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Full Length Research Paper

Isolation of phosphate solubilizing bacteria from tropical soil

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Phosphate solubilizing microorganisms were isolated from the Typic Paleudalf and the Pellustert. Isolates obtained were tested for their ability to solubilize calcium phosphate, iron phosphate, and rock phosphate in Pikovskaya (1948) cultural medium. Most of the isolates were able to solubilize Fe-P, and then the Ca-P medium but little rock phosphate. There were no differences in solubility of inorganic phosphates between the isolates from the different soils types. The colonies of the isolates produced yellow halo zone on agar plates that bromothymol blue had been added indicating the production of weak organic acid.

Keywords: Phosphate solubilizing bacteria, Pikovskaya medium, Paleudalf, rock phosphate.

INTRODUCTION

The need to increase agricultural production from a steadily decreasing and degrading land resource base has placed strain on agro ecosystems (Tilak, 2005). To maintain and improve agricultural productivity, the current strategy is to use chemical fertilizers. Yet, many synthetic fertilizers contain acids, such as sulphuric and hydrochloric acids, that tend to increase the acidity of the soil, reduce the soil's beneficial organism population and interfere with plant growth. Bio fertilizers on the other hand add nutrients to soil, are environmentally friendly and offer alternatives to chemical fertilizers. One form of bio fertilizer is the use of phosphate solubilizing bacteria that are able to convert insoluble P forms into soluble phosphate forms for crop improvement. Some of the insoluble P forms are mineral phosphate forms such as apatite, hydroxyapatite, oxyapatite and mineral phosphate found associated with the surface of hydrated oxides of Fe, Al,and Mn that are poorly soluble and assimilable.

Many bacteria genera have the capacity to convert insoluble P forms into soluble P for the use of plants and other organisms in soil. Such bacteria genera include *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aerobacter, Flavaobacterium* and *Erwin*. Some of the isolated phosphate solubilizing organisms are, *Pseudomonas putida, Erwinia herbicola, Rahnella aquatitis, Enterobacter agglomerans, Pseudomonas cepacia, Pseudomonas aeruginosa, Serratia marcescens, Klebsiella pneumonia, Pseudomonas striata, Burkholderia cepacia, Rhizobium sp., Bacillus sp., Aspergillus niger, Penicillium sp.*etc. These microorganisms have the genes for mineral phosphate solubilization and thus exhibiting the Mps^+ phenotype (mineral phosphate solubilizing phenotype).

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Phosphate solubilizing bacteria solubilize different forms of inorganic insoluble phosphates. *Pantoea agglomerans* was isolated from an iron-rich ore, and cultured on various insoluble inorganic phosphates media, including tri-calcium phosphate [$Ca_3(PO_4)_2$], iron phosphate ($FePO_4$), aluminum phosphate ($AlPO_4$), and rock phosphate. *Pantoea agglomerans* solubilized tri-calcium phosphate more efficiently than $FePO_4$ and $AlPO_4$ (Sulbaran *et al*., 2009). Other bacteria isolates were able to solubilize P from tricalcium phosphate and rock phosphate, but not from aluminum phosphate and $FePO_4$ (Panhwar *et al.,* 2009; Pérez *et al*., 2007 and Chen *et al*., 2006). On the otherhand, Chang and Yang (2009) noted that some phosphate solubilizing microorganisms could solubilize aluminum phosphate, iron phosphate, and hydroxyapatite. Feng *et al*. (2009) isolated phosphate solubilizing microorganisms that could readily solubilize $Ca_3 (PO_4)_2$ and $Ca_8 (PO_4)_6 H_2O$ phosphate but could not solubilize ($Ca_{10} (P0_4)_6$-Fe_2 phosphate. It will be interesting to know what dictates the isolates preference for solubilizing a particular type of inorganic phosphate form or what makes an isolate solubilize one form of phosphate more than another form of phosphate.

