuca leucadendron* L	Mannitol ashby	BBG13	*Azotobacter chroococcum*
C	Rhizosphere of *Acasia mangium* Willd.	YEMA congo red	CBT1	Unidentified
S: 2º 9' 36"	Nodule of *Acasia mangium* Willd.	YEMA congo red	CBT2	*Rhizobium* sp.
and	Soil	Mannitol ashby	CBT3	Unidentified
E:106º 9'	**Rhizosphere of *Melastoma malabathricum* L**	**Caseres**	**CBT4**	***Azospirillum lipoferum***
4"	Rhizosphere of *Acasia mangium* Willd.	Caseres	CBT5	*Azospirillum* sp.
	Rhizosphere of *Acasia mangium* Willd.	Mannitol ashby	CBT6	Unidentified
D	Soil	Caseres	DBT1	Unidentified
S: 2 º 9'	Soil	Caseres	DBT2	Unidentified
36"	Soil	Mannitol ashby	DBT3	Unidentified
and	Soil	YEMA congo red	DBT4	Unidentified
E: 106º 9'	Soil	Mannitol ashby	DBT5	Unidentified
4"	Soil	Natrium Agar	DBT6	*Enterobacter cloaceae*

Table 2. Analysis of the Bacterial Population in the tin Mining soil

Analysis	Analysis methods	Type of soil sample			
		A	B	C	D
Total Bacteria Population (CFU/gram soil)	TPC (NA)	$10^6 - 10^7$	$10^5 - 10^6$	10^4 - 10^5	$10^3 - 10^4$

Note: A = fertile soil from Cibinong, B = Soil from post-mining reclaimed turned into Bangka Botanical Garden, **C =** Soil from post-tin mines has been abandoned two years, D = soil from tin mining is still mined

Functional Characterization of Isolates: Nitrogen Fixation Ability. The results showed that the functional characterization analysis of 25 isolates, 20% positive had nitrogenase activity (Figure 1). Nitrogen is a crucial nutrient for plants but could not be absorbed without the aid of NFB in the rhizosphere. The diversity of NFB in the rhizosphere depends on the host plant.

The growth of 25 isolates was tested in test tubes containing semi-solid medium of nitrogen-free bromthymol blue (NFB). Only six isolates formed a circle mist like a ring below the surface of the medium after 3 days of incubation at room temperature (Figure 1). The ring formation was caused by the nitrogenase activity of NFB. Similar results were reported that is, the activity of NFB in producing nitrogenase was indicated by the formation of circular fog rings below the surface of NFB semi-solid medium [34]. The obtained nitrogenase activity-producing isolates included *Azospirillum* sp.1, *Bacillus weihenstephanensis*, *Azospirillum* sp 2, *Azospirillum* sp 3, and *Azospirillum lipoferum*. The formation of nitrogenase indicates that these six bacterial species are NFB. Characteristic properties (nitrogen fixation ability, produce IAA, and Acc-diamininase) are found in several bacteria such as *Azotobacter* and *Azospirillum*. The isolates were transferred back to the Caseres medium to further confirm if they are NFB. If the grown single colony had irregular round shaped, reddish color, and elevation as well as flat, smooth, shiny surface with flat edges, then the bacteria are indeed NFB [16]. *Bacillus* is a group of phosphate-solubilizing bacteria that also exhibits nitrogenase activity. The present result is consistentwith that in the study who reported that *Bacillus* is a producer of nitrogenase [35].

The results showed that the functional characterization analysis of all isolates (25 isolates), 100 % produced IAA. (Table 3). IAA is a growth hormone needed by the plant for growth. The isolated bacteria were able to produce IAA in the group of PGPR. All isolates (25 isolates) grown in the liquid medium containing 200 ppm

precursor L-Tryptophan produced IAA after incubation for 0, 24, 48, and 72 hours (Table 3). According to the

