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Isolation and identification of phosphate solubilizing bacteria from non saline soils of coastal region in Bangladesh

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Abstract: The ability of soil microorganisms to convert insoluble forms of phosphorus to an available form is an important trait in plant growth-promoting bacteria for increasing crop yields. The study was conducted for the isolation of phosphate solubilizing bacteria from non-saline soils of different locations of tidal floodplain region of Bangladesh, their identification and to explore the ability of phosphate solubilization. Three phosphorus solubilizing bacteria were isolated and purified. The isolates were preliminary identified on the basis of their morphology and biochemical characteristics. All the phosphorus solubilizing bacterial isolates decreased pH of the culture medium by producing some organic acids and they were able to solubilize tricalcium phosphate remarkably in broth culture. Based on the results, it can be concluded that the isolates possess great potential to be developed as phosphatic biofertilizers to enhance soil fertility and plant growth. However, their performance under green house and field conditions should be assessed before being recommended for biofertilizer production and commercial applications.
Key words: Phosphorus solubilizing bacteria, biofertilizer and non saline soils.

Introduction

Agriculture is a major sector of Bangladesh economy and over 30% of the net cultivable land is in the coastal area. The coastal area includes tidal, estuaries and river floodplains in the south along the Bay of Bengal. Agricultural land use in these areas is very poor, which is roughly 50% of the country average.

Phosphorus is one of the major nutrient elements needed in adequate quantity in available form for the growth and reproduction of plants. Pierre (1938) referred to it as the "master key" element in crop production. It is associated with several vital functions and is responsible for several characteristics of plant growth such as utilization of sugars and starch, photosynthesis, nucleus formation andcell division, fat and albumen formation, cell organization and the transfer of heredity. The role of phosphorus in increasing yield and sometimes improving the quality of crops is well known. Phosphate solubilizing bacteria and phosphorus fertilizer has also the great influence on crop production (Sattar, 2004). Low recovery (10-30%) of phosphorus from applied fertilizer due to its fixation characteristics in the soils coupled with recent steep hike in prices of phosphatic fertilizers have further complicated the problems of its use by the farmers. In this context it is needed to have a comprehensive approach to phosphorus application for sustainable crop production and to enhance its use efficiency (Singh and Sharma, 1994). Certain phosphate dissolving microorganism still could be used as a means to improve the efficacy of triple super phosphate and unavailable soil phosphorus (Gaur, 1990 and Dubey, 2000). A lot of research has been done in other countries (Kim *et al.,* 1998 and Dupononis *et al.,* 1998) but only a few reports are available on the beneficial effect of phosphate dissolving microorganisms isolated from Bangladesh soils with different crops (Sattar, 2001). Phosphate-Solublizing Bacteria (PSB) solublize the insoluble phosphorus compounds through the release of phosphatase enzymes and organic acids. Organic acids and acid phosphatases play a major role in the mineralization of organic P in soil. In particular, soil microorganisms are effective in releasing P from inorganic and organic pools of total soil P through solubilization and mineralization (Hilda *et al*., 1999). In recent years, it has come to be established that soil microorganisms increase the availability of phosphate to the plants not only by mineralizing organic phosphorus compounds but also by rendering inorganic phosphorus compound more available to the plants (Gerretsen, 1948; Arora and Gaur, 1979; Sundaro Rao, 1965; Sattar and Gaur, 1986). Therefore, the present research work was undertaken for the isolation and identification of P-solubilizing microorganisms from non-saline soil and to prepare their mother cultures for the preparation of phosphaticbiofertilizer is a first step and to investigate the effect of these microorganisms for enhancing crop production and sustainable agriculture.

The specific objectives of the research were to isolate the potential phosphate solubilizing bacteria from selected soil, and to develop mother culture of phosphate solubilizing bacteria.

Materials and Methods

The experiment was conducted at the laboratory of Department of Soil Science and central laboratory, Patuakhali Science and Technology University, Dumki, Patuakhali during July 2014 to June 2015.

