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Isolation, Characterization of PSB stains from rock phosphate and their potential as Biofertilizer

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Abstract

Rock phosphate (RP) is an important alternative source of phosphorus (P) and phosphorus solubilizing bacteria (PSB) are responsible for the efficient use of this valuable source. In vitro study was conducted in the microbiology laboratory of Soil Science PMAS-AAUR to assess the ability of PSB strains to solubilize Phosphorus from Rock Phosphate amended broth medium. For this purpose, different phosphorus solubilizing strains were first isolated from Rock Phosphate and characterized by Gram testing and API 20 E-Kit. The strains were named as *Pseudomonas* after characterization. These strains made halo zone around its colony, which confirmed them as P-solubilizer. Among the various P-solubilizers, best strains were picked and their efficiency was tested in the broth culture. The drop of pH and P-solubilization were the key factors to be observed. An inverse relation between drop of pH and solubilized P was noticeable. The drop of pH was due to the productions of various organic acids and was measured by pH meter while mineralization of soil P was due to acid phosphatase. The drop of pH and Phosphorus was measured by Spectrophotometer at 880 nm. The individual application of these strains showed great variation in their capability to solubilize RP and same results were seen when applied in soil. However, the co-inoculation of the strains showed significant ability to solubilize Rock Phosphate. The results of the current study confirm that PSB have great potential to be used as a bio fertilizer.

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Introduction

Phosphorus is one of the major essential macronutrients for plants growth and development. It plays a vital biochemical role in photosynthesis, respiration, energy storage and transfer, cell division, cell enlargement and several other processes in the plants. It is applied to the soil in the form of phosphatic fertilizers. Availability of phosphorus for plant utilization is limited. As inorganic phosphate applied to the soil as chemical fertilizer, it is immobilized rapidly and becomes unavailable to plants (Akhtar *et al.,* 2010).Among the alternative P sources, the most important is locally available rock phosphate (Khan *et al.,*2009). Since phosphorus availability from the phosphate reserves under neutral and alkaline conditions is negligible, phosphate solubilizing microbes have special significance for enhancing the solubility of locked phosphate reserves in the soils (Patil *et al*., 2002).

Majority of the soils throughout the world are P-deficient (Muhammad, 2012). The concentration of bioavailable P in soil is very low getting to the level of 1.0 mg kg^{-1} (Goldstein, 1994). Phosphorus solubilizing microorganisms (PSM) play a vital role in soil P dynamics and subsequent availability of phosphate to plants (Richardson, 2001). Suboptimal levels of P can lead to a 5–15% loss in the yield of plants. The PSB can be used as bio fertilizer, which are environment friendly and are the possible way to increase the efficiency of P fertilizers. Phosphorus bio-fertilizer may help increase the availability of accumulated phosphate by solubilization process for crop production (Hinsinger, 2001).

Repeated and non-judicious applications of mineral P fertilizers could lead to the loss of soil fertility, disturbance to microbial diversity and their associated metabolic activities, and reduced yield of crops (Toro, 2007). This has led to the search for environment-friendly and economically feasible alternative strategies for improving crop production in low or P deficient soils. Natural rock phosphates have been recognized as a valuable alternative to P fertilizer through the process of mineralization/solubilization by microbes.

The main objectives of the study were to isolate, characterize and find out the most efficient strains of PSB and use them as a Biofertilizer.

Materials and methods

Different strains of phosphate solubilizing bacteria were isolated from rock phosphate. It was a laboratory scale study and whole of the work was undertaken in the laboratories of Department of Soil Science &SWC, Pir Mehr AliShah-Arid Agriculture University, Rawalpindi (PMAS-AAUR)

Sample preparation

Rock phosphate samples were collected from the Kakul area located in Abbottabad district Khyber Pakhtunkhwa. Visual impurities were removed and RP samples were brought to the laboratory in ice box. Soon after arrival in the laboratory, samples were passed through 2 mm sieve and stored in the refrigerator for further processing and use in the studies.

Characterization of rock phosphate

Suspension of RP (1:1 of RP and distilled sterilized water) was prepared. The pH of RP was measured using pH meter (McLean, 1982) which was recorded as 8.1. The $CaCO_3$ contents were determined by the procedure based on CH_3COOH consumption (Khan *et al*., 2011). By this procedure $CaCO_3$ was noticed as 25% in RP. Total P content in rock phosphate was determined by digestion with $HClO_4$ and was found as 14%. Available P was measured by spectrophotometer (Olsen and Sommers, 1982). The recoded available P in RP was as followed 37.10 ppm.