Impurities inside rock phosphate or in the crystal lattice of rock phosphate can affect the ability of microorganisms to solubilize it. Some rock phosphates contain lanthanum in the crystalline lattice thus affecting microorganisms that solubilize it. Relatively, higher concentration of lanthanum inhibited fungal growth and delayed rock phosphate solubilization activity. *Penicillium oxalicum* was more sensitive to lanthanum than *Aspergillus niger* on rock phosphate solubilization (Wang *et al*., 2004).

Application of bacterial inoculants as bio fertilizers resulted in improved plant growth, increased yield, improved plant available P and also releasing indole acetic acid and gibberellic acid that caused growth and elongation of plant cell (Bashan *et. al*., 1998; Vessey, 2003; Illmer and Schinner, 1992; Peix *et al*., 2001, Tripura *et al*., 2007). Using phosphate solubilizing microorganisms on plants may not influence plant phosphate uptake (Poonguzhali *et al*., 2008; Fernández *et al.,* 2007) and in some cases the total P for plants decreased (Suh and Kwon, 2008). But, phosphate-solubilizing bacteria of the genera *Bacillus* and *Cellulomonas* caused high available phosphate, and increased total P uptake in plants. The phosphate solubilizing bacteria and plant growth promoting rhizobacteria together could reduce phosphorous fertilizer application by 50% (Jilani *et al*., 2007; Yazdani *et al*., 2009). The type of bacteria present or inoculated and soil properties are some of the factors that influence P availability to plant (Smyth and Sanchez, 1982).

The objective of this paper is to isolate phosphate solubilizing bacteria in the tropical environment and to study their ability to solubilize the different forms of inorganic insoluble phosphates.

MATERIALS AND METHODS

Site description and Soil sampling

Two different soil samples namely Kokofu and Akuse series were collected from the Agriculture Research Centre, Kade in the Eastern Region and the Agricultural Research Centre, Kpong found in the Accra Plains of Ghana. According to the USDA system of soil classification Kokofu is classified as Typic Paleudalf whilst Akuse series is classified Typic Pellustert (USDA). Kokofu series is found in a semi-deciduous forest with 1,480 mm per annum of rainfall, and drainage being moderately well drained. On the other hand, Akuse series has short-grass savanna vegetation with 900 mm per annum of rainfall, and drainage being imperfectly drained. Soil samples were collected from 0-20 cm of depth and brought to the Department of Soil Science, University of Ghana where analyses were carried out.

Laboratory procedures

Analyses were performed on air-dried soil fractions (<2 mm). The soil pH was measured potentiometrically in 1:2 (W/V) suspension of water and in 0.01M $CaCl_2$. Organic carbon was determined by the Walkley and Black (1934), Cation exchange capacity (CEC) and exchangeable basic cations Ca^{2+}, Mg^{2+}, K^+ and Na^+ (1 M NH4OAc at pH 7) were determined. The total soil nitrogen was determined by the Kjeldahl method and available P by Watanabe and Olsen (1965). Exchangeable Ca^{2+} and Mg^{2+} were measured by Atomic Absorption Spectrometry (AAS), whilst K^+ and Na^+ were determined by flame photometry.

Isolation of Rhizosphere bacteria that produced organic acid

Nerica 2 rice seeds were planted in pots for 3-4 weeks after which the rice plants were uprooted and the rhizosphere soil was obtained. First, the root system was separated from the bulk soil by shaking gently. The remaining soil attached to the root was separated from the roots by shaking more vigorously. Soil still adhering to the roots defined as rhizospheric soil was removed using a brush. With the rhizospheric soil, serial dilutions were made with Ringers solution. The composition of the Ringers solution is as follows, NaCl- 2.15 g; KCl - 0.075 g; $CaCl_2$ - 0.12g; $NaS_2O_3.5H_2O$- 0.5 g per litre of distilled water and pH of the medium was adjusted to 6.6. An aliquot of the final dilution was plated on PTYG (Peptone, tryptone yeast extract and glucose) agar plate with bromothymol blue being added. The agar plates were left to incubate for 4-7 days at room temperature. The

Table 1. Some Physiochemical properties of Akuse and Kokofu series from 0-20 cm depth of soil.

