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Isolation and Screening of Phosphate Solubilizing Bacteria from Paddy Rhizosphere Soil

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ABSTRACT

Phosphorus is one of essential macro-minerals for the growth and development of plant. The main of this study to isolate the PSB and screened for its solubilization efficiency on Pikovskaya's agar and broth at different incubation period. Totally 40 PSB were isolated from paddy rhizosphere soils of Raichur and Koppal districts. PSB isolates were studied for zone of solubilization, solubilization efficiency, solubilization index, ph change titrable acidity and phosphatase activity. The solubilization zone, efficiency and index were highest on 6th day of incubation period. From that 10 isolates were found to be good phosphate solubilizer. Among 10 isolates, isolate PPSB-21 showed highest zone of solubilization (18.4 mm) phosphate solubilization efficiency (253.84%) and solubilization index (3.53) on Pikovsakaya's medium. These efficient isolates can be used as biofertilizer.

Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops in the world belongs to the family Poaceae. Rice is the important food grain and staple food for over the billions of people in most of the countries particularly in East Asia. It's a major part of balanced diet and rich source of energy and carbohydrate. It is extensively grown food crop in 114 countries across the world, occupying area of about 163.20 million hacters of farm lands with annual production of over 758.9 million tonnes with a productivity of 4448 kg ha^{-1} per hectare.

Phosphorus is one of essential macro-minerals for the growth and development of plant

(Schachtman *et al.,* 1998). It is a major component in ATP, the molecule that provides energy to the plant for such processes as photosynthesis, protein synthesis, nutrient translocation, nutrient uptake and respiration. In addition, P has been observed to increase root growth and influence early maturity, straw strength, crop quality and disease resistance (Deepak Kumar, 2011). The available phosphorus in soils of India is generally poor. Efficiency of P fertilizer is around 10-25 % throughout the world (Isheword, 1998).

India, majority of the phosphorus provided in the form of chemical fertilizer. Inorganic P occurs in soil, mostly in insoluble mineral complexes, these insoluble, precipitated forms cannot be absorbed by plants (Rengel and Marschner, 2005). Large amount of phosphorus applied as fertilizes enter into immobile pool through chelating action with highly reactive Al^{3+} and Fe^{2+} in acidic soil (Gyaneshwar *et al.,* 2002). In nature, wide range of microbial biosolubilization mechanisms exist which are necessary to maintain global cycle (Whitelaw, 2000).

Phosphorus solubilizing bacteria a group of beneficial bacteria play a key role in soil Phosphate solubilization (Abd-Alla, 1994) there by increasing the bioavailability of soil P for plants (Zhu *et al.,* 2011). Phosphate solubilization occurs by production of organic acids and released by microorganisms; this release also decreases soil pH (Rodriguez *et al.,* 2006). Organic acids solubilize insoluble P either by decreasing the pH or by the complexing the cation which is bound to the P (Vassilev *et al.,* 2006). The organic acids such as succinic acid, malic acid, propionic acid and oxalic acid are released by Phosphorus solubilizing bacteria (Panhwar *et al.,* 2012), which chelate the cation bound to phosphate and being converted to soluble forms through their hydroxyl and carboxyl groups. Large number of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter*, *Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus, Rhizobium* and *Serratia* have reported to enhance plant growth by with their different plant growth promoting activities including phosphate solubilization (Kumar *et al.,* 2012).

Materials and Methods

Collection of soil sample

A total of forty soil samples from rhizosphere of paddy were collected from different locations of Raichur and Koppal district of Karnataka by adopting standard soil sampling methods described by Jackson (1973). Soil samples were collected in sterilized polythene bags. The Polythene bags were properly tied; labeled and at most care was taken to avoid contamination. Soil samples were stored in refrigerator at $4^{o}C$ for the isolation of phosphate solubilizing bacterial isolates.

Analysis of soil chemical properties

Air dried samples were analysed for pH, EC, OC, N, P, K and Zn content estimation by following standard procedures.

Isolaton of PSB from paddy rhizosphere soil

One gram of soil from each sample was suspended in 9 ml blank sterile distilled water and serially diluted up to 10^{-6} homogenization of soil was carried out by keeping it on shaker. The dilutions were plated on Pikovskaya's agar medium Pikovaskaya's medium (Pikovskaya's, 1948) contains (grams/liter); Glucose, 10.0gm; $Ca_3(PO_4)_2$, 5.0 gm; $(NH_4)_2SO_4$, 0.5 gm; NaCl, 0.2 gm; $MgSO_4.7H_2O$, 0.1gm, pH 7.3±2. These plates were kept at 28±2 $^{\circ}C$ for 7 days in order to isolate the PSB. The bacterial colonies exhibiting the clearance zone around the colonies were selected, purified, sub-cultured and stored on the slants of Pikovskaya's agar for further studies.