Isolation and screening of efficient PSB strains

The isolation of phosphorus solubilizing bacteria (PSB) from rock phosphate was done by following the serial dilution technique. For isolation, rock phosphate samples were serially diluted from 10^{-4} to 10^{-6} containing Pikovskaya's agar medium and incubated at 28°C (Wollum II, 1982). Colonies showing halo zones were picked and purified by sub culturing on Pikovskaya agar medium.

Biochemical Characterization of PSB Strains

Morphological characters

Suspension of each purified culture was prepared and poured on plates having solid media by spread plate method. The inoculate plates were incubated at 25°C till the appearance of colonies. Morphological characters of colonies like size, shape, color and elevation were measured as (Goenadi *et al.,* 2000).

Microscope characters

The isolated strains were heat fixed called as smear. Crystal violet was flooded for one minute and washed gently by tape water. Then the smears were exposed to Gram's iodine for one minute and washed and drained carefully. 95% alcohol was applied for 30 seconds and washed. Finally the smears were washed and drained with 0.25% safranine for 30 seconds and examined under microscope. It focused on shape, size and gram staining. Pink colored bacteria were named as Gram –ve while purple colored were named as Gram + ive.

Solubilization index

For testing the P – solubilizing capability of PSB strains, each PSB culture was poured on Pikovskayaagar's plate containing insoluble tricalciumphosphate. The plates having culture were incubated for seven days at 28°C.Solubilization index was measured by Edi-Premono formula (Edi-Premono *et al.,* 1996).

SI= Colony diameter + Halozonediameter/Colony diameter. The most efficient PSB strains were characterized by biochemical tests (Smibert and Krieg, 1994).

Biochemical tests with API 20 E kit

Biochemical characterization was done by using the API 20 E Kit which tested the PSB strains.

Determination of soluble p from rock phosphate broth culture

Bioavailability of the solubilized P was determined through inoculation of known P containing rock phosphate containing broth media with PSB. Sterilized broth media containing rock phosphate containing broth media was quantitatively poured into autoclaved 50 ml capped tubes with subsequent inoculation with two PSB strains (screened on SI basis) alone and in combination.

These tubes were shacked placed on reciprocal shaker at 300 rpm. On each 10 days interval, tubes were taken into laminar flow unit for recording pH and available P content. Available P content of broth culture was determined by taking 1 ml in sterile distilled water (containing about 1x 103 CFU) was added to sterile 100 ml Pikovskaya's broth (PB) medium in 250 ml conical flask and be kept on shaker.

The pH of the culture was recorded at 10 days interval for 4 week by digital pH meter. PSB cultures were grown in PB for with continuous shaking (reciprocating shaker at 150 cpm) at 35°C. Broth P was determined by Ascorbic acid method (Watanabe and Olsen, 1965). One mL of broth sample extract was be taken in 50 mL conical flask and 9 mL of distilled water + 2.5 mL of freshly prepared color reagent [12 g ammonium molybdate + 250 mL distilled water and 0.2908 mg antimony potassium tartrate in 1000 mL of 5N H_2SO_4 (148 mL conc. H_2SO_4 L^{-1})].

Both the solutions were mixed and the volume was raised up to 2 liter. 140 mL solution of this mixture was added to 0.74 g ascorbic acid and stirred gently.

The optical density of the blue color was measured at 880 nm by spectrophotometer. The concentration of available P (ppm) was determined by graph constructed by the readings from of known P standard solutions.

Statistical analysis

Statistical comparison of different PSB strains for P-solubilization efficiency, PSI, pH and available- P was undertaken. These parameters were measured at 0, 10, 20 and 30 days of incubation. Data were subjected to analysis of variance through MSTAT-C statistical computer programmed (MSTAT-C, 1988) and mean values were compared with least significance difference (Steel *et al.,* 1997).

Results and discussion

Isolation, purification and characterization of PSB

Phosphate solubilizing bacteria were isolated from rock phosphate employing serial dilution technique.

From the various PSB isolates, two best strains were selected on the basis of solubilization index for further characterization and incubation studies.

The characterization of PSB was done by the following tests.

Table 1. Identification of PSB through biochemical characterization using API 20 kit.

Tests	Reaction	Color PSB_1	Color PSB_2	Result PSB_1	Result PSB_2
ONPG	Beta galactosidase	Yellow	Yellow	+	+
ADH	L-arginine	Red	Orange	+	+
GLU	Glucose Fermentation	yellow	yellow	+	+
ARA	Arabinose fermentation	Blue green	Blue	_	_
LDC	Lysine Decarboxylase	Red	Orange	+	+
ODC	Ornithine Decarboxylase	Orange	Red	+	+
VP	Sodium pyruvate	Blue	Blue	_	_
MAN	D-manitol	Blue	Blue	_	_
INO	Inositol	Blue green	Blue	_	_
SOR	Sorbitol	Blue	Blue	_	_
RHA	Rhamnose	Blue	Blue	_	_
SAC	Sucrose	Yellow	Yellow	+	+
MEL	Mllibiose	Blue	Blue green	_	_
ARA	Arabinose	Blue	Blue	_	_
CIT	Citrate utilization	Blue grreen	Blue grreen	_	_
H2S	H_2 production	Colorless	Colorless	_	_
URE	Urease	Red	Red	+	+
TDA	Trytophane	yellow	Yellow	_	_
IND	Indole production	Pink	Pink	+	+
OX	Cytochrome oxide	Pale green	Yellow	_	_
NO_2	NO_2prodution	yellow	Yellow	+	+

