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Phosphate Solubilization by Endophytic Bacteria isolated from *Oryza sativa*

Madhusmita Borah[1], Pompi Das[2], Susanta Sarmah Pathak[2], Robin Chandra Boro[2] and Madhumita Barooah[2*]

[1]Centre for Biotechnology and Bioinformatics, Dibrugarh University, Dibrugarh, Assam, India
[2]Department of Agricultural Biotechnology, Assam Agricultural University, Jorhat-13, Assam, India
**Corresponding author*

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A B S T R A C T

Endophytic bacteria isolated from the *Oryza sativa* plant were evaluated for phosphate solubilisation. Five of these isolates that showed good phosphate solubilising activity were characterised at molecular level and tested for phosphate solubilisation using three different sources viz tricalcium phosphate (TCP), aluminium phosphate ($AlPO_4$) and iron phosphate ($FePO_4$). Based on 16s rRNA gene sequence analysis the isolates were identified as *Pantoeaa ananatis*, *Pseudomonas putida*, *Brevibacillus agri*, *Bacillus subtilis* and *Bacillus megaterium*. The isolates showed maximum phosphate solubilizing activity on the 6th day of incubation (DAI) with a concomitant decrease in the pH of the medium. *Bacillus subtilis* (LP31 L03) showed highest phosphate solubilising activity (57.58±0.65, 6.10±0.65, 7.65±0.30 µg/ml) in TCP, $AlPO_4$ and $FePO_4$ respectively. The isolates under study were able to easily solubilize TCP followed by $FePO_4$. Among the sources tested, $AlPO_4$ was least solubilised. The decrease in pH of the medium inoculated with *Bacillus subtilis* (LP31 L03) was recorded as 4.02±0.12, 3.33±0.19 and 3.8±0.14 in TCP, $AlPO_4$ and $FePO_4$ respectively on 6th DAI. The study reports that endophytic isolates have good phosphate solubilizing activity and indicates and can be good candidate strains for developing an effective phosphate solubilizing biofertilizer.

Introduction

Phosphorus is the most essential key element in the nutrition of plants, next only to nitrogen (N). It is one of the structural components of nucleic acids, phospholipids and adenosine triphosphate (ATP) and plays a vital role in most of the important metabolic processes in plant including photosynthesis, respiration, signal transduction, energy transfer and macromolecular biosynthesis (Khan *et al.*, 2010). Phosphorous is abundantly present in soil, mostly in the form of insoluble mineral complexes which the plants cannot directly absorb (Rengel and Marschner, 2005). Only 0.1 % of the total phosphorous exists in a soluble form available for plant uptake (Zou *et al.*, 1992). Under natural conditions phosphorous rapidly precipitates as sparingly soluble complexes of different kinds of phosphates. In acid agricultural soils phosphorous occurs in complexes like variscite ($AlPO_4.2H_2O$) and strengite ($FePO_4.2H_2O$) that are very resistance to solubilisation (Richardson 2001). The availability of phosphorous depends on its solubility which is strongly influenced by the plant physiology and soil microorganisms. These factors influence the solubilisation of insoluble forms of phosphorous such as

tricalcium phosphate ($Ca_3PO_4)_2$, aluminium phosphate (Al_3PO_4), iron phosphate (Fe_3PO_4) (Sharma *et al.,* 2013). Phosphate solubilizing bacteria constitute about 1-50% of the total population of cultivable microorganisms in the soil (Khan *et al.,* 2009). Among the phosphate solubilising microorganisms bacteria are the most effective (Afzal *et al.,* 2008). Bacterial endophytes living within the tissues of higher plants are known to aid in plant growth and promotion by production of phytohormones and solubilising complex minerals like phosphorous present in soil (Vendan *et al.,* 2010). This trait can be of interest when developing an efficient phosphate solubilising biofertilizer. The introduction of beneficial bacteria as efficient inoculants in the soil can be considered an important strategy for sustainable agricultural management as is likely to be more environmental friendly and cost effective than chemical fertilization (Souza *et al.,* 2015; Alves *et al.,* 2004; Adesemoye *et al.,* 2009; Hungria *et al.,* 2010, 2013).