Screening of PSB

Each of the isolates was screened for their ability to solubilize Tri phosphate Phosphate (TCP) present in the Pikovaskaya's medium. A loopful of bacterial culture was placed on the centre of the same agar plates and incubated for 28±2^{o}C for 6 days. The solubilization zone, efficiency and index were calculated at different incubation period. The phosphate solubilization efficiency was calculated (Kannapiran and Ramkumar, 2011).

$$SE\ \% = \frac{SZ - CD}{CD} \times 100$$

Where, SE = Solubilization efficiency (%), SZ = Solubilization zone (mm), CD= Colony diameter (mm).

Solubilization index on the solid media was calculated considering the ratio of the total (colony + halo zone) diameter and colony diameter (Edi-Premono *et al.,* 1996).

Quantitative estimation of available phosphorous in Pikovsakaya's broth *in vitro*

The PSB isolates were tested invitro by estimating available phosphorus in the Pikovskaya's broth supplemented with TCP as a substrate. Cultures of PSB were inoculated to 100 ml of Pikovskaya's broth in 150 ml conical flask in triplicates with uninoculated controls and incubated for 10 days at 30 ^{0}C and centrifuged at 10000 rpm for 10 minutes. Then separate the supernatant from cell growth and insoluble phosphate. The clear supernatant was collected in 100 ml volumetric flasks. The available phosphorus in the filtrate was estimated by method of Olsen, 1954.

pH change

Estimation of change in pH of the broth due to the growth of PSB was measured with a pH meter at different incubation period of 3rd, 6th, and 9th day **(**Parimal *et al.*, 2015).

Titrable acidity

In order to study the titrable acidity of culture medium, 5 days old cultures were centrifuged at 1000 rpm for 10 min. 10 ml culture filtrate was taken in a 50 ml conical flask. 5ml of supernatant was added with few drops of phenolphthalein indicator and titrated against 0.01 N NaOH solution. The end point of titration was determined as pink colour. The titrable acidity was expressed as ml of 0.01 N NaOH consumed per 5 ml of culture filtrate **(**Ponmurugan and Gopi, 2006).

Phosphatase activity

In order to study the phosphatase activity in response to the phosphorus enrichment, culture filtrate were centrifuged and subjected to estimate phosphatase activity following the standard procedure (Tabatabai and Bremner, 1969).

Results and Discussion

Total 40 PSB were isolated from 40 rhizospheric sample of rice from different paddy growing regions of Raichur and Koppal districts. Among 40 isolates, ten isolates showed the remarkable zone of solubilisation (table 1). Based on clear zone formation around colonies on Pikovsakaya's medium the solubilization efficiency and index calculated (table 1)

Hallow zone of solubilization was ranged from 18.5 mm to 12.5mm Plate-1 shows maximum zone of solubilization. There was a correlation with incubation time and zone size. It was also observed that increasing in the incubation time, increases the in the zone size of each isolates. Percent Solubilization efficiency was ranging from 80.95 to 253.84% from 3rd to 9th day of incubation period. The SE% increased with incubation period. The solubilization index based on colony diameter and halo zone for each PSB isolate is presented in Table 1. The results showed that, among the 10 efficient isolates, the solubilization index varied from 1.80 to 3.22. The solubilization efficiency index enhanced with incubation period. The diameter of clear halo zone formed by the bacterial isolates has the direct correlation with the phosphate solubilization efficiency.

All the ten isolates were able to solubilize the insoluble phosphorus in Pikovaskaya's broth at different incubation period and were represented in table 2. Among ten isolates, available phosphorous content was ranged from 31.92 to 171.84mg/L. All the isolates were recorded decreased pH with increased incubation period. Among ten isolates, the reduction in the pH varied from 6.12 to 3.15. The drop of pH was due to the production of organic acids.

The titrable acidity of the culture medium was measured. All ten isolates showed values in the range of 0.30% to 0.65%. The titrable acidity increased with incubation period. The variability in the phosphatase activity of soil ranged from minimum of 16.28µmoles/g/hr to maximum of 42.40µmoles/g/hr with the application of PPSB-21 and PPSB-5 respectively (table 3). There was a positive correlation between the phosphate solubilizing capacity and phosphatase activity.