Gram Test

Gram test was performed to check whether PSB strains were gram positive or negative. In this test both strains retained pink color and were categorized as gram negative.

P-Solubilization Test

For the solubilization test, PSB strain were cultured on Pikovskaya's agar medium and placed in incubator at 28°C for seven days.

The formation of halo zone around the bacterial colony confirmed them as P solubilizer (Seshadari *et al.*, 2000). Both the tested strains were found as P solubilizer.

API, 20- E Kit

Different tests were carried out by using this kit. Both strains were identified as member of *Pseudomonas* genus after comparing with Burgey's manual of determinative bacteriology.

Table 2. Effect of selected PSB strains on solubilization index.

PSB Strains	Days				Mean
	0	10	20	30	
Control (T_0)	0.00 i	0.00 i	0.00 i	0.00 i	0.00 D
PSB_1 (T_1)	0.00 i	1.29 h	3.26 ef	4.32 c	2.22 C
PSB_2 (T_2)	0.00 i	2.17 g	3.08 f	3.88 d	2.28 B
PSB_1+PSB_2(T_3)	0.00 i	3.46 e	6.34 b	8.20 a	4.5 A
Mean	0.00 D	1.73 C	3.17 B	4.1 A	_

All the mean values fallowed by same letters are not significantly different (P≤ 0.05, LSD= 0.0147).

Effect of PSB on broth acidity and p- solubilization

Results indicate that solubilization index varied with the different day's interval and applied strains. The treatment (PSB_1+PSB_2) showed maximum solubilization index of 3.46 at day 10, however minimum was noticed in PSB_1. Controversially at 20th day minimum solubiliztion index was noticed in PSB_2. White Law., (2000); Rashid *et al*., (2004) reported that generally solubilization index increases with days preceding but increase is not always with the same pace. Solubilization index of PSB_1+PSB_2 increased 2.88 from 10 to 20th day while 1.88 from day 20-30.

Table 3. Effect of PSB on Broth Ph.

PSB Strains	Days				Mean
	0	10	20	30	
Control (T_0)	7.00 a	7.00 a	7.00 a	7.00 a	7.00 A
PSB_1 (T_1)	7.00 a	6.32 b	5.72 d	5.45 e	6.12 B
PSB_2 (T_2)	7.00 a	5.96 c	5.16 f	4.82 g	5.73 C
PSB_1+PSB_2(T_3)	7.00 a	5.78 d	4.89 g	4.38 h	5.51 D
Mean	7.00 A	6.27 B	5.69 C	5.41 D	_

All the mean values fallowed by same letters are not significantly different (P≤.05,LSD= 0.07).

These results are in agreement to Joseph and Jisha (2009) and Alam *et al.* (2002) who noted such increase in solubilization index of PSB strains at different day's interval. Results revealed that solubilization index of PSB_1, PSB_2 and PSB_1+PSB_2 at 30th day was 4.32, 3.88 and 8.20, respectively. Solubilization index of PSB_1+PSB_2 was significantly higher with the mean value 4.5 and was statistically significantly different from PSB_1andPSB_2.

Table 4. Effect of PSB on soil pH.

PSB Strains	Days				Mean
	0	10	20	30	
Control (T_0)	7.67 a	7.67 a	7.67 a	7.67 a	7.67 A
PSB_1 (T_1)	7.67 a	7.46 b	7.35 b	7.02 d	7.39 B
PSB_2 (T_2)	7.67 a	7.36 b	7.19 c	6.86 e	7.27 C
PSB_1+PSB_2(T_3)	7.67 a	7.10 cd	6.86 e	5.75 f	7.09 D
Mean	7.67 A	7.40 B	7.27 C	7.09 D	_

Effect of PSB on broth pH

Change in pH by PSB isolates in broth medium was determined by using pH meter. Maliha *et al* (2004) and Chen *et al* (2005) reported that PSB strains secrete various organic acids which drop the pH of the mediums resulting in P- sollubilization. Among the selected PSB strains, PSB_1 showed minimum change in pH from 0-10 days while maximum change was observed in PSB_1+PSB_2. At 20th day of incubation, the difference in level of pH drop between PSB2 and PSB_1+PSB_2 was significant depicting its higher efficiency to decrease pH Alam *et al.,* (2002) also found a significant drop in pH of broth medium at regular interval.

