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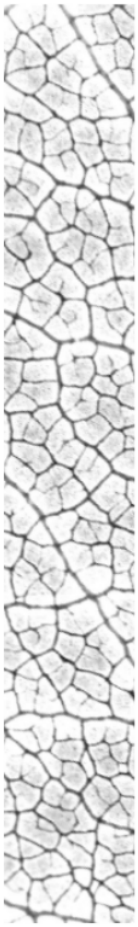
Submission date: 20-Mar-2023 11:16AM (UTC+0700)
Submission ID: 2041331384
File name: on-of-indigenous-phosphate-solubilizing-microbe-_Nurul_Husna.pdf (561.08K)
Word count: 6750
Character count: 35731



EurAsian Journal of BioSciences
Eurasia J Biosci 13, 267-276 (2019)

Microbial selection of indigenous phosphate solubilizing microbe of tidal land as an inoculant in biochar

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Abstract

This study was aimed to obtain phosphate solubilizing microorganism (PSM) isolates, which can be tolerant high Al and Fe content and low pH, to be utilized as an inoculant in biochar. Microbial isolation of indigenous PSMs was carried out on intertidal zone, with samples from four typological landscape (A, B, C, and D) collected from four villages in Delta Telang, Banyuasin, South Sumatra. The isolates that produced clear zones were purified and assayed with varying levels of $AlPO_4$, $FePO_4$, and pH. The selected PSM isolate was further tested to evaluate the phosphate-dissolving ability with liquid Pikovskaya media. The microbes obtained were three bacterial isolates and one phosphate-solubilizing fungus, which were identified as *Paenibacillus alvei, Burkholderia cepacia, Acinetobacter baumannii,* and *Penicillium variabile*. The identified microbes were tolerant to Al and Fe at concentrations up to 1500 mg L^{-1} and pH 3.0. Mixed cultures of the four isolates on liquid Pikovskaya media were able to dissolve $Ca_3(PO_4)_2$ at 57.45 mg L^{-1}, $AlPO_4$ at 13.98 mg L^{-1} and $FePO_4$ at 8.46 mg L^{-1}. PSM viability on four types of biochar after three months of storage ranged from log population of 1.57 x 10^8–1.73 x 10^9 (log population 8.20–10.23 CFU g^{-1}). The highest PSM viability was obtained in coconut shell biochar.

Keywords: biochar, phosphate-solubilizing microbe, Tidal Wetland, tolerant

Husna N, Budianta D, Munandar, Napoleon A (2019) Microbial selection of indigenous phosphate solubilizing microbe of tidal land as an inoculant in biochar. Eurasia J Biosci 13: 267-276.

INTRODUCTION

Agricultural prospects can be applied in the intertidal zone to support increased agricultural production and food sovereignty. Based on typology, the intertidal zone in Indonesia has few types, i.e., peatland that is approximately 10,890,000 ha (54.26%), acid sulfate land that is 6,670,000 ha (33.24%), potential area that is 2,070,000 ha (10, 31%) and saline soil that is 440,000 ha (2.19%), while in the South Sumatra intertidal zone is an agricultural area with 961,000 ha (Alihamsyah 2004, BBSDLP 2014, Ghazanfarpour 2013). Common problems with potential and acid sulfate lands in the intertidal zone are the toxicity of aluminum (Al) and iron (Fe), low macronutrient content (especially phosphorous (P) due to fixation by Al and Fe), low pH, the presence of pyrite and the deficiency of divalent cations such as calcium (Ca) and magnesium (Mg), which make the land less productive (Harahap et al. 2014, Koesrini and Anwar 2017, Masganti et al. 2017, Noya et al. 2014, Suwanda and Noor 2014, Türkyürek et al. 2015).

Using biochar as soil enhancers offer a solution to overcome the constraints of soil fertility in the intertidal zone (Annisa and Nursyamsi 2016, Masulili 2010). Biochar is an organic material that is processed by heating with very little oxygen (pyrolysis). The application of biochar has been widely carried out and has a positive impact on improving the chemical and physical properties of soil. Biochar does not interfere with the carbon-nitrogen balance, but it does help increase the availability of water and nutrients for plants. Biochar decreases soil density, exchangeable Al and Fe, and increases porosity, water content, organic carbon (C), phosphate, cation exchange capacity (CEC), potassium (exchangeable K) and exchangeable Ca (Dang et al. 2016, Gul et al. 2015, Kuppusamy et al. 2016, Zheng et al. 2018).