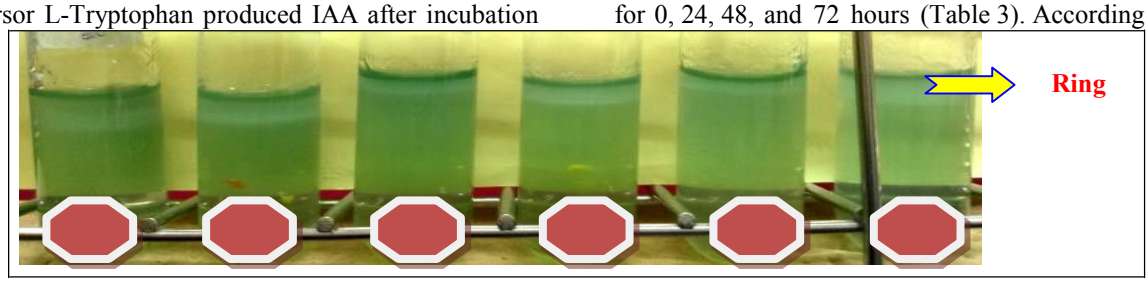

Figure 1. Nitrogenase Activity of Nitrogen Fixation Bacteria

Table 3. Production of IAA by Indigenous Isolates after Incubation for 0–72 Hours

Isolates	IAA production (ppm)			
	0 hour	24 hour	48 hour	72 hour
BBG1	0.339 ^{cde}	2.118 ^{gh}	3.248 ^{e}	4.391 ^{gh}
BBG2	0.388 ^{ef}	1.667 ^{fg}	2.394 ^{cd}	3.779 ^{f}
Azospirillum spp1 BBG3	0.203 ^{b}	1.064 ^{bcde}	1.890 ^{c}	5.400 ^{jk}
Azotobacter paspalii BBG4	0.315 ^{cd}	3.439 ^{i}	3.994 ^{fg}	1.348 ^{c}
BBG5	0.364 ^{de}	2.248 ^{h}	3.836 ^{f}	5.467 ^{k}
Bacillus weihenstephanensis BBG6	0.230 ^{b}	1.221 ^{cdef}	2.048 ^{c}	2.806 ^{e}
Klebsiella sp. BBG7	0.439 ^{gh}	1.264 ^{cdef}	0.558 ^{a}	0.303 ^{a}
Azospirillum spp2 BBG8	0.364 ^{de}	0.970 ^{abcd}	2.276 ^{cd}	5.133 ^{jk}
Azospirillum spp3 BBG9	0.476 ^{h}	0.794 ^{abcd}	2.679 ^{d}	3.803 ^{f}
BBG10	1.021 ^{j}	1.291 ^{def}	3.218 ^{e}	4.697 ^{hi}
BBG11	0.364 ^{de}	1.476 ^{ef}	4.321 ^{fgh}	4.924 ^{ij}
BBG12	0.415 ^{fg}	3.658 ^{i}	4.812 ^{h}	10.303 ^{m}
Azotobacter chroococcum BBG13	0.339 ^{cde}	0.806 ^{abcd}	0.330 ^{a}	0.303 ^{a}
CBT1	0.342 ^{cde}	0.918 ^{abcd}	1.218 ^{b}	1.564 ^{cd}
Rhizobium sp.CBT2	0.315 ^{cd}	3.942 ^{i}	16.709 ^{i}	29.603 ^{n}
CBT3	0.303 ^{c}	0.879 ^{abcd}	1.118 ^{b}	4.036 ^{fg}
***Azospirillum lipoferum* CBT4**	**0.670** ^{i}	**4.994** ^{j}	**19.773** ^{j}	**31.730** ^{n}
N Azospirillum spp4 CBT5	0.318 ^{cd}	0.773 ^{abc}	0.461 ^{a}	0.303 ^{a}
CBT6	0.333 ^{cd}	0.700 ^{ab}	0.542 ^{a}	0.303 ^{a}
DBT1	0.148 ^{a}	0.500 ^{a}	0.352 ^{a}	0.303 ^{a}
DBT2	0.303 ^{c}	2.488 ^{h}	3.258 ^{e}	1.991 ^{d}
DBT3	0.430 ^{gh}	1.555 ^{ef}	4.658 ^{h}	3.218 ^{e}
DBT4	0.624 ^{i}	1.591 ^{f}	4.042 ^{fg}	0.845 ^{b}
DBT5	0.324 ^{cd}	3.736 ^{i}	4.027 ^{fg}	1.824 ^{cd}
Enterobacter cloaceae DBT6	0.339 ^{cde}	1.261 ^{cdef}	4.488 ^{gh}	6.824 ^{l}