Literature analysis and chemical considerations of biological phosphate solubilization have shown that the commonly used source for phosphate solubilisation is tricalcium phosphate (TCP). This is relatively a weak and unreliable as universal selection factor for isolating and testing phosphate-solubilizing bacteria (PSB) (Bashan *et al.,* 2012). This study reports the isolation of potential phosphate solubilising endophytic bacteria from *Oryza sativa* along with their solubilisation efficiency of three metals of phosphate compounds *viz*. $Ca_3(PO_4)_2$ (TCP), $FePO_4$ and $AlPO_4$.

Materials and Methods

Isolation of phosphate solubilising endophytes

To isolate phosphate-solubilising endophytes, different paddy cultivars were collected from Lakhimpur district of Assam. Leaves, stems, and roots of healthy and disease free plants were selected for the study. The whole plant was washed with tap water to remove attached debris. Subsequently, leaves, stem and roots were separated and cut into sections 2-3 cm long and washed thoroughly with double distilled water followed by rinsing with 70% ethanol. The sample was sterilized with 0.1% $HgCl_2$ and then washed with sterile distilled water for 10 times to remove any traces of the surface sterilizing agents (Gagne *et al.,* 1987). One gram of the sample was homogenized in 10 ml of distilled water to prepare stock solution of tissue homogenate. After appropriate serial dilution the sample was inoculated in Tryptic soya agar (TSA) plates and incubated at 30^{o}C for 48-72 hours. The bacterial colonies that appeared were transferred to PVK agar, $AlPO_4$ agar, $FePO_4$ in which $FePO_4$ and $AlPO_4$ were replaced as the phosphorous resource in the medium, respectively. The cells were cultured at 30^{o}C for 48-72 hours. Strains that could grow in all insoluble phosphate media were selected for further research.

16S rRNA gene amplification and sequencing

Genomic DNA was extracted from five selected isolates as per standard phenol–chloroform method (Sambrook and Russel, 2001). The 16S rRNA gene was amplified from the extracted genomic DNA using the universal forward primer 27F and the reverse primer 1492R (Lane, 1991). The amplification was carried out in a reaction with a final volume of 25 µl containing 1 µl (0.5–10 ng) of total DNA, 1µl (20 pico M) of the forward primer, 1 µl (20 pico M) of the reverse primer, 2.5 µl (2.5mM of each) dNTP mix, 2.5 µl of 10x PCR buffer, 1µl (1U) of Taq DNA polymerase. A negative control (PCR mix without DNA) was included in all PCR experiments. The PCR reaction conditions were set for 94ºC for 4 min,

followed by 30 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 1 min and extension at 72ºC for 2 min, before a final extension at 72ºC for 7 min. The PCR products thus obtained were sequenced. The forward and reverse sequences obtained were assembled using the Codon Code Aligner software. Nucleotide sequence identities were determined using the BLAST tool from the National Center for Biotechnology Information (NCBI). Partial sequence data for the 16S rRNA genes have been deposited in the Gen Bank and Gene bank accession numbers have been provided to these sequences.

Quantification of phosphate solubilisation in liquid culture

Quantitative analysis of solubilization of tri-calcium phosphate, aluminium phosphate and iron phosphate in liquid medium was carried out following the protocol described by Nautiyal (1999). The test isolates were inoculated in 50 ml of Ca–P, Al–P, and Fe–P broth respectively and incubated for 10 days at 30^{o}C. After every two days 10 ml of the cultures were centrifuged at 10,000 rpm for 10 min. One ml of supernatant was mixed with 10 ml of chloromolibidic acid and the volume was made up to 40 ml with distilled water. 1 ml cholorostannous acid was added and the volume was made up to 50 ml with distilled water. Absorbance of the developing blue colour was measured at 600 nm wave length with UV- VIS spectrophotometer (Spectroquent Pharo 300). The amount of soluble phosphorus was detected from the standard curve of KH_2PO_4. Periodic estimation of culture pH of the medium was performed.

Statistical analysis

Experimental data were analyzed statistically using MS Excel and SPSS 22.0 software. Significance of variance among the data was calculated by ANOVA analysis and difference between means was compared by LSD tests. The level of significance was set at ≤0.05.