Soil Property	Akuse series	Kokofu series
sand	560	355.25
silt	90	269.75
clay	350	375
texture	Sandy clay loam	Clayey
pH	7.5	4.5
Organic carbon (%)	1.44	1.70
Total nitrogen (%)	0.06	1.51

composition of the PTYG agar medium is as follows peptone - 0.25 g; tryptone - 0.25 g; yeast extract - 0.6 g; glucose - 0.5 g; $MgSO_4.7H_2O$ - 0.03 g; $CaCl_2.2H_2O$ - 0.003 g; bromothymol blue - 0.025 g and agar 15 g all dissolved in 1 litre of distilled water.

Isolates that produced organic acids had yellow colonies. These colonies were picked and streaked and restreaked on new PTYG agar medium that had bromothymol blue to obtain pure colonies.

Culturing of isolates

Isolates that produced organic acid were selected from the PTYG agar plates of the various soil samples. To test the ability of these isolates to solubilize different forms of inorganic phosphates viz rock phosphate, iron phosphate and calcium phosphate, the isolates were grown in the Pikovskaya (1948) culture medium with the pH adjusted to 6.6. The sole source of P in the culture medium was the different forms of inorganic P introduced. These were then incubated in an orbital shaker at a temperature of 28^{o}C for 14 days. Growth was determined by the turbidity of the media. The composition of the Pikovskaya medium (Piks medium) is as follows glucose - 10 g; $Ca(PO4)_2$ or the other form of inorganic phosphate - 5g; $(NH_4)_2SO_4$ - 0.5 g; NaCl - 0.2 g; $MgSO_4.7H_2O$- 0.1g; KCl - 0.2 g; yeast extract - 0.6 g; $MnSO_4$ - 0.04 g; $FeSO_4$.$3H_2O$ - 0.04 g all were dissolved in 1 liter distilled water. After the incubation period, the pH of the culture medium was determined. The number of replicates being three and the experiment was set up in a completely randomized design manner. Control culture media was set up for each source of inorganic phosphate where no microorganism was introduced into the culture medium and it was also replicated thrice. It was incubated under the same condition as other treatments. The culture medium was filtered after 14 days of incubation, using Whatman filter paper # 42 after which an aliquot of 10 ml of each filtrate was discharged into 50 ml volumetric flasks followed by color development using the Watanabe and Olsen's method (1965). Color was developed against the standard and available phosphorus was then determined using the spectrophotometer.

RESULTS

Physiochemical properties of soils

Akuse series has a pH of 7.5 which is neutral (Table 1). The organic carbon is medium and total nitrogen content is low. The texture of the Akuse series is sandy clay loam. On the otherhand, the pH of the Kokofu series is 4.5 which is very strongly acidic (Table 1). The organic carbon content is medium and total nitrogen of the soil is high (Landon, 1984). The data indicate that the nitrogen content in the Akuse series 0.06% which is very low and therefore the soil will respond to nitrogen fertilizers during cultivation. The texture of the Kokofu series is clayey from the surface to the depth of 20 cm.

Total available P produced by isolates from Akuse series in the various culture media

All the isolates solubilized most P from Fe-P followed by Ca-P and the least solubilization occurred with rock phosphate (Table 2). In the Pikovskaya + Fe-P medium, PSB 2 produced the highest P of 1843 µg ml^{-1} followed by PSB 1 that produced 1780 ug ml^{-1} in the same medium, however, differences in phosphate production in that medium was not significant between the two isolates.

Furthermore, PSB 2 solubilized Fe-P, rock phosphate and Ca-P yielding some amount of phosphate in Piks medium + Ca-P. PSB 3 performed poorly in solubilizing Ca-P and rock phosphate but performed better in solubilizing Fe-P. This illustrates the solubilization preferences of isolates for the different forms of inorganic P forms. Thus in a mixed culture medium of PSB 1 and PSB 2, it is likely that Fe-P, Ca-P and rock phosphate will be solubilized efficiently.