Collection and preparation of soil samples: Soil samples were collected from selected non saline areas of Charfashion upazila in Bhola district under AEZ 18 (Young Meghna Estuarine Floodplain) of Bangladesh.Ten surface soil samples were collected from each location. The samples of each location were mixed to make composite sample. For the convenience of discussion, the samples were referred to soil no. 1, soil no. 2 and soil no.3 as shown in Table 1. Some portions of collected soil sample were kept in refrigerator at 4^0C for isolation of bacteria. The rest portions of soil samples were then air dried ground to pass through a 2 mm sieve andthen mixed to from a composite sample.

Table 1. Designation and location of soil sampling sites

Designation	Location
Soil no.1	Aslampur, Charfashion, Bhola
Soil no.2	Ginnagor, Charfashion, Bhola
Soil no.3	Usmangonj, Charfashion, Bhola

Culture media: Pikovskaya's media (Pikovskaya, 1948) was used for culture of phosphate solubilizing bacteria (PSB).

Method of isolation: Enrichment culture technique (in liquid medium) was used for isolation of bacteria.

Modified Pikovskayamedia with the above composition and three successive transfers were made at seven days interval to enrich the culture.

Colony isolation: It was done by following the standard procedure.

Estimation of bacterial population: Count of viable cells of heterotrophic bacteria in coastal soil was made following the drop plate method of Miles and Misra (1938). Then the viable cells were calculated by the following formula stated by Somasegaran and Hobben, 1985. Number of cells/ml (CFU/ml) = [{(Number of colonies) X (Dilution factor)}÷ (Volume per drop)].

Purification of isolates: 9 isolates of each PSB were taken from respective cultured media and streaked on respective prepared plate's media. The streaked plates were incubated at 28^0C for 2-4 days. Repeated streaking was done until purification.

Preparation and preservation of mother culture: Purified isolates of PSB were transferred into Pikovskaya'sslanting media, preserved for further study. Slant method is one of the simplest methods.

Identification of phosphate solubilizing bacterial isolates: To identify the isolates some tests were done.

Motility test: Motility test was done following the hanging drop method (Vincent, 1980).

Gram reaction test: Gram reaction test was done by Hucker and Conn's method (1923).

Starch hydrolysis: The ability of each of the PSB iolates to hydrolyze starch was observed by streaking the strains on starch medium. Seven days after incubation when sufficient growth of the culture occurred, the plates were flooded with iodine solution. Development of white areas along the colonies indicated hydrolysis of starch and formation of blue colored "starch iodine complex" was indicated nonhydrolysis of starch.

Gelatin Hydrolysis: Tubes of nutrient broth with 25 percent gelatin were inoculated and incubated for 7 days at 37^0C. The tubes were then kept in the refrigerator to determine whether they would resolidify. Liquefaction is the positive test for gelatin hydrolysis. Podder (1977) reported that none of the isolates from chickpea used in his study could hydrolyse gelatin.

pH test: The growth responses of the PSB isolates were investigated in the Pikovskaya's broth having 5 levels of pH. The pH levels 4.0, 5.0, 6.0, 7.0 were created adding HCl solution and 8.0 adding NaOH as required. Triplicate flasks containing the defined lequid medium were autoclaved at 121^0C and 15 Ibs for 20 minutes. After cooling the flasks were arranged as per two-factor complete randomized design with three replications. Then the medium was inoculated with the PSB strains.

Bromotyhmol blue test: 5 ml of 0.5% bromothymol blue solution was thoroughly mixed with one liter of Pikovskaya's and then autoclaved and plated. Cultures of different PSB isolates were streaked on the plates and incubated at 30^0C and examined.

Catalase test: 3% of H_2O_2 solution was prepared. 1 drop of distilled water was taken on clean glass slide. Then colony of isolates was transferred on water and suspension was made. 1 drop of 3% of H_2O_2 solution is was placed on suspension and observed.

Screening of isolates for solubilization of insoluble phosphate compounds: With a view to study the relative efficiency of the 9 phosphate isolates solubilizing bacterial (PSB) strains, a series of laboratory incubation experiments for quantitative estimation of phosphate solubilized was set up as follows

Plan of the experiment

l. 25.0 mg P_2O_5 as tricalcium phosphate

2. Number of cultures tested: 10 with one control

3. Replications: 3

Statistical analyses: Data collected on different parameters under study were statistically analyzed to ascertain the significance of the experimental results. The analysis of variance was performed and means were compared by Duncan's Multiple Range Test (DMRT) for interpretation of results. The significance of the difference between the pair of means was evaluated at 1% level of significance using MSTAT-C computer package programs.

