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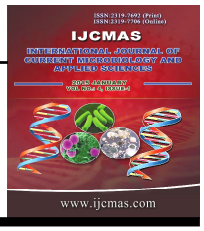

Original Research Article

Isolation, identification and characterization of phosphate solubilizing bacteria (PSB) isolated from economically important crop plants

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A B S T R A C T

There are various types of soil microbes which can solubilize the fixed form of P and make it available to plants. Such organisms are called Phosphate solubilizing bacteria (PSB). In the present study, PSB were isolated, identified and characterized. Further, biology of the selected strains was also studied. The study revealed that the population level of PSB was higher in the rhizosphere soils of cluster bean. The selected strains were identified as Bacillus and Pseudomonas spp. The isolated strains were characterized under in vitro conditions. The selected strains were differed in P solubilization zone formation, pH change, phosphatase activity, organic acids production and P solubilization. PSB strains could grow well at the temperature ranged from 28ºC to 35ºC. All the strains were differed in utilization of different carbon, nitrogen, amino acid, vitamin sources.

K e y w o r d s

PSB,
Crop plants,
Isolation,
Identification,
Characterization

Introduction

Phosphorus is an essential element for plant development and growth. Plants acquire P from soil solution as phosphate anions. However, phosphate anions are extremely reactive and may be immobilized through precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, depending on the particular properties of a soil. In these forms, P is highly insoluble and unavailable to plants. As the results, the amount available to plants is usually a small proportion of this total (Khan *et al.*, 2010).

Phosphate solubilizing microorganisms (PSM) play a significant role in making phosphorus available to plants by bringing about favourable changes in soil reaction in the soil microenvironment leading to solubilization of inorganic phosphate sources. Some microorganisms associated with different pant rhizosphere are able to solubilise inorganic insoluble P salts. *Pseudomonas* and *Bacillus* are two important genera of soil bacteria with promising activity of phosphate solubilisation (Reyes *et al.*,1999; Yadav and Tarafdar, 2011).

The majority of the isolated organisms are bacterial organisms, although several fungi are also known to solubilize phosphates. These bacteria and fungi have the potential

to be used as biofertilizers. Their role in increasing the soil nutrient value is of utmost importance. Their application to crop fields has resulted in an increased yield of several crops, such as cereals, legumes, fibers, vegetables, oils, and other crop plants (Silini-Cherif, 2012; Viruel *et al*., 2011; Khalimi *et al*., 2012).

The objective of this research work is to isolate the PSB strains from the rhizosphere soil of different crop plants such as such as bhendi, chilly, cluster bean and tomato. Further, the isolated strains were identified and characterized and also studied their biology.

Materials and Methods

Collection of soil samples

Rhizosphere soils were collected from the bhendi, chilly, cluster bean and tomato. The soil samples were air dried under shade and used for the isolation and enumeration of PSB.

Isolation and enumeration of PSB

Isolation and enumeration of PSB were carried out by dilution plate technique using hydroxy apatite medium.

Identification of PSB

The isolated bacterial strains were identified using standard biochemical tests as listed in the Bergey's Manual of Determinative Bacteriology (Krieg and Dobereiner, 1984).

Characterization of PSB

Characterization of PSB was done under *in vitro* by measurement of halo zone, determining the pH change of the medium, estimation of available P Olsen *et al.* (1954), phosphatase enzyme (Eivazi and Tabatabai, 1977) and organic acids (Sperber, 1958).

Utilization of carbon, nitrogen, amino acid and vitamin sources

The utilization of different carbon, nitrogen, amino acid and vitamin sources by PSB isolates were estimated in LB broth. Filter sterilized carbon, nitrogen, amino acid and vitamin sources were inoculated aseptically into the sterile medium at 1 per cent level.

The PSB cultures were inoculated at the rate of 1.0 ml and incubated at room temperature. The growth was observed by the turbidity of the broth read at 560 nm.

Result and Discussion

Isolation and population dynamics of PSB

The population level of PSB was higher in the rhizosphere soil collected from cluster bean followed by tomato. Based on the solubilization zone production in the solid medium, two isolates from each crop plant were isolated and totally 8 PSB strains were selected.

These 8 strains were sub cultured regularly and used for further studies (Table 1). Gaind (1987) reported that the PSB strains were isolated using the Pikovskaya's medium based on the formation of halo zone around these microorganisms.

These findings were also supported by Ahmad and Jha (1967) that the phosphobacteria were identified by noting the solubilizing zone formed around the bacterial colony. Further, Kundu *et al.* (2002) were isolated 73 PSB strains from the rhizosphere of different crops. Out of 73 PSB isolates, 11 isolates showed better zone of P-solubilization on solid medium.