Table.1 Screening of PSB isolates for zone diameter, solubilization efficiency and solubilization index at different incubation period on Pikovsakaya's agar media

S. No.	PSB isolates	Zone diameter	Solubilization efficiency%			Solubilization index		
		6th day	2nd day	4th day	6th day	2nd day	4th day	6th day
1	PPSB1 (LGR-2)	12.5	80.95	98.33	127.27	1.80	1.98	2.27
2	PPSB5	17.5	136.61	178.46	222.80	2.36	2.78	3.22
3	PPSB7	15.8	113.23	158.33	216.00	2.23	2.58	3.16
4	PPSB11	16.3	102.89	149.43	201.85	2.12	2.47	3.01
5	PPSB15	12.5	82.53	106.89	140.38	1.82	2.06	2.40
6	PPSB21	18.4	143.47	198.36	253.84	2.43	2.98	3.53
7	PPSB25	13.0	88.33	110.52	150.00	1.86	2.10	2.50
8	PPSB28	12.9	101.61	139.62	186.66	2.01	2.39	2.86
9	PPSB35	14.5	90.00	127.77	178.84	1.91	2.17	2.68
10	PPSB39	12.9	98.33	133.96	180.43	1.98	2.33	2.80
	G R -8	16.5	120.58	165.07	119.81	2.31	2.65	3.21

Table.2 Quantitative estimation of available phosphorous in Pikovsakaya's broth *in vitro* and effect of PSB isolates on pH change of Pikovsakaya's broth

S. No.	PSB isolates	Amount of P released (mg/L)			Change in pH		
		3rd day	6th day	9th day	3rd day	6th day	9th day
1	PPSB1	44.89	82.53	121.44	6.12	5.88	5.24
2	PPSB5	63.90	104.94	159.90	3.76	3.58	3.32
3	PPSB7	55.63	93.31	148.53	4.98	4.21	3.96
4	PPSB11	58.59	99.54	151.59	5.03	4.75	4.10
5	PPSB15	39.43	79.05	132.43	5.25	4.83	4.28
6	PPSB21	64.84	110.67	171.84	3.54	3.37	3.15
7	PPSB25	35.67	76.53	128.67	5.95	5.14	4.87
8	PPSB28	50.23	90.68	140.23	5.30	4.96	4.42
9	PPSB35	48.13	87.62	124.13	6.08	5.84	5.13
10	PPSB39	31.92	71.21	118.92	5.65	5.08	4.24
	G R -8	60.32	102.07	155.51	3.90	3.70	3.40

Table.3 Effect of PSB on titrable acidity of Pikovsakaya's broth and phosphatase activity of rhizosphere soil

S. No.	PSB isolates	Titrable acidity (%)	Phosphatase activity (µ moles/g/hr)
1	PPSB1	0.30	16.28
2	PPSB5	059	37.05
3	PPSB7	0.50	31.10
4	PPSB11	0.49	28.72
5	PPSB15 -1)	0.41	25.58
6	PPSB21	0.65	42.40
7	PPSB25	0.35	18.25
8	PPSB28	0.38	21.39
9	PPSB35	0.32	13.47
10	PPSB39	0.45	23.55
	R. strain	0.52	33.00
	Control	0.07	3.35

Plate.1 Isolation of PSB on PVK media

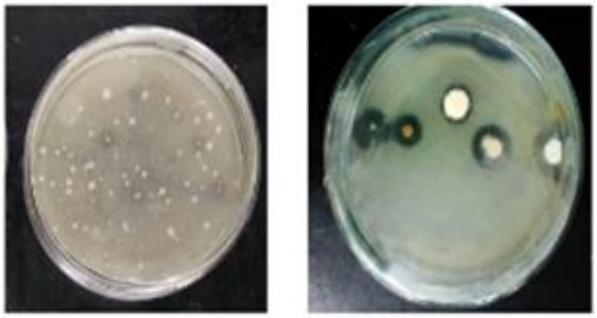

Plate.2 Screening of PSB isolates for solubilization efficiency on PVK media

Rice is the important food grain and staple food for over the billions of people in most of the countries particularly in East Asia. It's a major part of balanced diet and rich source of energy and carbohydrate. Phosphorus is one of the essential macro-minerals for the growth and development of plant (Schachtman *et al.*, 1998). It is a major component in ATP, the molecule that provides energy to the plant for such processes as photosynthesis, protein synthesis, nutrient translocation, nutrient uptake and respiration (Deepak kumar, 2011). But after application, a considerable amount of them are rapidly transferred into less available forms by forming complexes with Fe, Ca and Al cations before roots have a chance to absorb it (Alam and Ladha, 2004).