Results and Discussion

Phosphorus solubilizing bacteria: The present research work was conducted to achieve two major objectives - firstly, isolation and characterization of phosphorus solubilizing bacteria from non-saline soil of coastal region and secondly, preparation of mother culture for biofertilizer production.

Estimation of PSB population: Bacterial populations of collected soils were determined (Plate 1) and presented in Table 2. The results showed that the highest populations of PSB(2.0*10^6) was found in soil no. 1 (Aslampur) and the lowest populations of PSB (1.3*10^6) was found in soil no. 2 (Ginnagor).

Plate 1. Estimation of bacterial population

Table 2. Bacterial population of phosphorus solubilizing bacteria

Location	**P** Solubilizing Bacteria ($CFUg^{-1}$)
1. Aslampur, Charfashion, Bhola	1.8*10^6
2. Ginnagor, Charfashion, Bhola	1.6*10^6
3. Usmangonj, Charfashion, Bhola	1.7*10^6

Isolation of phosphorus solubilizing bacteria from non saline soil of coastal region: Three phosphorus solubilizing bacterial isolates were isolated from non-saline soils of coastal region. They were designated as P_1, P_2 and P_3, respectively (Table 3). These findings are in good agreement with several researchers. Suryakala (2004) isolated plant growth promoting rhizobacteria belonging to fluorescent *Pseudomonas* from rice, wheat, pigeon pea, groundnut and chilli crops.

Table 3. List of PSB isolates from non saline coastal areas

Isolate name	Location
P1	Aslampur, Charfashion, Bhola
P2	Ginnagor, Charfashion, Bhola
P3	Usmangonj, Charfashion, Bhola

Morphological characteristics of isolate: Morphological characteristics of the isolates i.e. colony morphology has been presented in Table 4 and Table 5. The colony characteristics of isolates did not vary widely. Morphologically the isolates were found irregular, circular and round shaped, small to large size, slightly raised elevation, odour less, entire to undulated margin, smooth surface, viscous consistency and whitish color on Picovskaya's medium (1948).

Table 4. Colony characteristics of PSB isolateson agar plate

Isolate	Shape	Size	Elevation	Odour
P1	Irregular	Medium	Slightly raised	Odour less
P2	Circular	Small	Slightly raised	Odour less
P3	Round	Large	Raised	Odour less

Table 5. Colony characteristics of PSB isolates on agar plate

Isolate	Margin	Surface	Opacity	Colour	Consistency
P1	Undulate	Smooth	Opaque	Whitish	Viscous
P2	Entire	Smooth	Opaque	Whitish	Viscous
P3	Undulate	Smooth	Opaque	Whitish	Viscous

Microscopic characteristics of the isolates: The Microscopic characteristics of the phosphorus solubilizing bacteria isolates were examined under light microscope and were found short rods, circular, round, Gram negative (Plate 2) and motile. Vincent *et al*. (1980) stated that Phosphorus solubilizing bacteria was gram negative, rod shaped and generally motile. The isolates produce circular, low convex to convex, mucous and opaque white. Phosphorus solubilizing bacteria isolates are presented in Table 6. All the isolates were found Gram negative and motile. The shape of phosphorus solubilizing bacterial isolates is presented in Table 7. The shape of the isolated bacteria P_1, P_2 and P_3 were found circular, round and shot rod, respectively.