Identification of PSB

Based on the biochemical and morphological tests, PSB were identified at genus level. Among 8 PSB strains, 5 strains (BDP 1, CP 1, CP 2, TP 1 and TP 2) were identified as *Bacillus megaterium*, 2 strains (CBP 1and CBP 1) as *Pseudomonas putida* and 1 strain (BDP2) as *Pseudomonas fluorescence* (Table 2).

Pseudomonas putida: They were Gram negative and motile and growth was strictly aerobic and spores were absent in all pseudomonads. *P. putida* were positive to oxidase, catalase, arginine dihydrolase, acid from glucose and growth on citrate agar and negative to growth at 41ºC, gelatin hydrolysis, starch hydrolysis and denitrification.

Pseudomonas fluorescens: They were Gram negative, motile and growth was strictly aerobic and not producing spores. On King's B medium, they produced fluorescein, a water soluble pigment. Cells positive to oxidase, catalase, lipase activity, arginine dihydrolase, gelatin hydrolysis, acid from glucose, and urease and negative to growth at 41ºC and starch hydrolysis.

Bacillus megaterium: Growth strictly aerobic and produced endospores. Cells were Gram positive and motile in nature. Growth in glucose agar was mucoid. They were positive to catalase, acid from glucose, casein hydrolysis, gelatin hydrolysis, starch hydrolysis, citrate utilization and nitrate reduction and negative to anaerobic growth, gas from glucose, VP test, indole production and growth with lysozyme.

Gaur *et al*. (1973) studied the bacterial cultures morphological, cultural and physiological and biochemical characteristics using the manual of microbiological methods and identified the organism *Bacillus* sp., using Bergey's manual of Determinative Bacteriology. Frietas and Germida (1990) isolated the phosphate solubilizing microorganisms such as *Pseudomonas* a*eruginosa, P. cepacia, P. fluoresence* and *P. putida* from the rhizosphere of wheat and *Bacillus licheniformis, B. mycoides, B. megaterium* from the rhizosphere of paddy. The morphological and biochemical characters of phosphobacteria were found to be gram negative with rod shaped and non motile characteristics. The organism showed positive results for indole production, methyl red and catalase test (Amutha *et al.* 2014).

Characterization of PSB strains

Among 8 strains, CBP 2 to be superior in forming halo zone of P solubilization followed by BDP2 and CBP 1 (Plate 1). All the PSB strains brought down the pH in the liquid medium. The maximum pH reduction was noticed in CBP2 with tricalcium phosphate (TCP) as phosphate source. Among 8 PSB strains, the strains CBP 2 (12.9 0.1 N NaOH consumed) and CBP1 (8.7 0.1N NaOH consumed) were good in organic acid production in the presence of TCP. Estimation of phosphatase activity indicated that the activity by PSB was highest with strain CBP2 and least with TP 2. The P solubilization potential of selected strains of PSB was tested *in vitro* by estimating available phosphorus in the liquid medium. The results indicated that a wide variation in the phosphate solubilization capacity of different strains in PSB. Among 8 PSB strains, CBP 2 (45.96ppm) released more phosphorus in the medium followed by CBP1 (45.4ppm) with TCP as phosphate source (Table 13).

The clear or halo zone was formed due to the solubilization of insoluble phosphates by acidification of association of either proton

extrusion or organic acid secretion (Darmwall *et al.,* 1989). The mechanism of solubilizing insoluble phosphate by soil microbes was on their ability to secrete organic acids. The organic acids bring down soil pH resulting in the dissolution of insoluble forms of phosphate (Hegde and Dwivedi, 1994). PSM produced the phosphatase enzyme which solubilized the phosphate in the aquatic environment (Alghazalli*et al*., 1986). It was suggested that the phosphate solubilizing activity in liquid medium ranged from 11% to 72% of total phosphate solubilization (Dave and Patel, 1999).

Biology of PSB strains

The PSB strains preferred temperature ranging from 20ºC to 35ºC and above and below which the growth was retarded. PSB strains could grow well at temperature 28ºC to 35ºC (Table 4). The PSB strains utilized different types of chemical compounds as carbon source. The utilization of different types of carbons sources varied from strain to strain. Most of the PSB strains were preferred well glucose, fructose as carbon source and maltose and lactose were moderately utilized. Further, starch and sucrose were poorly utilized by all strains (Table 5).

It was observed that PSB strains moderately utilized nitrogenous compounds such ammonium sulphate, ammonium nitrate, ammonium chloride, potassium nitrate and urea (Table 6). All the aminoacids were found to be supported the growth of PSB strains. Amino acids like leucine and isoleucine were preferred more compared to other amino acids (Table 7). Among different types of vitamins tested, ascorbic acid was utilized more by all PSB strains. Thiamine, biotin and myoinositol were moderately utilized. Further nicotinic acid poorly utilized by all PSB strains (Table 8).