Biochar has an ideal pore size for soil microbial growth (Vanek et al. 2016). The presence of biochar in the soil can be useful for bacteria, fungi, and other soil microbes. Chen et al. (2013) reported that biochar microbes are protected from external conditions, such as pH, toxic materials and competition with other microbes. These advantages make biochar as a soil

Received: October 2018
Accepted: December 2018
Printed: May 2019

enhancer and carrier agent (Egamberdieva et al. 2017, Hale et al. 2015, Kheiry et al. 2013) beneficial microbes such as phosphate-solubilizing microbes (PSMs).

PSMs are capable of dissolving P in the soil, which is fixed by Al and Fe in acidic soils and Ca in alkaline soils, by producing organic acids, such as formic acid, acetic acid, citric acid, propionic acid and fumaric acid (Behera et al. 2014, Li et al. 2016). These organic acids react with Ca^{2+}, Fe^{3+} and Al^{3+} ions which bind P to a stable form (chelate) so that bound P is exempt by organic acids and can be used for plants (Behera et al. 2014). Dissolution of P by microbes that do not produce organic acids occurs through the release of protons (H+ ions) in the process of respiration, assimilation of ammonium (NH^{4+}) and competition between organic anions and orthophosphates on colloidal surfaces (Illmer and Schinner 1995). Dissolution of P by PSMs also occurs due to chelation and exchange reactions, which are caused by decreased rhizosphere pH due to the presence of organic acids (Khan et al. 2014, Wei et al. 2017).

PSMs are naturally present in the soil, but their ability to dissolve P depends on their type, adaptability, and ability to live in different environments, i.e., pH, P availability and organic C (Khan et al. 2014, Li et al. 2016, Liu et al. 2014). If PSMs from one soil inoculated another soil, they might not necessarily maintain the same ability to dissolving phosphate. Studies involving PSMs capable of adapting to various agricultural land agroecosystems, such as the intertidal zone, are still required for further observations.

The indigenous PSM utilization is one way to increase the viability and the ability to dissolve phosphate in intertidal zones. Through this research, we hope to find PSMs with physiological characteristics that are capable of adapting to tidal lands, such as tolerance to high Al and Fe content and low pH conditions and high viability as an inoculum material for biochar. The use of PSMs and biochar simultaneously is expected to create a soil enhancer that improves the chemical, physical and biological fertility of the soil, which will increase the productivity of the intertidal zone in a sustainable manner.

MATERIALS AND METHODS

This study was conducted in Palembang, South Sumatra from May to November 2017 at the Microbiology Laboratory of the Biology Department, Faculty of Mathematics and Natural Sciences, Sriwijaya University, Inderalaya and at the Laboratory of Ida Bajumi University Palembang. Samples were taken from four landscape typologies. The locations were Sukatani village (typology A = always inundated by tidal water), Mulyasari village (typology B = only inundated by large tides), Banyu Urip Selatan village (typology C = not inundated, depth of water surfaces < 50 cm) and Banyu Urip Utara village (typology D = not inundated, bottom of water surfaces > 50 cm), all within the Tanjung Lago Sub-district, Banyuasin District, South Sumatra, Indonesia. Samples were collected by the composite sampling method (Hyde et al. 2009) from 5 points in every location. As much as 200 g of each sample was put into a plastic bag and stored in a cool box. Sampling was done randomly of 20 cm depth within root zones of rice and corn plants.

Phosphate-solubilizing Microbe Isolation

Isolation was carried out using Pikovskaya medium containing 5 g $CaPO_4$, 0.5 g $(NH_4)_2SO_4$, 0.2 g NaCl, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 10 g glucose, 0.5 g yeast extract, 20 g agar powder, 0.01 g $MnSO_4$ and 0.01 g $FeSO_4$ in 1 L distilled water. Ten grams of soil was put into a 250 ml Erlenmeyer flask containing 90 ml of sterile 0.85% NaCl solution and incubated in a shaker at 120 rpm for 2 hours. Dilutions were made in a series of 10^{-1} to 10^{-6} of the soil extract, from which 1 ml was taken and poured onto a sterile Petri dish, and then 15 ml of Pikovskaya agar media; plates were incubated for 72 hours at 30°C. PSM growth was indicated by the formation of colonies surrounded by a clear zone (halo zone). One loopful of bacteria was taken and streaked aseptically on a new Pikovskaya agar plate for purification and incubated for 72 hours at 30°C.