Results and Discussion

Characterization of phosphate solubilizing bacterial isolates

Five endophytic bacterial strains isolated from different parts of the rice plant were screened for phosphate solubilizing activity. The 1500 bp region of the 16s rRNA gene showed sequence similarity to *Pantoea ananatis*, *Pseudomonas putida*, *Brevibacillus agri*, *Bacillus subtilis* and *Bacillus megaterium* (Table 1).

Bacterial dissolution of phosphate and change in pH of the medium

Bacillus subtilis LP31 L03 showed maximum phosphate solubilising activity when TCP was used as the source. Although the isolates, *Bacillus subtilis* LP31 L03 and *Pantoea ananatis* RNS 02 solubilized almost equal amount of phosphate (26.08 ± 0.51 µg/ml) on the second days of incubation, *Bacillus subtilis* LP31 L03 displayed the highest solubilizing activity by the sixth day of incubation (56.80 ± 0.98 µg/ml of phosphate) which was significantly higher than all other isolates under study. The efficiency of all the isolates to solubilise phosphate decreased gradually reaching its zenith on the 10th day (Fig. 1).

Phosphate solubilisation of $FePO_4$ was less as compared to TCP. Most of the isolates solubilised similar amount of phosphate when $FePO_4$ was used as a source until second day of incubation. Solubilization efficiency of the *Bacillus subtilis* LP31 L03 isolate reached its peak (7.06 ± 0.72 µg/ml) on 6 DAI, which was quite similar the solubilizing efficiency by *Pantoea ananatis* RNS 02 (6.25±0.08

µg/ml) and *Pseudomonas putida* RNS 04 (6.68 ± 0.50 µg/ml) (Fig. 2).

The isolates showed similar solubilisation pattern when aluminium phosphate was used as a source of phosphate. *Brevibacillus agri* RNS 05 showed significantly high solubilisation efficiency (2.46 ±0.18 µg/ml) on 2 DAI. However, *Bacillus subtilis* LP31 L03 displayed higher solubilizing capacity from 4 DAI onwards and continued to reach the maximum (6.24±.0.08 µg/ml phosphate) on 6 DAI (Fig. 3).

As the phosphate solubilisation efficiency of the isolates increased, there was a decrease in the pH of the medium. This was found to decrease to its minimum level on the sixth day, beyond which it increased with concomitant lowering in phosphate solubilisation of the isolates. In the medium inoculated with *Bacillus subtilis* LP31 L03, pH significantly reduced to 4.02±0.12, 3.33±0.19 and 3.8±0.14 with TCP, $AlPO_4$ and $FePO_4$ respectively. The pH was most reduced in the medium containing Aluminium phosphate. On the 8DAI, the pH of the medium slightly increased with a decrease in solublization of available phosphate. At the end of ten days the pH of the media were recorded to be 5.0±0.16, 4.0±0.17, 4.5±0.10 in TCP, $AlPO_4$ and $FePO_4$ respectively (Fig. 4).

Plant growth and yield are essentially dependent on the availability of minerals which they directly or indirectly acquire from soil in the soluble ionic forms. Soil contains 0.5% phosphorus, mostly in the form of insoluble mineral complexes which plants cannot absorb (Rengel and Marschner, 2005), only 0.1 % of the total phosphorous exists in a soluble form available for plant uptake (Zou *et al.*, 1992). Phosphate solubilization is a common trait among plant-endophytic bacteria. Phosphate-solubilising bacteria are able to solubilize bound phosphorous from organic or inorganic molecules, by secretion of organic acids and phosphatases thereby making it readily available for the plant (Kim *et al.*, 1998).These organic acids thus produced can chelate the cation bound to phosphate with their hydroxyl and carboxyl groups (Kpomblekou and Tabatabai, 1994).