Overall changes in pH of broth medium caused by different strains was noticed as 1.55 units (7.00-5.45), 2.18 units (7.00- 4.37) and 2.66 units (7.00-4.37) by PSB_1, PSB_2 and PSB_1+PSB_2 isolates respectively. Among the applied treatments, the treatment (PSB_1+PSB_2) showed maximum drop in pH and this drop was observed at day 30. The drops of pH in PSB broth culture has been reported by several researchers like Rashid *et al.* (2004) and Panhwar *et al.,* (2009), who found PSB very effective in lowering the pH of the broth medium. Present study results are in line with their findings as they said about PSB's inoculation result in change of pH broth medium.

Table 5. Effect of PSB on soil phosphorus (µgg^{-1})

PSB Strains	Days				Mean
	0	10	20	30	
Control (T_0)	8.78 g	8.78 g	8.78 g	8.78 g	8.78 D
PSB_1 (T_1)	8.78 g	9.14 g	10.45 f	15.13 d	10.79 C
PSB_2 (T_2)	8.78 g	10.28 f	13.53 e	18.71 b	12.83 B
PSB_1+PSB_2(T_3)	8.78 g	12.82 e	16.86 c	23.19 a	15.50 A
Mean	8.78 D	10.20 C	12.35 B	16.43 A	_

All the mean values fallowed by same letters are not significantly different (P≤ 0.05, LSD=0.047).

Phosphorus content in broth

Results reveal that all the PSB (strains) solubilized phosphorus from 0-10th day at varying rates having significant difference among them. Though the quantity of P solubilized by PSB_2 at 20th day was much greater than PSB1 but rate of solubilization was less as compared to PSB_1.

Fig. 1. Halo zone formation by PSB_1

The quantity of P solubilized by PSB_1+PSB_2, PSB_2 and PSB_1 figured as 198.81 µg mL^{-1}, 174.08µg mL^{-1} and 168.60µg mL^{-1}, respectively. Overall inoculated PSB_1+PSB_2 proved to be most efficient treatment solubilizing phosphorous in broth culture. The results are inconformiting with Sundra *et al* (2002) who reported similar results of individual and co-inoculation of PSB strains in liquid media. They suggested co inoculation of PSB strains works better than individual application of PSB strains.

Solubilization of Soil Phosphorus

Results revealed that all the strains solubilized phosphorus but the rate of solublization varied among them. Phosphorus Solubilization was found increasing with the day's interval during incubation. All the strains showed better P-Solubilization potential as compared to control. The co-inoculated treatment (PSB_1+PSB_2) effectively solubilized 12.82 µgg^{-1}Pat day 10; it is followed by PSB_2. Similarly at day 20, maximum quantity of P was solubilized by PSB_1+PSB_2 and PSB_2 while minimum was solubilized by PSB_1. Significant difference was also observed at day 30 in solubilized P rate between PSB_1 and PSB_2 but the amount differed between them. Total quantity of P solubilized by PSB_1, PSB_2 and PSB_1+PSB_2 was recorded as 6.71 µgg^{-1}, 10.29 µgg^{-1} and 14.04 µgg^{-1} respectively from 0-30 days.

Overall, the treatment T3 (PSB_1+PSB_2) dual inoculation proved best in soil P availability. At day 30 maximum solubilized quantity of phosphorus was recorded which was due to the action of co inoculated strains PSB_1+PSB_2. These results match with the work done by Wani *et al* (2007) who found dual inoculation very effective for the availability of soil P. Overall an increasing trend of P solubilization with increase in incubation days is reported in said study, which is in accordance with the finding of Fernandez *et al* (2007).

Fig. 2. Halo zone formation by PSB_2

Correlation coefficients between different parameters of selected psb strains

Data analysis gave a significant positive correlation (r = 0.96) between a colony diameter and colony + halo zone formation of isolates Fig.1& Fig. 2. Colony diameter was found to be highly positively correlated with solubilization index and available P. Colony + halo zone diameter appeared significantly negatively correlated (r = -0.95) with pH and significantly positively with solubilization index and available P.

However it showed significantly negative correlation with colony diameter. A highly negative correlation (r = -0.97) between pH and available P was observed. These findings matched with Keneni *et al* (2010), who found a significant negative correlation (r = -0.93) between pH and available P but are in contrary with the findings Narsian *et al* (1995) who found no correlation between pH and available P. Solubilization index and P solubilized showed a strong correlation with each otheras supported by the results of Chen *et al* (2005)who found a positive correlation between Solubilization index and P-solubilized.

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