Note: N umber in the same group followed by the same letter in the same columns are not significantly different ($p < 0.05$) as determined by Duncan's test

availability of suitable precursor primarily affects the microbial secretion of secondary metabolites; so, the addition of tryptophan could increase the production of IAA because tryptophan is a precursor for the IAA pathway [36]. The average yield of IAA produced ranged from 0.148 ppm to 31.73 ppm. Isolate DBT1 produced the lowest amount of IAA (0.14, 0.500, 0.352, and 0.303 ppm, respectively) after incubation for 0, 24, 48, and 72 hours. Isolate CBT4 produced the highest amount of IAA (0.60, 4.994, 19.773, and 31.73 ppm, respectively) after incubation for 0, 24, 48, and 72 hours. DBT1 isolates from active tin mining soil was unidentified, and CBT4 isolates from the rhizosphere of *Melastoma malabathricum* L were identified as *Azospirillum lipoferum*. About 80% of microbial isolates, particularly strains of *Azospirillum* [37] that live

in the rhizospheres of plants, had the ability to synthesize and release auxin (IAA) as secondary metabolites [38]. The concentration of indolic compound formed by bacteria will stimulate the formation of lateral and adventitious roots, which could increase the absorption of nutrients, including phosphate [38].

Functional Characterization of Isolates: Phosphate Solubilizing Ability. The functional characterization analysis of the 25 isolates indicated 56% dissolved P and 56% produced PMEase (Table 4). The reaction of halo zone and solubilization index (SI) in the solid Pikovskaya media after incubation for 5 days and dissolving P in the liquid media of Pikovskaya after 3–9 days of incubation with P tricalcium phosphate sources were investigated.

As shown in Table 4, the 14 isolates obtained reacted positively and the cells were surrounded by a clear zone (halo zone). The formation of halo zone around the isolate colony is an indication of dissolving P attached to "tricalcium phosphate" as a source of P in the solid Pikovskaya medium. The phosphate solubilization index (SI) was the highest in *Azospirillum lipoferum* CBT4 (6.00) and the lowest in isolate DBT6 (2.50). SI value is an indication of the amount of P that can be released by bacteria [39]. *Azospirillum lipoferum* CBT4, which had an SI value of 6.00, could dissolve the highest amount of P in the liquid Pikovsakaya medium, i.e., 5.154, 11.562, and 6.671 ppm after incubation for 3, 6, and 9 days, respectively; the optimum P dissolution was detected after 6 days of incubation. The dissolution of P bound by bacteria in the liquid Pikovskaya medium was affected by aeration and duration of incubation; however, in solid media, the incubation did not always increase the size of the clear zone. Stated that bacteria that can remove inorganic phosphate from $Ca_3(PO_4)_2$ in the liquid medium could dissolve P to be available to plants. In the present study, *Azospirillum lipoferum* CBT4 was identified NFB and phosphate solubilizing bacteria [40]. Reported that NFB genera, such as *Azotobacter*, *Azospirillum*, and *Rhizobium* could dissolve P bound by forming a clear zone around the colony [41]. According to the success of P dissolution by bacteria that dissolve fine particles from $Ca_3(PO_4)_2$ bonds rely on temperature, humidity, pH, food supply, and environmental conditions for microbial growth in liquid culture [14]. The mechanism associated with the ability of bacteria to produce PMEase enzymes and organic acids, such as succinic acid, acetic acid, propionic acid, glycolic acid, fumaric acid, oxalic acid, lactic acid, and ketoglutarate acid has important role in mineralizing organic P present in soil [14]. PMEase is a phosphatase enzyme that is involved in mineralization of organic phosphates.