For the solubilization of Ca-P, PSB 1 produced the highest available P of 1727 µg ml^{-1} followed by PSB 5 (890

Table 2. Phosphorus solubilization potential of five different PSB isolated from Akuse series in different cultural media

Treatments	Piks medium + Ca-P (µgml^{-1})	Piks medium+ Fe-P (µgml^{-1})	Piks medium+ RP (µgml^{-1})
PSB 1	1727	1780	21.7
PSB 2	801	1843	48.0
PSB 3	404	1042	18.9
PSB 4	802	937	32.9
PSB 5	890	1360	22.9
*Control	110	127.6	4.2
LSD(0.05)	**32.78**	**277.4**	**37.61**
Mean P Produced	**924.8**	**1392.4**	**28.88**

*Control was made with Pikovskaya (1948) culture medium with no isolate added.

Table 3. Phosphorus solubilization potential of five different PSBs from the Kokofu series in different cultural media

Treatments from Kokofu series	Piks medium + Ca-P (µgml^{-1})	Piks medium+ Fe-P (µgml^{-1})	Piks medium+ RP (µgml^{-1})
PSB6	1680	1058	14.5
PSB7	1334	1605	29.1
PSB 8	1703	1277	34.5
PSB 9	835	1868	16.5
PSB 10	553	1125	14.7
Control	328.3	159.6	3.1
LSD(0.05)	**32.78**	**277.4**	**37.61**
Mean P produced	**1221**	**1386.6**	**21.86**

µg ml^{-1}), PSB 4 (802 µg ml^{-1}), and isolate 2 (801µg ml^{-1}) respectively (Table 2). There were significant differences in treatments in the same medium ($p<0.05$).

In Piks medium + rock phosphate, the highest available P of 48 µg ml^{-1} was recorded by PSB 2 followed by PSB 4 (32.9 µg ml^{-1}), PSB 5 (22.9µg ml^{-1}), and PSB 1(21.7 µg ml^{-1}) correspondingly (Table 2). However, there were no significant differences in treatments in Piks medium + RP ($p > 0.05$).

Total available P produced by PSBs from the Kokofu series in the various culture media

Generally, isolates from the Kokofu series solubilized more P from the media containing Ca-P, Fe-P and the least solubilization occurred with medium containing rock phosphate (Table 3). PSB 9 produced the highest amount of available P of 1868 µgml^{-1} in the Piks medium+ Fe-P whilst PSB 8 produced highest available P (1703 µgml^{-1}) in the Ca-P medium and the medium containing rock phosphate (34.5 ugml^{-1}). Thus, PSB 9 solubilized more Fe-P than solubilizing Ca-P and rock phosphate in culture medium. PSB 6 on the other hand performed well in solubilizing Ca-P but poorly in solubilizing rock phosphate.

In a mixed culture medium of PSB 9 and 8, most of the inorganic forms of insoluble P are likely to be solubilized. Whilst PSB 9 solubilizes effectively Fe-P and moderately solubilizes Ca-P, PSB 8 will solubilize the rock phosphate and Ca-P effectively.

A comparison of Tables 2 and 3 shows that isolates obtained from the Akuse series generally solubilized Fe-P and rock phosphate producing more available phosphate in culture solution than isolates obtained from the Kokofu series. Isolates obtained from the Kokofu series on the otherhand solubilized more Ca-P in culture medium than isolates from the Akuse series.

Table 4. pH values recorded from the various media containing Isolates from Akuse series

Isolates from Akuse series	pH		
	Piks medium + Ca-P	Piks medium + Fe-P	Piks medium + RP
PSB 1	5.4	4.8	4.3
PSB 2	4.8	4.7	3.8
PSB 3	4.2	5.3	4.4
PSB 4	4.7	5.0	4.2
PSB 5	4.5	4.9	4.7
Control	6.5	5.4	5.9
Mean pH	**4.72**	**4.94**	**4.28**

Table 5. pH values recorded from various media containing Isolates from Kokofu series