Temperature tolerance and utilization of carbon, nitrogen, vitamin and amino acid sources varied within the selected strains of PSB. These variations are mainly due to the nature of strains and differences in the species of respective genus. The optimum temperature for growth of PSB preferred a temperature range of 30 - 35°C (Gaind and Gaur, 1991). Phosphate solubilization was greatly influenced by the tolerance of temperature in the culture medium. A range of 30ºC - 35ºC was more favorable for rock phosphate dissolution by PSB in the alkaline soils of tropics (Narula *et al.,* 1995).

There was a marked difference in the carbohydrate utilization between the species of PSB (Patil *et al*., 2001). Dave and Patel (2003) experimentally proved that N sources such as ammonium sulphate and ammonium nitrate were best for the solubilization of rock phosphate. All the amino acids and vitamins supported the growth of PSB strains. The preference of amino acids and vitamins differed from strains to strains for PSB.

The phosphorus applied to soil cannot be fully utilized by the plants due to its chemical fixation. However, some microorganisms are able to solubilize it and make available to the plants. Such organisms are called as phosphate solubilizers. Several soil bacteria, particularly those belonging to the genera *Pseudomonas* and *Bacillus* possess the ability to solubilize the insoluble phosphate into soluble form. From this present study, the solubilization is only based characteristic feature of PSB such P solubilization zone production in the solid medium, pH change of the liquid medium and phosphatase activity, organic acids production and ability to P liberation.

Table.1 Population level of PSB in different crop plants

Crop Plants	Population level (x 10^5 /g soil dry wt.)	PSB isolates and Code No.
Bhendi	6.23	BDP1 BDP2
Chilly	6.12	CP1 CP2
Cluster Bean	18.21	CBP1 CBP2
Tomato	11.28	TP1 TP2

Table.2 Identification of PSB

PSB Strains	Identified PSB strains
BDP1	*Bacillus megaterium*
BPP2	*Pseudomonas fluorescence*
CP1	*Bacillus megaterium*
CP2	*Bacillus megaterium*
CBP1	*Pseudomonas putida*
CBP2	*Pseudomonas putida*
TP1	*Bacillus megaterium*
TP2	*Bacillus megaterium*

Table.3 Characterization of PSB strains

PSB strains	Solubilization zone (mm)	pH Reduction	Organic acid (0.1 N NaOH Consumed)	Phosphatase activity (µ mole/ml/hr)	Available P (ppm)
BDP1	2	5.6	3.1	20.8	43.0
BDP2	4	5.1	3.7	23.3	44.8
CP1	2	4.7	6.2	20.9	45.0
CP2	2	4.7	6.7	21.0	45.08
CBP1	4	4.9	8.7	22.9	45.4
CBP2	5	4.5	12.9	26.1	45.96
TP1	2	4.7	7.0	19.6	44.68
TP2	2	4.7	7.4	19.3	44.36

Table.4 Temperature tolerance of PSB Strains

PSB Strains	Temperature				
	5ºC	15ºC	28ºC	35ºC	50ºC
BDP 1	+	++	+++	+++	-
BDP 2	+	++	+++	+++	-
CP 1	+	++	+++	+++	-
CP 2	+	++	+++	+++	-
CBP 1	+	++	+++	+++	-
CBP 2	+	++	+++	+++	-
TP 1	+	++	+++	+++	-
TP 2	+	++	+++	+++	-

- → No growth
+ → Poor growth
++ → Moderate growth
+++ → Best growth

Table.5 Utilization of carbon sources by PSB strains

Strains	Glucose	Lactose	Maltose	Sucrose	Starch	Fructose
BDP 1	+++ (1.265)	++ (1.096)	+++ (1.109)	++ (0.834)	++ (0.650)	+++ (1.243)
BDP 2	+++ (1.285)	++ (1.087)	+++ (1.197)	++ (0.800)	++ (0.603)	+++ (1.286)
CP 1	+++ (1.326)	++ (1.038)	+++ (1.321)	++ (0.325)	++ (0.567)	+++ (1.331)
CP 2	+++ (1.314)	++ (1.035)	+++ (1.297)	++ (0.291)	++ (0.576)	+++ 1.365)
CBP 1	+++ (1.304)	++ (1.352)	+++ (1.294)	++ (1.363)	++ (0.091)	+++ (1.341)
CBP 2	+++ (1.285)	++ (1.382)	+++ (1.275)	++ (1.334)	++ (0.027)	+++ (1.439)
TP 1	+++ (1.360)	++ (1.037)	+++ (1.261)	++ (0.325)	++ (0.802)	+++ (1.295)
TP 2	+++ (1.331)	++ (1.038)	+++ (1.296)	++ (0.731)	++ (0.813)	+++ (1.306)