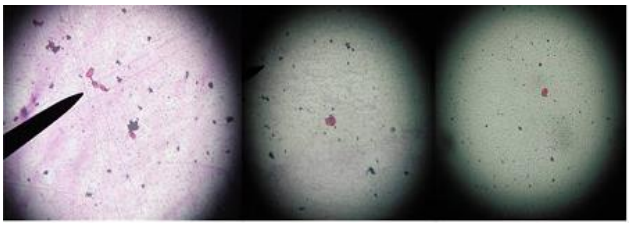

Plate 2. Microscopic view of Gram staining test

Table 6. Morphological (Microscopic) characteristics of PSB isolates

Isolate	Shape	Gram reaction	Motility
P1	Circular	Gram negative	Motile
P2	Round	Gram negative	Motile
P3	Shrot rod	Gram negative	Motile

Biochemical tests: Carbohydrate utilization

Results of carbohydrate utilization by the isolates are presented in Table 7. The sign of carbohydrate utilization was observed from the growth and fermentation characteristics of the isolates in a given carbohydrate medium and the variation in growth was identified by measuring the optical density of the media. It was observed that the isolates P_1, P_2 and P_3 showed heavy, minimum and medium growth in mannitol and sucrose, respectively. It was also observed that all the isolates showed minimum growth in glucose and produced gas in carbohydrate media used. Similar results were observed by Podder (1977).

Table 7. Carbohydrate utilization and fermentation by the strains

Strain	Sucrose	Glucose	Mannitol
P1	+++	+	+++
P2	+	+	+
P3	++	+	++

+++ = Heavy growth, ++ = Medium growth and + = Minimum growth

Gelatin hydrolysis: Results in Table 8 showed that all the isolates had the capacity to hydrolyze gelatin.

Table 8. Gelatin hydrolysisby the strains

Isolates	Gelatin hydrolysis
P1	(+)
P2	(+)
P3	(+)

(+) = hydrolytic/, (-) = nonhydrolytic

Growth on different pH levels: The growth responses of the PSB isolates were investigated in the Pikovskaya's broth having 5 levels of pH. The pH levels 4.0, 5.0, 6.0, 7.0 were created adding HCl solution and 8.0 adding NaOH as required. Results in the Table 9 showed that all isolates viz., were medium to heavy growers at pH 6, pH 7.0 and pH 8.0. In case of pH 4 and 5 they were found slight to minimum grower.

Table 9. Effectof different pH on PSB isolates in Picovskaya's medium

Isolate	Different pH				
	4	5	6	7	8
P1	-	+	++	++	++
P2	+	+	++	++	+++
P3	-	-	++	+++	++

+++ = Heavy growth, ++ = Medium growth , + = Minimum growth, - = Slight growth and - - = No growth

Starch hydrolysis: P_1, P_2 and P_3 of the isolates gave positive results for starch hydrolysis (Table 10). Halos developed around the bacterial colonies (Plate 3). Bergey's Manual of Determinative Bacteriology, 1974 was referred for the identification of the bacterial cultures. Podder (1977) also noted that the isolates from chickpea failed to cause hydrolysis of starch.

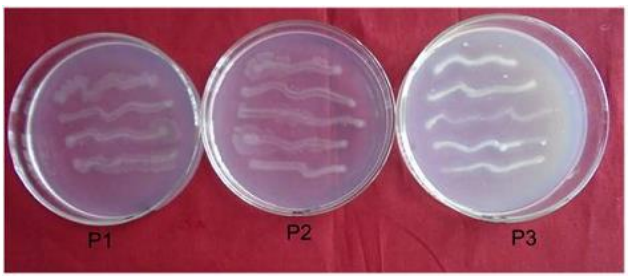

Plate 3. Growth of PSB isolates on Picovskaya's media with starch

Table 10. Physiological characteristics of isolated PSB strains

Isolate	Starch Hydrolysis test	Catalase test
P_1	+	+++
P_2	+	+
P_3	-	++

+++ = Rapid evolution of oxygen (O_2), + = Slow evolution of oxygen (O_2), ++ = Medium evolution of oxygen (O_2)

Catalase test: Table 10 showed that all of the tested isolates gave positive results for catalase test. All of the isolates produced bubbles within a few seconds (Plate 4). Bergey's Manual of Determinative Bacteriology (1974) was referred for the identification of the bacterial cultures.

Plate 4. Catalase test of PSB isolates

Bromothymol blue test: 5 ml of 0.5% bromothymol blue solution was thoroughly mixed with one liter of Pikovskaya's and then autoclaved and plated. Cultures of different PSB isolates were streaked on the plates and incubated at 30^0C and examined. Results in the Table 11 showedthat all isolates viz., P_1, P_2 and P_3 were found yellow in colour. The growth of the all isolates viz., P_1, P_2 and P_3 develops yellow color (Plate 5) that results acidic in nature.