Phosphate-solubilizing Microbe Selection

The excellent PSMs in this study had to contain the following criteria for further studies: 1) tolerant of high concentrations of Al and Fe, 2) tolerant of low pH and 3) capable of dissolving phosphate from various P sources.

Tolerance assay for Al and Fe

One loopful of pure cultures were taken and put into Pikovskaya liquid media with P in the form of aluminum phosphate ($AlPO_4$) at 0.5 g L^{-1}, then were homogenized for 2 hours. A dilutions series from 10-1 to 10-5 was made from the solution, and 1 ml of each dilution was aseptically transferred into a sterile Petri dish, followed by the addition of 15 ml of Pikovskaya agar media. The dishes were then stored in an incubator for 72 hours at 30°C. The calculation of the total population was carried out using the plate count method. PSM survival was then assayed for its resistance to $AlPO_4$, which was given gradually at 500 mg L^{-1}, 1000 mg L^{-1}, 1500 mg L^{-1}, and 2000 mg L^{-1}.

PSMs were assayed to evaluate Fe-resistant capabilities in the same as testing for Al resistance. One loopful of culture was taken and put into Pikovskaya liquid media with P in the form of ferric phosphate ($FePO_4$), which was given gradually at increasing concentrations of 500 mg L^{-1}, 1000 mg L^{-1}, 1500 mg L^{-1}, and 2000 mg L^{-1}. PSMs that survived were the bacteria that were tolerant of $AlPO_4$ and $FePO_4$.

Tolerance assay for low pH

PSMs that were tolerant to 1500 mg L^{-1} $AlPO_4$ and $FePO_4$ were placed into adjusted-Pikovskaya liquid

Table 1. Microbial morphology of phosphate-solubilizing microbes from the tidal land in 4 landscape typologies in Sukatani village, Mulia Sari, Banyu Urip Utara and Banyu Urip Selatan

Isolate	Colony Forms	Colony Colors	Clear Area Diameters (mm)
STB_1	Irregular	Yellowish white	2.5
$BUUB_1$	Round	Yellowish white	5
$BUUB_2$	Round	White	1
$BUUB_3$	Round	Yellowish white	5.5
$BUSB_1$	Round	White	10
$BUSB_2$	Round	White	1
$BUUJ_1$	Round	White with little brown in the center	6
MSJ_1	Stringy	Milk white	11

media. The pH was adjusted to 4.5 by adding 37% HCl solution, then was spread on solid Pikovskaya media in a Petri dish and incubated for 72 hours at 30°C. After that, the total microbial population was counted. The pH of the media was then gradually reduced to 4.0, 3.5 and 3.0 by adding 37% HCl solution. PSMs that survived in the medium with the lowest pH was the PSM isolates selected to be assayed for their ability to dissolve phosphate in liquid media.

Phosphate-dissolving ability assay

A quantitative assay for the ability to dissolve phosphate in liquid Pikovskaya media was prepared independently for each P source using a completely randomized design (with three replications). The results obtained were analyzed using the analysis of variance assay (ANOVA), and further using the Least Significant Different (LSD) test (p = 0.05).

The phosphate-solubilizing microbial isolates included a Control (without PSM), as well as BUUB1, BUUB3, BUSB1, BUUJ1 and mixed cultures. The source of P was $Ca_3(PO_4)_2$, $AlPO_4$ and $FePO_4$. The 10^8 cell mL^{-1} of phosphate-solubilizing microbial inoculum was added in 100 mL of sterile Pikovskaya medium with each of the P sources.

Inoculants were incubated in a shaker (130 rpm) for 14 days, and the pH was measured at the beginning and end of incubation period. Cultures were centrifuged at 7,200 rpm for 10 minutes, and the supernatant was analyzed to determine the dissolved P content using a spectrophotometer.

Microbial Viability Assay in Biochar

Biochar was made by heating the raw materials of agricultural waste, such as rice husk, corn cobs, coconut shells and palm bunches, using the pyrolysis technique for 8 hours at 600°C. Then, the biochar was chilled overnight, mashed (± 1 mm) and sterilized by autoclaving at 120°C for 15 minutes. Furthermore, four types of biochar were inoculated with selected PSMs with a population density of > 10^{11} CFU mL^{-1}. Inoculated biochar was stored at room temperature for three months, and every month samples were taken to evaluate their viability by calculating the PSM population using the plate count method.