Table 4. P Solubilization in Solid and Liquid Media of Pikovskaya with $Ca_3(PO_4)_2$ Source After Incubation for 3–9 Days

Isolate	P Solubilization with $Ca_3(PO_4)_2$ source				
	Solid medium		Liquid medium (ppm)		
	Halozone	SI (5 day)	3 day	6 day	9 day
BBG1	-	-	-	-	-
BBG2	-	-	-	-	-
Azospirillum spp1 BBG3	-	-	-	-	-
Azotobacter paspalii BBG4	+	3.67	5.003^{de}	10.368^{bc}	3.819^{ab}
BBG5	+	3.67	5.072^{de}	11.008^{d}	4.349^{cde}
Bacillus weihenstephanensis BBG6	-	-	-	-	-
Klebsiella sp. BBG7	-	-	-	-	-
Azospirillum spp2 BBG8	-	-	-	-	-
Azospirillum spp3 BBG9	-	-	-	-	-
BBG10	+	5.00	5.085^{de}	10.214^{bc}	3.909^{abc}
BBG11	+	3.67	4.691^{bcde}	11.505^{e}	4.507^{de}
BBG12	+	4.00	4.647^{bcd}	10.449^{bc}	4.193^{bcd}
Azotobacter chroococcum BBG13	-	-	-	-	-
CBT1	+	3.67	4.636^{bcd}	10.092^{ab}	3.986^{abc}
Rhizobium sp.CBT2	+	3.67	4.227^{ab}	11.008^{d}	4.621^{de}
CBT3	+	4.00	5.072^{de}	11.008^{d}	4.763^{e}
***Azospirillum lipoferum* CBT4**	**+**	**6.00**	**5.154^{e}**	**11.562^{e}**	**6.671^{f}**
Azospirillum spp4 CBT5	-	-	-	-	-
CBT6	+	3.67	4.484^{abc}	9.606^{a}	3.672^{a}
DBT1	+	3.67	4.908^{d}	10.650^{cd}	4.026^{abc}
DBT2	-	-	-	-	-
DBT3	-	-	-	-	-
DBT4	+	2.50	4.073^{a}	10.449^{bc}	4.225^{bcd}
DBT5	+	3.67	4.784^{cde}	11.116^{de}	4.371^{cde}
Enterobacter cloaceae DBT6	+	3.67	4.756^{cde}	10.001^{ab}	4.369^{cde}

Note: Number in the same group followed by the same letter in the same columns are not significantly different ($p < 0.05$) as determined by Duncan's test

Functional Characterization of Isolates: phosphomonoesterase Activity. All isolates had phosphomonoesterase enzyme activity (PMEase) in acid and alkaline reactions after 3–9 days of incubation (Table 5). *Azospirillum lipoferum* CBT4 showed the highest PMEase activity in the acid and base media compared with the other isolates. The isolates produced 0.941 µmol nitrophenol/mL/hour of PME-ase bases after 3 days of incubation, and the amount decreased after 6 and 9 days of incubation. Moreover, 0.670 µmol of nitrophenol/mL/hour of PME-ase acid was produced after 6 days of incubation, and the amount decreased after 9 days. The lowest of acids and bases PMEase were found in isolates BBG11 and CBT1. The highest PMEase of acid and bases was lowere than the PMEase of acids bases (14.5830 and 13.4219 mg/L) produced by *Azospirillum* in the study [42]. The PMEase activity increased in acids and bases during incubation due to the induction process. According [43], the induction process occurs because of the limited amount of P in Pikovskaya media and bacteria requires a high P for survival in the liquid Pikovskaya media.

Recapitulation on the functional characterization of the isolates obtained one superior isolate (*Azospirillum lipoferum* CBT4) for dissolving inorganic phosphate compounds into organic phosphate compounds and for producing PMEase enzymes and IAA hormones. This isolate was also used for the bioassay test to promote the growth of *S. bicolor* by indigenous NFB on post tin mining soil.

Bioassay in Greenhouse. Plant shoot length, plant dry weight, and bacterial population in the growth media (soil in the pot) were evaluated 45 days after planting. The effects of indigenous *Azospirillum lipoferum* CBT4 as PGPR on *S. bicolor* L Moench seedlings in soil of post tin mining are shown in Tables 6 and 7.