Isolates from Kokofu series	pH		
	Piks medium + Ca-P	Piks medium + Fe-P	Piks medium + RP
PSB 6	4.8	4.4	4.3
PSB 7	3.6	3.9	3.5
PSB 8	3.4	3.3	3.5
PSB 9	5.0	5.6	4.0
PSB 10	3.4	3.8	4.1
Control	6.4	5.7	5.9
Mean pH	**4.04**	**4.2**	**3.88**

Changes in pH of culture solution during incubation of isolates in different phosphate sources

At the end of the 14 days incubation for the isolates from the Akuse series, the mean lowest pH occurred in the Piks medium + RP followed by Piks medium + Ca-P and then Piks medium + Fe-P (Table 4). Also, PSB 2 lowered the medium pH from 6.5 to 3.8 when rock phosphate was used as the phosphate source (Table 4). All the other isolates lowered the pH of the culture media at the end of the 14 days of incubation period as compared to the respective control medium.

In the Kokofu series, the mean lowest pH occurred in the Piks medium + RP and this was followed by the Piks medium + Fe-P and lastly the Piks medium + Ca-P (Table 5). PSB 8 lowered the pH of all the culture media introduced into. In comparing the ability of isolates from the Akuse series to that of the Kokofu series influencing the pH of the media, isolates from Kokofu series generally lowered more the mean pH of the culture media regardless of the source of inorganic phosphate used than isolates from the Akuse series.

A positive relationship with r of 70% existed between culture medium pH and available phosphate in the medium at the end of the incubation period for the media that isolates from the Akuse series had been introduced (Figure. 1). However for the media where isolates from the Kokofu series had been introduced, an insignificant correlation coefficient of 28 % existed between the culture medium pH and the available phosphate in the medium (Figure. 2).

DISCUSSIONS

The results from study carried out showed that isolates obtained from the two different types of soil solubilized inorganic phosphate forms in the order Fe-P > Ca-P > rock

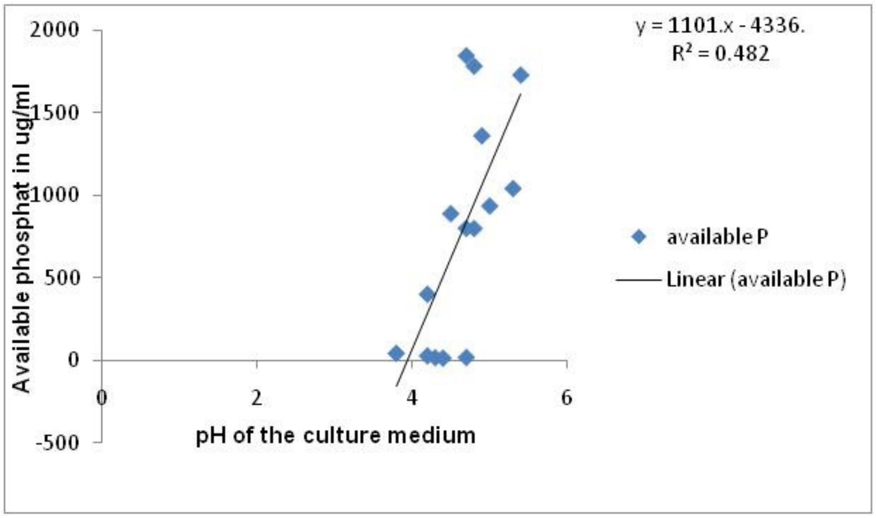

Figure 1. Relationship between available phosphate and culture medium pH of isolates from Akuse soil (r = o.70).