Tolerant Microbial Identification

The bacterial identification method was based on biochemical characters using BD Phoenix™ tools. The automatic microbiology system BD Phoenix™ was used to identify genus and species from a microorganism (bacteria), based on the ability of fermented sweets. This system consists of fluorogenic and chromogenic substrates. When bacteria come into contact with the substrate, they cause with a positive (+) reaction, or they create an adverse reaction (-) when there is no contact. Meanwhile, when positive results and negative reactions are combined, the bacteria will be automatically identified by BD Phoenix™ by comparing them to baseline data, while identifying fungi includes microscopic observations, which are then described and matched with fungi identification books (Gilman 1971).

Data Analysis

Data was analyzed in a descriptive way, phosphate solubilizing ability assayed with ANOVA, and further with LSD (p = 0.05).

RESULTS AND DISCUSSION

Isolation and Characterization of Phosphate-solubilizing Microbes

Based on the isolation results from the four sample locations, it was obtained six bacterial isolates and two fungal isolates showed clear zones as an indication of phosphate dissolution. The eight isolates were $STTB_1$, $BUUB_1$, $BUUB_2$, $BUUB_3$, $BUSB_1$, $BUSB_2$, $BUUJ_1$ and MSJ_1, as shown in **Table 1**. The number of phosphate-solubilizing bacteria (**Fig. 1**) found more than that of phosphate-solubilizing fungi. According to Alexander (1977), the population of phosphate-solubilizing microorganisms from the bacterial group was higher than the fungi group. The community of phosphate-solubilizing bacteria (**Fig. 1**) can reach 12 x 10^6 organisms per gram of soil, while phosphate-solubilizing fungi only range from 20 x 10^5 to 1 x 10^6 per gram of soil.

The clear zone (halo zone) is the initial sign in determining PSMs ability to dissolve phosphate. A full clear zone is qualitatively considered an indication that the PSMs can dissolve phosphate in a media. This clear area is formed because of the dissolution of phosphate from an insoluble source that is present in the media due to organic acids produced by microbial colonies. The time needed for growth, the color and the size of colonies, as well as the amount of clear area, varies depending on the type of PSM tested. However, formation broader and more precise clear regions

Fig. 1. Phosphate-solubilizing microbes on Pikovskaya media shows a clear zone. Fungi (left) and Bacteria (right)

Table 2. Phosphate solubilizing microbial population from tidal land of four villages in the Telang area of Banyuasin District, South Sumatra

Location/Typology Area	Soil pH	Population (CFU g^{-1})
Suka Tani (A)	3.99	1.2×10^4
Mulia Sari (B)	4.49	3.8×10^4
Banyu Urip Selatan (C)	4.54	3.7×10^5
Banyu Urip Utara (D)	4.39	2.1×10^5

indicate a higher solubility of phosphate in the media, so that the colonies can be selected or isolated as PSM isolates or strains to be further analyzed.

Generally, the color of the bacterial or fungal colonies obtained is white or yellowish-white, in line with the results of Gemici et al. (2015) research on acid sulfate soils in Kalimantan tidal swamps, where he found organisms that produced orange, yellow and white colonies. Bacterial colonies and fungi have unique properties in a dense medium, where the colony's morphology can be described as dots, round or circular, filamentous and irregular (Dwijoseputro 2005). The effectiveness of dissolving P was shown by the formation of clear zones around the colonies of isolates grown on Pikovskaya agar media. According to Fankem et al. (2006), discoloration in the zone around the colonies that is supposed to be transparent shows a decreasing pH in the area and acidification of media related to the P dissolution process.

Phosphate-solubilizing Microbial Population

The phosphate solubilizing microbial population from the rhizosphere of rice and corn plants on four landscape typologies, i.e., Suka Tani Village (typology A), Mulia Sari Village (typology B), Banyu Urip Selatan Village (typology C) and Banyu Urip Utara Village (typology D), ranged from 1.2×10^4 to 3.7×10^5 CFU g^{-1} (**Table 2**).

According to Tate (1995), in acidic soil the microbial population in the soil 10^4 CFU g^{-1}. One environmental factor affects bacterial growth is the presence of substrate. Root plant exudates are expected to affect the population and variety of phosphate-solvent microorganism in the root surrounding. Sabarudin (2004), mentioned that pH also affects the phosphate-solvent microorganism population, related to nutrients that required by microorganism to survive. Purnomo et al. (2000) reported that higher pH allows the suitable environment for soil phosphate-solvent microorganism to grow optimally.