Under such conditions PSB play fundamental role in biogeochemical phosphorous cycling in natural and agricultural ecosystem. Extensive use of chemicals as fertilizer improves the plant health and productivity but disturbed the ecological balance of soil and resulted in nutrient depletion. This has necessitated the search for alternate source of this element. The use of PSB in agriculture practice is not only offset high cost of manufacturing phosphatic fertilizers but also make availability of insoluble P fertilizer.

The maximum halo zone was found with PPSB-21 with zone diameter of 18.5 mm. The results showed that, among the 10 isolates, the PSB-21 isolate showed a maximum solubilization efficiency and index of 253.84 and 3.53 respectively on 9th day of incubation period over other bacterial strains. The solubilization efficiency enhanced with incubation period. The diameter of clear halo zone formed by the bacterial isolates has the direct correlation with the phosphate solubilization efficiency. The percent solubilization increased with incubation period. Similarly, Ngomle *et al.*, (2014) isolated ninteen PSB. The P-solubilization efficiency was found maximum in UBPS-22 (28 mm) followed by UBPS-19 (25 mm) and UBPS-18(24 mm) respectively within 72 hrs of incubation in Pikovskaya's medium. Similar outcomes have been reported by many workers *viz*. Frateme *et al.*, (2014), Deepak *et al.*, (2018).

Among ten isolates PPSB-21 recorded the highest available phosphorous content of 171.84mg/L on 9th day of incubation. All the ten isolates were able to solubilize the insoluble phosphorus in Pikovaskaya's broth at different incubation period it may be due the abilities of the isolates to solubilize inorganic phosphate by the production of organic acids. Similarly, Karpagam and Nagalakshmi (2014) isolated 8 potent isolates, 3 strains showed high soluble phosphate production of 0.37mgL^{-1}, 0.30mgL^{-1} and 0.28mgL^{-1} in broth culture. Similar work was carried out by Manivannan *et al.*, (2011), Buddhi and Min-Ho (2013), Gandhi *et al.*, (2014), Manouchehr *et al.*, (2016), Amit *et al.*, (2017) and Deepak *et al.*, (2018).

PPSB-21 showed highest reduction in pH of 3.15 on 9th day of incubation. The drop of pH was due to the production of organic acids. Other reason like microbial respiration may also be involved for drop in pH. Tensingh and Jemeema (2015) observed the pH change of upto 4.6 by the *Bacillus sp*. Amit *et al.*, (2017) isolated 8 PSB isolates, among 3 isolates showed lower pH ranging 3.08 ±0.08 to 3.82 ± 0.12. Similar results were obtained by Oliveira *et al.*, (2009), Buddhi and Min-Ho (2013). Those bacteria that produced halo zones around colonies in PVK medium were able to produce organic acids in broth culture. This result is in accordance with Ogut *et al.*, (2010). This is also in agreement with Mehta *et al.*, (2001), Chen *et al.*, (2006), Ponmurugan and Gopi (2012), Studies related to the production of organic acids have shown that citric and oxalic acids were two major organic acids produced by PSB (Alam *et al.*, 2002).

Among all ten isolates, highest titrable acidity of 0.65% was expressed by PPSB-21. This might be due to reduction in the pH and secretion of organic acids. Results showed that strong positive correlation was found between titrable acidity and P solubilization. PSB produce phosphatase enzymes in soil, the activity of enzymes leads phosphate solubilization. There was a positive correlation between the phosphate solubilizing capacity and phosphatase activity. Phosphate solubilization occurs by production of organic acids and released by microorganisms; this release also decreases soil pH (Rodriguez *et al.*, 2006). Organic acids solubilize insoluble P either by decreasing the pH or by the complexing the cation which is bound to the P (Vassilev *et al.*, 2006). The organic acids such as succinic acid, malic acid, propionic acid and oxalic acid are released by Phosphorus solubilizing bacteria (Panhwar *et al.*, 2012), which chelate the cation bound to phosphate and being converted to soluble forms through their hydroxyl and carboxyl groups. Phosphate-solubilizing bacterial strains isolated were identified by biochemical tests; the isolates PPSB-21 and PPSB-5 were identified as *Pseudomonas sp.* and *Bacillus sp.*

In conclusion, our study demonstrated that many of the bacteria had P solubilizing properties and the ability was not exclusive to specific genera, suggesting the importance of preliminary screening in vitro for a wide range of bacteria to characterize their potent P-solubilizing or mineralizing trait. The PSB population was found higher in rhizo-sphere when compared with non-rhizosphere soil. Isolated PSB strains were able to solubilize P, produce organic acids, and enzymes. These beneficial characteristics would be considered as potential biofertilizer of isolated PSB for rice production.

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