Fig.1 Phosphate solubilization in TCP

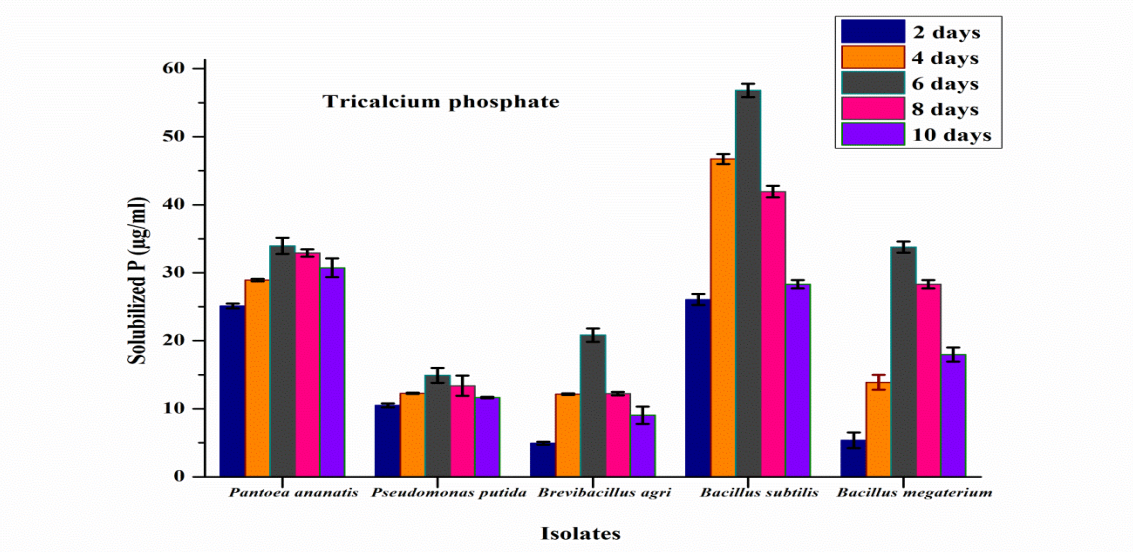

Fig.2 Phosphate solubilization in $FePO_4$

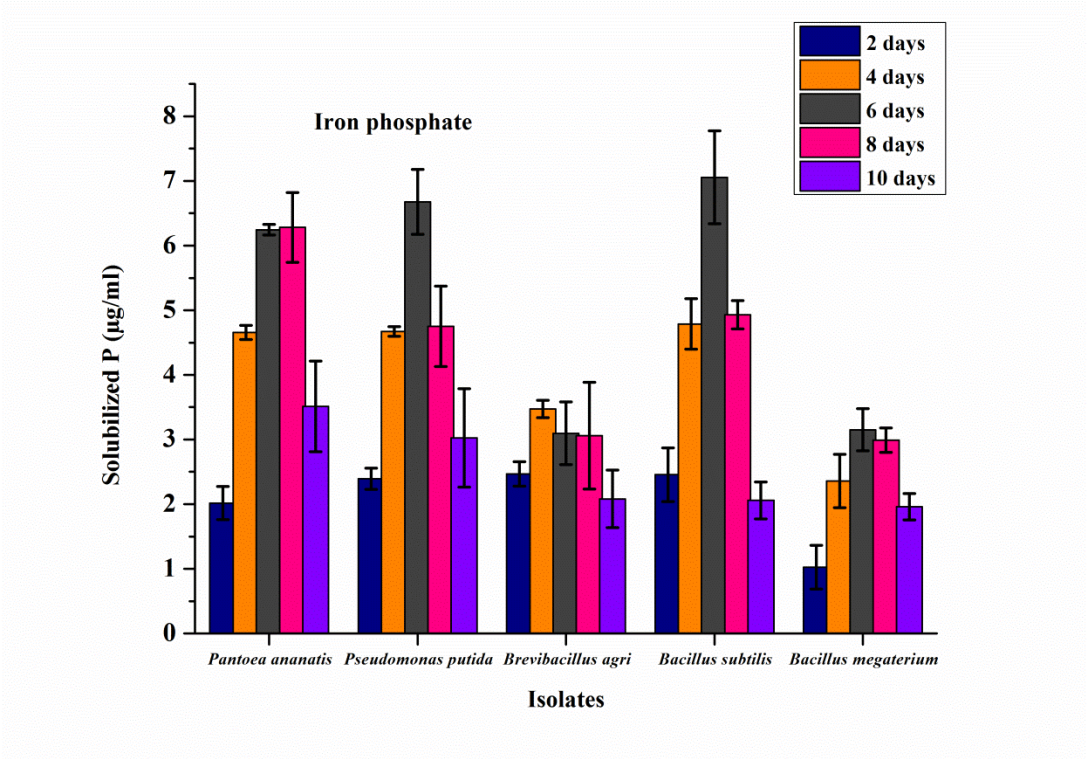

Fig.3 Phosphate solubilization in AlPO4

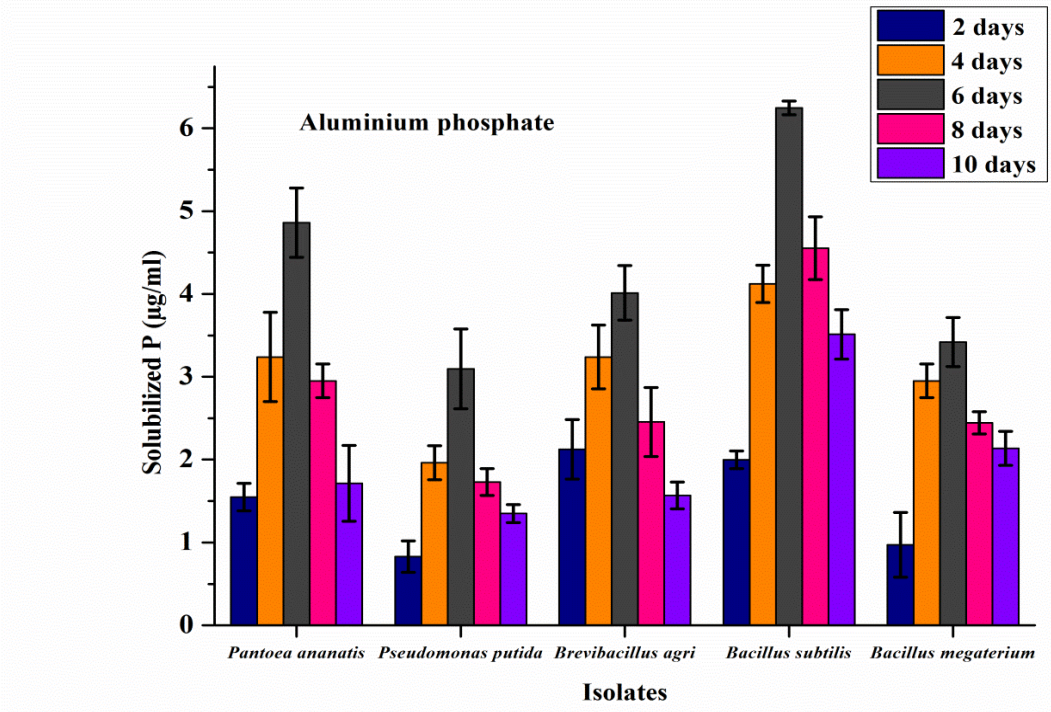

Fig.4 Dynamics of pH value in different media by *Bacillus subtilis*

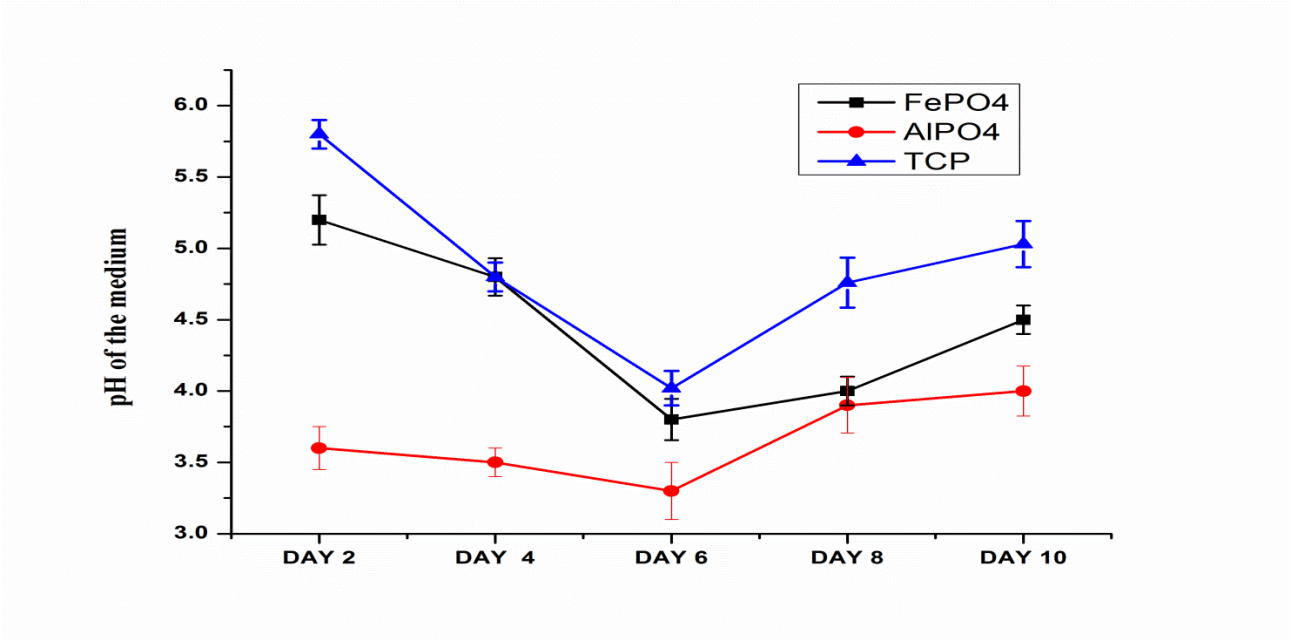

Table.1 Phosphate solubilizing bacteria with their NCBI accession numbers

S No.	Isolate Code	Isolation source	Sequence similarity	Similarity (%)	Acession No.