Tidal land have relatively low pH, Suryantini (2011) mentioned that there is tendency of decreasing phosphate-solvent microorganism population in the acidic soils. The acidity of the soil significantly affects the growth of bacteria; this is related to the function of phosphatase as a catalyst in various cell chemical reactions. Low pH results in reduced enzyme activity, destruction of proteins and stopping of enzyme activity (Pelczar and Chan 1986). Therefore, only bacteria that can adapt to low pH can survive. In addition to the low pH factor, the condition of the land and soil type also affects bacteria. The locations of the Suka Tani and Mulia Sari villages are typology A and B, respectively, which are always inundated. In this type of soil, there is an accumulation of organic matter under water saturation conditions, which causes an anaerobic state. It affects the population of aerobic bacteria; the reduced oxygen supply inhibits bacterial growth so that the density of bacteria decreases. On the other hand, the phosphate-solubilizing microbial community in the locations of the Banyu Urip Utara and Banyu Urip Selatan villages was higher because they were classified as relatively dry C and D typologies, respectively, so that the presence of phosphate-solubilizing microbes was both aerobic and anaerobic.

Table 3. Selection of PSM isolates treated with various concentrations of $AlPO_4$, $FePO_4$ and pH

Isolate Codes	$AlPO_4$ (mg L^{-1})			
	500	1000	1500	2000
STB_1	+	-	-	-
$BUUB_1$	+	+	+	-
$BUUB_2$	+	+	-	-
$BUUB_3$	+	+	+	-
$BUSB_1$	+	+	+	-
$BUSB_2$	+	-	-	-
$BUUJ_1$	+	+	+	+
MSJ_1	+	+	-	-
	$FePO_4$ (mg L^{-1})			
	500	1000	1500	2000
$BUUB_1$	+	+	+	-
$BUUB_3$	+	+	+	-
$BUSB_1$	+	+	+	-
$BUUJ_1$	+	+	+	+
	pH			
	4,5	4,0	3,5	3,0
$BUUB_1$	+	+	+	+
$BUUB_3$	+	+	+	+
$BUSB_1$	+	+	+	+
$BUUJ_1$	+	+	+	+

+ : Isolates that were able to survive
- : Isolates that were dead

Table 4. Phosphate content in the liquid media with the varied P sources (after 14 days incubation)

Isolates/Strain	P source		
	Ca3(PO4)2	AlPO4	FePO4
Control (without microorganism)	3,80 a	1,85 a	1,50 b
$BUUB_1$/*Paenibacillus alvei*	20,54 b	8,78 c	1,66 b
$BUUB_3$/*Burkholderia cepacia*	32,74 c	3,14 b	2,73 c
$BUSB_1$/*Acinetobacter baumannii*	36,76 d	10,24 d	0,52 a
$BUUJ_1$/*Penicillium variabile.*	18,90 b	8,92 c	3,90 d
Mixed cultures ($BUUB_1$,$BUUB_3$,$BUSB_1$,$BUUJ_1$)	57,45 e	13,98 e	8,46 e
BNT	1,67	0,50	0,24

Information: Data followed by same alphabetical letter in the same colomn have no significant differences (LSD 5%)

Microbial Selection of Phosphate-solubilizing Microbes based on their Resistance to Al, Fe and pH

The results showed that all phosphate-solubilizing microbial isolates were tolerant of 500 mg L^{-1} $AlPO_4$ (**Table 3**). With up to 1500 mg L^{-1} $AlPO_4$, four strains could still survive, i.e., $BUUB_1$, $BUUB_3$, $BUSB_1$ and one fungal isolate ($BUUJ_1$). The administration of 2000 mg L^{-1} $AlPO_4$ exposed only one strain that was able to survive, $BUUJ_1$. In further assays, all four isolates that were tolerant to the 1500 mg L^{-1} $AlPO_4$ were able to survive by giving $FePO_4$ up to 1500 mg L^{-1} concentrations. The four strains were also able to survive in medium with pH 3.0. Thus, the four isolates were selected for further assayed in phosphate dissolution.