Based on Table 6, the bacterial population in each pot containing soil types A, B, C, and D decreased in all treatments. The density of the bacterial population inoculated in the sorghum seed and soil after 45 days of incubation (harvest) of 10^9 CFU/g of soil/pot decreased to 10^7 CFU/g of soil. The population density of *Azospirillum* after harvest ranged from 0 to 7.53 × 10^7CFU/g of soil, with the highest population of 7.53 × 10^7CFU/g of soil on soil type A and the lowest population of 0.35 × 10^7 CFU/g of soil on soil type D. The average population density of *Azospirillum* per pot (soil A, B, C. D) was 10^7 CFU/g of soil. These results are higher than those in previous studies, which reported that the population density of the bacteria inoculated 10^9 CFU/g soil dropped to 10^5 CFU/g of soil after sorghum was aged for 30 days (unpublished).

Table 5. Phosphomonoesterase (PMEase) Enzyme Activity After Incubation for 3–9 Days

Isolat	Phosphomonoeterase (PMEase) enzyme activity (µmol nitrophenol/ml/hour), after incubation:					
	3 day		6 day		9 day	
	Acid	Basa	Acid	Basa	Acid	Basa
BBG4	0.214 ^{c}	0.154 ^{c}	0.343 ^{cd}	0.136 ^{cd}	0.309 ^{ef}	0.186 ^{c}
BBG5	0.128 ^{b}	0.128 ^{bc}	0.146 ^{a}	0.075 ^{ab}	0.101 ^{bc}	0.055 ^{a}
BBG10	0.083 ^{ab}	0.081 ^{ab}	0.141 ^{a}	0.104 ^{bc}	0.084 ^{ab}	0.056 ^{a}
BBG11	0.056 ^{a}	0.053 ^{a}	0.152 ^{a}	0.091 ^{abc}	0.139 ^{c}	0.123 ^{b}
BBG12	0.224 ^{c}	0.209 ^{d}	0.411 ^{e}	0.250 ^{e}	0.355 ^{f}	0.023 ^{a}
CBT1	0.069 ^{a}	0.069 ^{a}	0.124 ^{a}	0.045 ^{a}	0.051 ^{ab}	0.042 ^{a}
CBT2	0.327 ^{d}	0.327 ^{e}	0.339 ^{c}	0.242 ^{e}	0.337 ^{f}	0.265 ^{d}
CBT3	0.547 ^{e}	0.432 ^{f}	0.654 ^{fg}	0.271 ^{e}	0.596 ^{h}	0.497 ^{ef}
CBT4	**0.615** ^{f}	**0.491** ^{f}	**0.670** ^{g}	**0.377** ^{f}	**0.639** ^{h}	**0.437** ^{f}
CBT6	0.048 ^{a}	0.051 ^{a}	0.222 ^{b}	0.110 ^{bc}	0.266 ^{de}	0.227 ^{d}
DBT3	0.085 ^{ab}	0.099 ^{ab}	0.235 ^{b}	0.174 ^{d}	0.226 ^{d}	0.231 ^{d}
DBT4	0.048 ^{a}	0.050 ^{a}	0.607 ^{f}	0.353 ^{f}	0.046 ^{a}	0.016 ^{a}
DBT5	0.082 ^{ab}	0.068 ^{a}	0.427 ^{e}	0.378 ^{f}	0.447 ^{g}	0.403 ^{f}
DBT6	0.052 ^{a}	0.057 ^{a}	0.392 ^{de}	0.381 ^{f}	0.432 ^{g}	0.334 ^{e}

Note: Number in the same group followed by the same letter in the same columns are not significantly different ($p < 0.05$) as determined by Duncan's test

Table 6. Population of *Azospirillum lipoferum* in Soil Pot After Harvesting (45 days)

Treatments soil in the pot	Population of *Azospirillum lipoferum* in soil type (CFU/g soil/pot)			
	A	B	C	D
Control	0	0	0	0
NPK (Chemical Fertilizer)	0	0	0	0
CBT4 isolate *(Azospirillum lipoferum*)	**7.53 x 10^7**	**1.95 x 10^7**	**1.40 x 10^7**	**0.95 x 10^7**
NPK+ CBT4 isolate	3.45 x 10^7	1.85 x 10^7	0.80 x 10^7	0.35 x 10^7