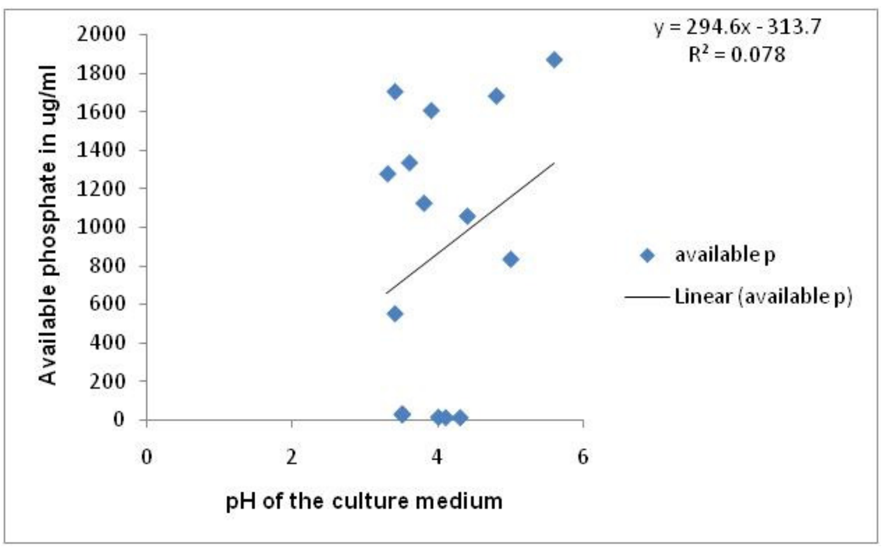

Figure 2. Relationship between available phosphate and culture medium pH of isolates from the Kokofu soil (r = 0.28)

phosphate, Fe-P was easily solubilized as compared to rock phosphate. Previous studies showed that most of the phosphate solubizing bacteria, easily solubilized calcium triphosphate as compared to iron phosphates (Panhwar *et al.,* 2009; Pérez *et al*., 2007 and Chen *et al*., 2006). Phosphate solubilizing bacteria obtained in this study, easily solubilized iron phosphates as compared to the other forms of phosphates probably illustrating the preference of these form of isolates to other PSBs obtained from elsewhere in the world. Expression of genes for mineral phosphate solubilizing phenotype $(Mps)^+$ led to the production of gluconic acid for most of the Gram negative strains isolated in other parts of the world. Gluconic acid biosynthesis is carried out by the glucose dehydrogenase (GDH) enzyme and the co-factor, pyrroloquinoline quinine (PQQ). It is likely that for the tropical strains isolated in this study, a different pathway is used where organic acids of high chelating ability are produced thus solubilizing iron phosphates more than calcium phosphate forms.

Also the results showed that generally, isolates from the Akuse series were able to solubilize more P than isolates from Kokofu series even though the differences did not appear significant. (Panhwar *et al.,* 2009; Pérez *et al*., 2007 and Chen *et al*., 2006).

Works done by Chen *et al*. (2006) shows that phosphorus solubilization activity of PSB is associated with the release of organic acid and a drop in the pH of the medium. Results of this experiment also showed similar activities; with a general drop in the pH values of the various cultural media. The release of organic acids by the isolates in the various cultural medium leads to the drop in the pH of the various cultural media. With this drop in pH, inorganic forms of phosphates can be solubilized to some extent. The ability of the isolates to solubilize Fe-P suggests that the some of the organic acids released by the isolates might have chelating abilities and thus complexing with iron in the medium. It is likely that the isolates used combinations of mechanisms to solubilize the various forms of inorganic phosphates and such mechanisms include are lowering of pH by acid production, ion chelation, exchange reactions and fermentation in the growth environment. Previous study characterized the rock phosphate having a low water soluble P of 0.001584 % and that reflected in the available P in the control medium. Probably the impurities associated with the rock phosphate prevented the isolates from solubilizing effectively the rock phosphate. This calls for a crystal analysis of the rock phosphate used.

CONCLUSION

Soluble phosphates react with clay, iron, and aluminum compounds in the soil. Through the process of phosphorus fixation, readily soluble forms of phosphates are converted to less available forms. This is one of the major challenges facing our local farmers in Ghana. Whiles, few farmers can afford chemical fertilizers majority of them cannot. The relevance of this experiment is to provide other options of making phosphorus available in the soil with the use of PSBs. Although isolates solubilized P from calcium phosphate, iron phosphate and rock phosphate and made P available in culture media, the best isolates need to be characterized further before introducing them to the farmers' field.

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