The microbe analyzer identified four isolates, including *Paenibacillus alvei* ($BUUB_1$), *Burkholderia cepacia* ($BUUB_3$), *Acinetobacter baumannii* ($BUSB_1$) and *Penicillium variabile* ($BUUJ_1$). Giving $AlPO_4$ and $FePO_4$ at doses up to 1500 mg L^{-1} at pH 3.0 was suitable for bacteria and phosphate-solubilizing fungi to survive, but in 2000 mg L^{-1} $AlPO_4$ and $FePO_4$, only phosphate-solubilizing fungi were able to survive. Goenadi et al. (2000) also mentioned that phosphate-solubilizing fungi prefer acid soils containing high Al and Fe. Fungi were able to dissolve $AlPO_4$ and $FePO_4$ better than bacteria, which were more effective at dissolving $CaPO_4$ in alkaline soils.

Paenibacillus alvei is a gram-positive bacterium with thick cell walls that are more resistant to environmental factors. These phosphate-solubilizing bacteria are facultative anaerobes and spore-forming in unfavorable environmental conditions. According to Pradhan et al. (2016), these bacteria could survive in extreme physical and chemical environments. Some species could live as saprophytes on the ground or survive as dormant spores. Likewise, *Burkholderia cepacia* is known as a phosphate-solubilizing bacteria that is commonly found in acidic soils and the rhizosphere of plants. This bacterium was able to dissolve phosphates from various phosphate sources, such as $CaPO_4$ in saline soils, as well as $AlPO_4$ and $FePO_4$ in acidic soils. *Acinetobacter baumannii* is a phosphate-solubilizing bacterium known to be resistant to antibiotics and a strong phosphate solubilizer.

On the other hand, *Penicillium variabile* is a phosphate-solubilizing fungi that is predominantly found in acidic soils in Indonesia. The three types of bacteria and the phosphate-solubilizing fungus could adapt to the administration of Al and Fe up to 1500 mg L^{-1} in low pH conditions. While four other bacterial isolates were unable to survive. Thus, these isolates have good prospects to be developed into phosphate-solubilizing inoculants.

Assay for the Potential Solubility of p by Isolates in Liquid Media

All isolates were able to dissolve phosphate from P sources $Ca_3(PO_4)_2$, $FePO_4$ and $AlPO_4$ on liquid Pikovskaya media (**Table 4**). Mixed cultures of the four isolates were able to provide the highest P solubility from all P sources, respectively 57.45 mgL^{-1}, 13.98 mgL^{-1}, and 8.46 mgL-1. Not all isolates were able to dissolve P well. *Acinetobacter baumannii* dissolved phosphate quite well from the P sources $Ca_3(PO_4)_2$ and $AlPO_4$, which was 36.76 mg L^{-1} and 10.24 mg L^{-1} respectively, but only able to dissolve $FePO_4$ 0.52 mg L^{-1}. The low solubility of $FePO_4$ was considered to be presented by microbial metabolic substances, which caused deposition of dissolved phosphorus and consumption by the bacteria itself. The same pattern was also shown by other isolates such as *Paenibacillus alvei*, *Burkholderia cepacia* and *Penicillium variabile*. It is following the results of the study by Chang and Yang (2009), which showed a higher solubility of $CaPO_4$, followed by $AlPO_4$ and $FePO_4$, by phosphate-solubilizing microorganisms in the same culture medium with the same phosphate source. Baydın et al. (2017) also states that $CaPO_4$ was more soluble than $AlPO_4$ and $FePO_4$ because the chemical bonds of $CaPO_4$ were weaker than Al and Fe. The ability of PSM to dissolve phosphate varies depending on the type and amount of organic acid

Table 5. pH measurement of Pikovskaya liquid media at 0 and 14 days of incubation

Isolates	P Source					
	$Ca_3(PO_4)_2$		$AlPO_4$		$FePO_4$	
	0	14	0	14	0	14
Control (without microbes)	5.9	5.8	4.6	4.5	5.1	5.1
$BUUB_1$/*Paenibacillus alvei*	5.9	4.1	4.6	4.1	5.1	3.6
$BUUB_3$/*Burkholderia cepacia*	5.9	4.5	4.6	3.2	5.1	3.0
$BUSB_1$/*Acinetobacter baumannii*	5.9	3.9	4.6	3.2	5.1	3.3
$BUUJ_1$/*Penicillium variabile.*	5.9	4.0	4.6	2.5	5.1	2.5
Mixed culture ($BUUB_1$, $BUUB_3$, $BUSB_1$, $BUUJ_1$)	5.9	3.5	4.6	2.5	5.1	2.5