Table.6 Utilization of nitrogen sources by PSB strains

Strains	Ammonium nitrate	Potassium nitrate	Ammonium chloride	Urea	Ammonium sulphate`
BDP 1	++ (1.147)	++ (1.135)	++ (1.308)	++ (1.327)	++ (1.186)
BDP 2	++ (1.141)	++ (1.161)	++ (1.264)	++ (1.323)	++ (1.191)
CP 1	++ (1.093)	++ (1.092)	++ (1.314	++ (1.292)	++ (1.103)
CP 2	++ (1.148)	++ (1.104)	++ (1.333)	++ (1.308)	++ (1.155)
CBP 1	++ (1.109)	++ (1.164)	++ (1.323)	++ (1.348)	++ (1.238)
CBP 2	++ (1.140)	++ (1.126)	++ (1.319)	++ (1.338)	++ (1.241)
TP 1	++ (1.156)	++ (1.137)	++ (1.282)	++ (1.336)	++ (1.146)
TP 2	++ (1.146)	++ (1.055)	++ (1.274)	++ (1.301)	++ (1.173)

Table.7 Utilization of amino acid sources by PSB strains

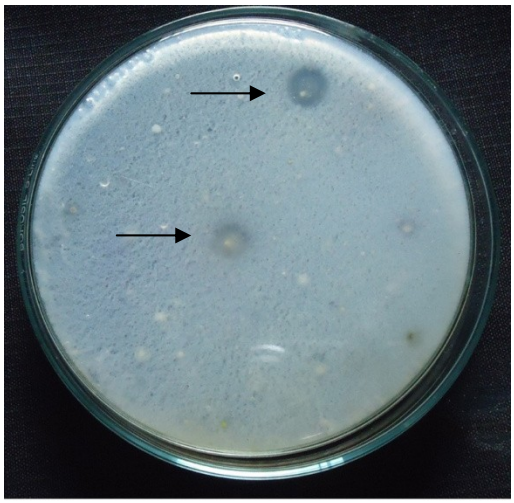
Strains	Leucine	Isoleucine	Alanine	Therionine	Cytosine
BDP 1	+++ (1.535)	+++ (1.540)	++ (1.101)	+++ (1.501)	++ (1.495)
BDP 2	++ (1.477)	++ (1.497)	++ (1.142)	+++ (1.533)	++ (1.473)
CP 1	+++ (1.538)	+++ (1.525)	++ (1.031)	++ (1.462)	++ (1.480)
CP 2	+++ (1.511)	+++ (1.577)	++ (1.074)	+++ (1.548)	++ (1.493)
CBP 1	+++ (1.519)	+++ (1.534)	++ (1.128)	+++ (1.546)	++ (1.489)
CBP 2	+++ (1.770)	+++ (1.434)	++ (1.079)	++ (1.445)	+++ (1.521)
TP 1	++ (1.496)	+++ (1.572)	++ (1.003)	+++ (1.504)	+++ (1.554)
TP 2	++ (1.482)	+++ (1.548)	++ (1.015)	++ (1.496)	+++ (1.550)

Table.8 Utilization of vitamin sources by PSB strains

Strains	Nicotinic acid	Thiamine	Ascorbic acid	Myoinositol	Biotin
BDP 1	+	+++	+++	+++	+++
	(0.997)	(1.144)	(1.388)	(1.289)	(1.135)
BDP 2	+	+++	+++	+++	+++
	(0.878)	(1.148)	(1.380)	(1.314)	(1.161)
CP 1	+	+++	+++	+++	++
	(0.894)	(1.129)	(1.354)	(1.250)	(1.092)
CP 2	+	++	+++	+++	+++
	(0.705)	(1.095)	(1.360)	(1.253)	(1.104)
CBP 1	+	+++	+++	+++	+++
	(0.948)	(1.107)	(1.415)	(1.297)	(1.164)
CBP 2	+	+++	+++	+++	+++
	(0.893)	(1.105)	(1.391)	(1.270)	(1.126)
TP 1	+	++	+++	+++	+++
	(0.803)	(1.089)	(1.357)	(1.277)	(1.137)
TP 2	+	+++	+++	+++	+++
	(0.847)	(1.137)	(1.387)	(1.317)	(1.055)

Values in parentheses indicate OD value
(+) – 0.0 – 0.5→ Poor growth
(++) – 0.51 – 1.0 → Moderate growth
(+++) – 1 .1 – 1.5 → Best growth

Plate.1 Solubilization zone produced by PSB



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