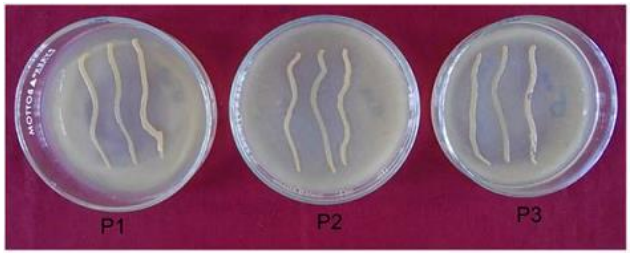

Plate 5. Effect of bromothymol blue on PSB isolates in Picovskaya's medium

Table 11. Effect of Bromothymol blue on PSB isolates in Picovskaya's medium

Isolate	Observation	Result
P1	Yellow colour	Acidic
P2	Yellow colour	Acidic
P3	Yellow colour	Acidic

Growth at different temperature conditions: All the isolates showed good growth at temperature 28^0C and 32^0C (Table 12). At 14^0C temperature the isolates P_1, P_2 and P_3 showed poor, medium and no growth, respectively. At 22^0C, all isolates exhibited medium growth.

Table 12 . Growth of isolates in different temperature conditions

Isolate	Growth in different temperature condition					
	14^0 C	22^0C	28^0C	32^0C	38^0 C	45^0C
P1	+	+ +	+ + +	+ + +	+ +	-
P2	++	+ +	+ + +	+ + +	+ +	-
P3	-	+ +	+ + +	+ + +	+ + +	-

- = No growth, + = Poor growth, ++ = Medium growth, + + + = Good growth

The isolates P_1 and P_2 exhibited medium growth and P_3 exhibited good growth at 38^0C.All the isolates exhibited no growth at 45^0C.Amarger *et al.* (1972) reported that freeze dried *R. meliloti* from old cultures survive better during storage at 30^0C than did freeze dried bacteria from young cultures. Deschodt and Strijdom (1976) observed that *Rhizobium meliloti* survived well at a temperature of 33^0C to 37^0C but *R. trifotii*, *R. japonicum* and a cowpea rhizobia survived less well at 37^0C.

Solubilization of insoluble phosphates by isolated bacteria: The relative efficiency of the isolates in solubilizing insoluble phosphates like tricalcium phosphate was investigated (Table 13). It was revealed that the phosphate solubilizing capacity varied between the isolates obtained from different rhizosphere soils.

Table 13. Solubilization of phosphorus by different bacterial isolates

Isolates Bacteria	Slubn. (mg P_2O_5)	Initial pH	Final pH	Net solubn. (mg P_2O_5)	% Solubn.
P1	20.04 a	5.78	3.98	9.405	37.62
P2	15.23 b	5.78	4.43	4.587	18.35
P3	14.20 c	5.78	4.85	3.560	14.24
Control	10.64 d	5.78	5.78		
CV	7.93				
SE	1.28				
Level of sig	**				

The results reviled that all the PSB isolates from different location of coastal region solubilize tricalcium phosphate as they solubilize a significant amount of P_2O_5. Among the isolates P_1 solubilize the highest amount of P_2O_5 (20.04 mg). The isolates P_3 solubilized the lowest (14.20 mg). Different amount of the solubilizing capacity of the isolates may be due to varied amount of organic acid in the liquid medium that decreased pH valueof the medium. Fankem*et al.*, (2006) stated that phosphate solubilization is the result of combined effect of pH decrease and organic acids production.

It is concluded from the present study that all the PSB isolates decreased the pH of the culture medium and showed the remarkable ability to solubilize tricalcium phosphate. Use of PSB as bioinoculants may increase the available phosphorus in soil. It helps to minimize the P-fertilizer application, reduce environmental pollution and promotes sustainable agriculture. Moreover, their performance under green house and field conditions should be assessed before being recommended for biofertilizer production and commercial applications.

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