1	RNS 02	Stem	*Pantoea ananatis*	99	KT380684
2	RNS 04	Stem	*Pseudomonas putida*	98	KX375413
3	RNS 05	Stem	*Brevibacillus agri*	99	KX375412
4	LP31 L03	Leaf	*Bacillus subtilis*	97	KX375411
5	LP31 L04	Leaf	*Bacillus megaterium*	98	KM350267

Supplementary Data. LSD (least square difference) Analysis

TCP	DAY2	DAY4	DAY6	DAY8	DAY10
RNS 02	25.12±0.36 a	28.94±0.18 a	33.94±1.18 a	33.76 ±0.83 a	30.74±1.37 a
RNS 04	10.51±0.27 b	12.27±0.08 b	14.90±1.10 b	13.39 ±1.48 b	11.66 ±0.10 b
RNS 05	4.93 ± 0.21 c	12.14±0.10 b	20.10±2.21 c	12.22 ±0.24 b	9.05 ± 1.27 c
LP31 L03	26.08±0.51 a	46.72±0.73 c	56.80±0.98 d	41.94 ±0.83 c	28.32 ±0.60 d
LP31 L04	5.37±1.15 c	13.89±1.08 d	33.76±0.83 a	28.3 ± 0.60 d	17.96 ±1.04e

$FePO_4$	DAY2	DAY4	DAY6	DAY8	DAY10
RNS 02	2.01±0.25 a	4.66±0.11 a	6.25±0.08 a	6.28±0.54 a	3.51±0.70 a
RNS 04	2.39±0.16 a	4.67±0.07 a	6.68±0.50 a	4.75±0.62 b	3.02±0.76 ab
RNS 05	2.46±0.18 a	3.47±0.13 b	3.10±0.48 b	3.06±0.82 c	2.08±0.45 b
LP31 L03	2.46±0.42 a	4.79±0.39 a	7.06±0.72 a	4.93±0.22 b	2.06±0.28 b
LP31 L04	1.02±0.33 b	2.35±0.41 c	3.15±0.32 b	2.98±0.18 c	1.96±0.20 b