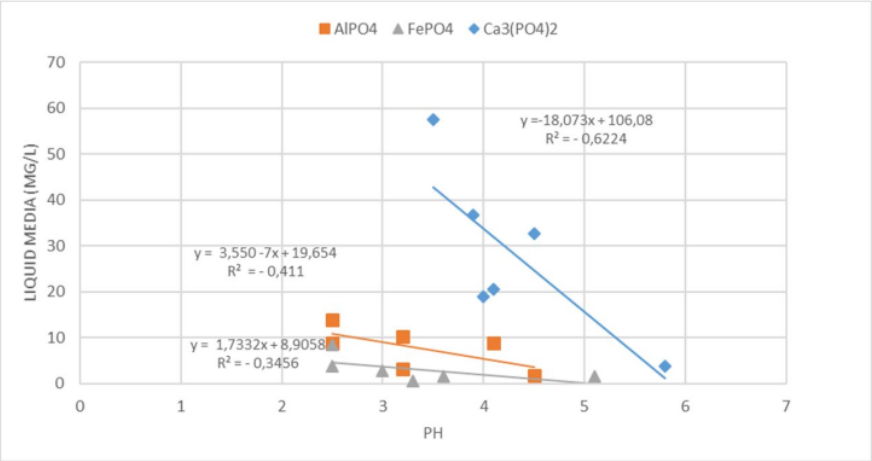

Fig. 2. Correlation between pH of liquid media and P dissolution with P sources $Ca_3(PO_4)_2$, $AlPO_4$ and $FePO_4$ after 14 days of incubation

produced, and the source of phosphate used. Based on the results of research by Chen *et al.* (2006) there were eight different types of organic acids produced by phosphate-solubilizing bacteria, i.e., citric acid, lactic acid, gluconic acid, propionic acid, succinic acid and three other unidentified kinds of acids. Organic acid was essential to dissolved phosphate because the organic acid was relatively abundant in carboxyl (-COO−) and hydroxyl (-O−) functional groups, which were negatively charged, and it is possible to form complex compounds with metal ions (cations), commonly called chelate (Wagner and Wolf, 1998). Organic acids chelated Al, Fe or Ca, resulting in phosphates being released from $AlPO_4$, $FePO_4$ or $CaPO_4$ bonds.

Organic acids that can release phosphate bonds, including gluconate acid, are the highest chemical property produced by phosphate-solubilizing bacteria. The four isolates, i.e., *Paenibacillus alvei*, *Burkholderia cepacia*, *Acinetobacter baumannii* and *Penicillium variabile*, all produced gluconate acid (Perez et al. 2007). Gluconic acid capability to solve phosphate is lower than citric or oxalic acid, therefore, the total of phosphate dilution is relatively lower. Acidic chemical compounds that have biggest until smallest stability constanta (log K) are: citric acid > oxalic > tartate > malate > lactate > gluconic > acetate > formic (Santosa 2007). The low phosphate content in this study is due to there is precipitation and re-dissolution towards obtained organic phosphate or anorganic within the cultures. On the 14th day, the phosphate concentration was changed, most likely due to the dissolve-phosphate (yield) was re-use by microorganism in the metabolism process as nutrition source (Illmer and Schinner 1995), and resulting the low content of phosphate in the growth medium.

Use of mixed cultures tends to provide better results than single isolates because the enzyme activity from each microbe can complement each other, allowing them to survive using available nutrient sources. The existence of more than microbes in the consortium (bacteria and fungi) could work synergistically so that the results were better than the non-consortium.

The increase in organic acids in the media was followed by a decreasing pH (**Table 5**). According to Widawati and Muharam (2012), this decrease in pH occurs due to oxidation, reduction, and competition of organic ligands, as well as the results of the synthesis of organic compounds being released into liquid inoculants (Pikovskaya media).