Table 7. Effect of *Azospirillum lipoferum* CBT4 Inoculants on Sorghum Seedlings (Gram)

Parameters	Fertilizer	Treatments			
		A			D
Plant length (cm)	Control	33.33 ^{a} (d)	30.67 ^{a} (c)	16.67 ^{a} (b)	**14.33 ^{a} (a)**
	NPK	37.50 ^{b} (c)	37.00 ^{b} (c)	21.00 ^{b} (b)	16.67 ^{b} (a)
	CBT4 isolate	**51.00 ^{d} (d)**	37.67 ^{b} (c)	29.67 ^{c} (b)	23.00 ^{c} (a)
	NPK+ CBT4 isolate	47.17 ^{c} (d)	44.60 ^{c} (c)	31.83 ^{d} (b)	27.67 ^{d} (a)
Leaf dry weight (gram)	Control	0.170 ^{a} (b)	0.170 ^{a} (b)	0.040 ^{a} (a)	**0.030 ^{a} (a)**
	NPK	0.330 ^{c} (c)	0.250 ^{b} (b)	0.04 ^{a} (a)	0.050 ^{b} (a)
	CBT4 isolate	0.260 ^{b} (c)	**0.450 ^{d} (d)**	0.20 ^{dc} (b)	0.06 ^{b} (a)
	NPK+ CBT4 isolate	0.39 d (d)	0.370 ^{c} (c)	0.17 ^{cb} (b)	0.13 ^{c} (a)
Root dry weight (gram)	Control	0.18 ^{a} (a)	0.16 ^{a} (a)	0.12 ^{a} (a)	**0.08 ^{a} (a)**
	NPK	0.33 ^{ab} (a)	0.37 ^{a} (a)	0.22 ^{ab} (a)	0.15 ^{a} (a)
	CBT4 isolate	**0.42 ^{b} (a)**	0.21 ^{a} (a)	0.48 ^{c} (a)	0.21 ^{ab} (a)
	NPK+ CBT4 isolate	**0.42 ^{b} (a)**	0.32 ^{a} (a)	0.49 ^{c} (a)	0.22 ^{ab} (a)

Notes: Number in the same group followed by the same letter in the same columns (black latter) and same rows (red latter) are not significantly different ($p < 0.05$) as determined by Duncan's test

The indigenous isolates of a post-tin mining soil survived with the assumption that the population did not decrease if returned (inoculated) back to the habitat (ground) of the bacteria where it came from. *Azospirillum lipoferum* was isolated from soil type C, which was post tin mining soil of a land abandoned for 2 years. This phenomenon could be due to several factors, such as bacteria undergo initial shock and have to adapt to the native habitat (soil type C) or a new habitat (soil type A, B, D); they took a long time to be able to survive in their new habitat. Bacteria need a certain time to adapt in response to a stimulus that was considered foreign or never encountered in previous habitat [3]. Another factor may be due to the physical properties (texture, moisture, pH, and aeration) and the chemical content of the soil (nutrients) that provide less support when the bacteria are inoculated into the soil. The availability of organic materials (macro nutrients and micro elements) in soil [32], pH, water content, soil texture [29], type of crop, and soil types [30] are barriers to the growth of bacteria in the soil. According to Wibowo [3], several species of bacteria can adapt and learn to grow in habitats with different temperatures, acidity, and extreme oxygen pressure; one of which is *Azospirillum* bacterium, which can survive (10^7 CFU/g of soil) in the soil in the experiment. Obaton [31] presented that the minimum limit of the bacteria population that can fertilize the soil was 10^7 CFU/g of soil. This finding was evidenced by *Azospirillum lipoferum* CBT4 bacteria inoculated in the soil and the sorghum plant at the environment of tin mining soil; although the bacteria population decreased, it effectively promoted the growth of sorghum seedlings (Table 7).