$AlPO_4$	DAY2	DAY4	DAY6	DAY8	DAY10
RNS 02	1.54±0.16 a	3.24±0.54 a	4.86±0.41 a	2.95±0.20 a	1.71±0.45 a
RNS 04	0.82±0.18 b	1.96±0.20 b	3.09±0.48 b	1.72±0.16 b	1.35±0.10 a
RNS 05	2.12±0.35 c	3.24±0.38 a	4.01±0.32 c	2.45±0.37 a	1.56±0.16 a
LP31 L03	1.99±0.11 ac	4.12±0.22 c	6.24±0.08 d	4.55±0.37 c	3.51±0.29 b
LP31 L04	0.97±0.39 b	2.95±0.20 a	3.42±0.29 bc	2.44±0.13 a	2.13±0.20 a

Mean with same letters in each column are not significantly different at P<0.05 according to LSD test

Among all the five isolates, *Pantoea ananatis*, *Pseudomonas putida*, *Brevibacillus agri*, *Bacillus subtilis* and *Bacillus megaterium* that could solubilise phosphate existing in the selective sources of phosphate (TCP, $AlPO_4$, $FePO_4$), it was *Bacillus subtilis* isolate that was most efficient in solubilizing maximum amount of all the sources of phosphate tested in this study. The bacterial isolates considered under the study were better able to solubilise TCP when compared to the $FePO_4$ and $AlPO_4$. Sources of phosphorous such a $FePO_4$ and $AlPO_4$ are less soluble than tricalcium phosphate in water (Bashan *et al.,* 2012). Earlier, Fifty-five endophytic PSB that were isolated from sap, leaves, and roots of maize were tested for their ability to solubilize tricalcium phosphate and produce organic acid. Partial sequencing of the 16S rRNA-encoding gene showed that the isolates were from the genus Bacillus and different species of Enterobacteriaceae. The phosphate solubilisation, out of the 336 bacteria isolated from rice plants, nearly 101 isolates belonging to the genera *Burkholderia, Cedecea, Cronobacter, Enterobacter, Pantoea* and *Pseudomonas* were able to solubilize tricalcium phosphate [$Ca_3(PO_4)_2$](Souza *et al.,* 2013). Phosphate solubilisation reached maximum on the 6 DAI with concomitant decrease in pH of the medium after which the pH gradually decreased. The decrease in the pH of the medium may be due to the secretion of organic acids and acid phosphatases by the isolates that helps in phosphate solubilisation (Illmer *et al.,* 1995). In organic acid production mechanisms, gluconic acid is regarded as the most frequent agent of mineral phosphate solubilization. It is reported as the principal organic acid produced by *Pseudomonas sp*. (Illmer *et al.,* 1992) and Erwinia sp. (Liu *et al.,* 1992). Ketogluteric acid is also reported as one of the major organic acids present in different phosphate solubilizing microbes (Puente *et al.,* 2004; Rodriguez *et al.,* 2006). Besides these two organic acids, glycolic, malonic, oxalic and succinic acids were also identified among phosphate solubilizers gluconic acid (GA) seems to be the most frequent agent. The production of 2-ketogluconic, oxalic, malic, lactic, succinic, formic and citric acid has also been reported in phosphate solubilization by *Bacillus* sp. (Chen *et al.,* 2006). Gaind and Gaur (1989) reported that the decrease in available phosphate after a period might be due to the deficiency in nutrients in the culture medium or refixation of solubilized phosphate with metal ions present in the broth. However, increase in the acidity of the medium also inhibits the growth of bacteria and decrease their phosphate solubilizing activity. Dhanya *et al.,* 2013 found *Bacillus subtilis* to show greater reduction in pH when inoculated in phosphate solubilising liquid media.

Prospecting for beneficial bacteria is an important criterion for the development of new and efficient inoculants for biofertilizer. The present study isolated rice endophytes *viz., Pantoea ananatis*, *Pseudomonas putida*, *Brevibacillus agri*, *Bacillus subtilis* and *Bacillus megaterium* that were able to efficiently solubilize different sources of phosphate *viz.* TCP, $AlPO_4$, and $FePO_4$. This

suggests that they could play a role in resource mobilization in nutrient-poor habitat. Among the isolates tested, *Bacillus subtilis* was found to be the most efficient phosphate solubilizer indicating their use in developing commercial phosphate solubilising biofertilizer.

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