There was a negative correlation between the pH of the culture medium and the soluble phosphorus content with the three P sources (**Fig. 2**). This decreasing pH is the basic principle in phosphate dissolution and may be related to the production of organic acids and the release

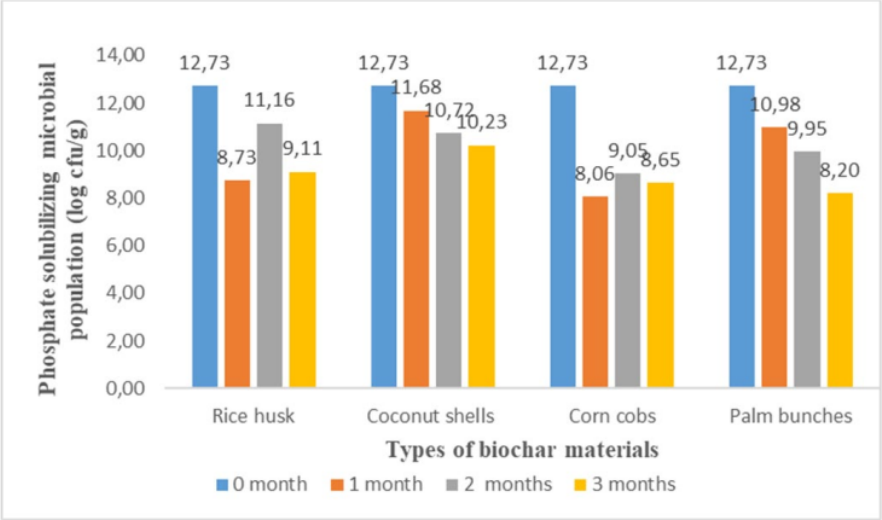

Fig. 3. Phosphate solubilizing microbial population during the 3 months storage period on several types of biochar materials

of protons (Lin et al. 2006, Spe[2]er 1958). It is in line with the study conducted by Rajkumar and Freitas (2008), which showed a negative correlation between phosphate solubility and a decreasing pH.

Microbial Viability Assay in Biochar

Mixed cultures of four selected isolates, i.e., *Paenibacillus alvei*, *Burkholderia cepacia*, *Acinetobacter baumannii* and *Penicillium variabile*, which were inoculated on four types of carrier materials in biochar stored at room temperature, had high viability until the third month (**Fig. 3**). The initial population of cultures of these isolates was 5.31 x 10^{12} CFU g^{-1} biochar. After three months of storage, the population of the strain was stable enough to meet the criteria for biological agents, which was > 10^7. It means that all carrier materials have suitable standards as a microbial carrier for phosphate-solubilizing microbes. One of the essential properties required of a carrier was its ability to maintain populations of microbial inoculants at a high concentration during the storage period.

Biochar has favorable characteristics as an inoculant carrier agent because of its high internal porosity, large specific surface area and the ability to adsorb organic compounds and bacteria (Chung et al. 2004).

Biochar as an inoculant carrier agent has shown high microbial viability. Research results by Santi and Goenadi (2010) showed that bioameliorans made from palm oil-bearing biochar, which was carbonated at 300–400°C for 8 hours with a shelf life of 3–9 months, could maintain a higher aggregate bacteria-keeping population of 10^8 CFU $gram^{-1}$ compared with compost and peat material. In this study, the highest microbial viability was obtained from a coconut shell biochar carrier, which had a population of 1.73 x 10^{10} CFU g^{-1} (log community 10.23 CFU g^{-1}).

CONCLUSION AND SUGGESTION

This study obtained that:

1. Isolates that were resistant to aluminium phosphate and ferric phosphate at concentrations of 1,500 mg L^{-1} and pH 3 were: $BUUB_1$ (*Paenibacillus alvei*), $BUUB_3$ (*Burkholderia cepacia*), $BUSB_1$ (*Acinetobacter baumannii*) and $BUUJ_1$ (*Penicillium variabile*).
2. Mixed cultures of the four isolates had the highest ability to dissolve P in liquid media with all three P sources, i.e., $Ca_3(PO_4)_2$ at 47.31 mg L^{-1}, followed by $AlPO_4$ at 13.98 mg L^{-1} and $FePO_4$ at 8.46 mg L^{-1}.
3. The four mixed isolates had high viability in four types of biochar (> 10^7) after three months of storage, but the carrier material made from coconut shell biochar gave the highest viability of 1.73 x 10^{10} CFU g^{-1} (log population 10.23 CFU g^{-1}).

Tolerant microbes obtained in this study can be assayed for their ability to dissolve phosphate through field experiments with plants to see its contribution in increasing P availability in other acid soils.

ACKNOWLEDGEMENT

Thank you to the Chancellor of Sriwijaya University through the 2017 Profession Research program which has helped in the form of financial aid in conducting research.

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