Based on Table 7, *Azospirillum lipoferum* CBT4 survived in the four types of soil (A, B, C, and D) and stimulated the growth of seedlings of sorghum to harvest (45 days). *Azospirillum* lived freely in the environment of diverse plants, including sorghum [44]. After 45 days, the sorghum plant had plant height, leaf dry weight, and root dry weight of 14.33–51.00 cm, 0.030−0.450 g, and 0.083−0.420 g, respectively. The highest value of plant height, leaf dry weight, and root dry weight were obtained in young sorghum plants that grew on soil type A inoculated with *Azospirillum lipoferum* CBT4 (51.00 cm), on soil type B inoculated with *Azospirillum lipoferum* CBT4 (0.450 gram), and on

soil type A inoculated with *Azospirillum lipoferum* CBT4 plus NPK (0.420 g) and *Azospirillum lipoferum* CBT4 (0.417 g). The lowest values were obtained in the control plants that grew in soil type D.

Azospirillum lipoferum CBT4 with a population density of 10^7 CFU/g of soil could promote the growth of plants in pots containing soil types A, B, C, and D up to 53%, 45.4%, 90.9%, and 60.5% when compared with control plants. Previous studies reported that the population density of *Azospirillum* 10^1 CFU/gram soil increased the harvest of cereals by 10%–30% [45] as well as by 75% in summer and 50% in spring [46]. The total population of these bacteria could increase the harvest yield of cereals in the fertile soil but inhibited the development of plant roots [47]. The total population of bacteria 10^7 CFU/gram soil also inhibited the growth of plant roots in the soil medium type D (0.147 g/pot). The level of optimization on the growth of seedlings of cereals and vegetables needed *Azospirillum* 10^1 CFU/gram soil [48], [49]. Seed corn plants required 10^7 CFU/gram soil [50], and tomato plants required 10^8 CFU /gram soil [51].

Azospirillum has been known for years as PGPR [52]. In certain environmental conditions and soil type, *Azospirillum* can positively influence the growth of plants. In the present study, *Azospirillum* adapted and survived in the medium with soil types A, B, C, and D and could promote the growth of sorghum seedlings. The mechanism of promoted sorghum growth by *Azospirillum* not be separated from N and P, and IAA hormone produced by *Azospirillum* [53]. Therefore, *Azospirillum* belongs to the PGPR group. Reported that PGPR can survive and grow in soils contaminated with heavy metals, thereby stimulating plant growth. In this experiment, *Azospirillum lipoferum* CBT4 was the best PGPR isolated from tin mining soil and had the potential for promoting the growth of sorghum plants by providing phosphate and IAA hormone [54,55]. Consistent with the present findings, reported that *Azospirillum* sp. stimulated the formation of new roots, promoted plant growth, and increased the dry weight of plants on marginal land [56].

Conclusion

This study successfully obtained 25 indigenous isolates on soil types B, C, and D, including rhizosphere bacteria with Ca-P solubilizing ability and IAA production. Eleven isolates were identified as PGPR, and eight isolates were identified as NFB [*Azotobacter paspalii* (BBG3: NFBnS), *Azospirillum* sp. (BBG4; NFBnS), *Bacillus weihenstephanensis* (BBG6), *Klebsiella* sp. (BBG7), *Azospirillum* sp. (BBG8: NFBnS), *Azospirillum* sp. (BBG9: NFB), *Azotobacter chroococcum* (BBG13; NFB), *Rhizobium* sp. (CBT2: NFB), *Azospirillum* sp. (CBT4: NFB), *Azospirillum lipoferum* (CBT5; NFB), and *Enterobacter cloacae* (DBT6)] and obtained 20% isolates had nitrogenase activity, 56% isolates which can P solubilizing, 56% isolates had PMEase activity, and 100% isolates coul produce of IAA. All of the isolates belong to the PGPR group. *Azospirillum lipoferum* CBT4 is the best potential PGPR isolate with the highest production of IAA hormone, dissolved P, SI, and PMEase, survived in tin mining soil, and increased *S. bicolor* growth on soil types A, B, C, and D. This isolate could be candidate for biofertilizer for sorghum in tin mining soil to support rehabilitation programs for plant growth in post tin mining soil.

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