Inorganic phosphate solubilization by *Pantoea* sp. KUP312, isolated from jute rhizosphere

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Abstract : Phosphorus is one of essential nutrients in plants. It is mostly present in the soil as fixed form that limits its bioavailability to plants. Phosphate solubilizing bacteria (PSBs) develop several mechanisms to solubilize phosphate. Organic acid production is one of such mechanisms to solubilize inorganic phosphate. In the present study, a PSB isolate KUP312 was recovered from the rhizosphere of a jute plant and was identified as *Pantoea* sp. based on 16S rDNA sequence analysis. The phosphate solubilization index on Pikovskaya agar medium was measured as 2.143. In Pikovskaya broth containing $Ca_3(PO_4)_2$, phosphate solubilization was increased with decrease in pH of the medium and maximum solubilized phosphate (2015 mg/l) was found after 3 days of incubation. With the increase of $Ca_3(PO_4)_2$ concentrations in broth, growth and acid production remained unaltered but phosphate solubilization was increased slightly and maximum solubilization was observed in presence of 10% of $Ca_3(PO_4)_2$. The isolate was also able to solubilize other inorganic phosphate salts viz., $Zn_3(PO_4)_2$, $Mg_3(PO_4)_2$, $AlPO_4$ and $FePO_4$. It could utilize and showed substantial level of P solubilization in presence of variety of carbohydrate sources viz., sucrose, maltose, mannitol, sorbitol, other than glucose. The isolate also able to produce different plant growth promoting factors like IAA (110 mg/ml), siderophore (131.69 nmole/ml) and NH_3. Hence, *Pantoea* sp. KUP312 might be used as biofertilizer to improve soil fertility in sustainable agricultural system.

Keywords **- Phosphate solubilizing bacteria, Identification by 16S rDNA sequencing, Pikovskaya medium, Lowering of pH, Production of plant growth promoting factors.**

1. INTRODUCTION

Phosphorus is the second most important plant nutrient only after nitrogen. It affects growth and metabolism of plant making up about 0.2% of plant dry weight (Schachtman et al. 1998). P is necessary for protein synthesis, signal transduction, energy transformations, respiration, and nitrogen fixation especially in legume plants (Khan et al. 2010). The plants obtain their P requirements from soil pool. The inorganic phosphates in soils are produced by weathering of parent rock and organic phosphates are derived from decayed plant, animal or microorganisms. However, P is one of the major growth limiting factors of plant because the concentration of soluble P in soil is very low normally at levels of 1 ppm or less (Goldstein 1994). The mobility of phosphate ions in the soil is very low due to their high retention and these get rapidly converted to less available forms by forming complex with Fe and Al in acid soils and Ca and Mg in alkaline soils (Toro 2007). To overcome this problem, farmers apply chemical phosphatic fertilizers which are again precipitated as insoluble forms and thus not available for plant growth, or quickly washed away by the rain waters, polluting rivers and ground waters (Shigaki et al. 2006). Indeed only 5% or less of the total amount of P in the soil is available for plant nutrition (Boronin 1998). Moreover, repeated and injudicious applications of chemical P fertilizers lead to the loss of soil fertility by disturbing microbial diversity, consequently reduces yield of crops and threaten to environment.

Phosphate solubilizing bacteria (PSBs) are the predominant rhizospheric microorganisms that might be used as one of the most significant alternatives to supplement P deficiency in plant (Mahmood et al. 2010; Mamta et al. 2011). Several mechanisms like lowering of pH by organic acid production, ion chelation and exchange reactions in the growth environment have been reported for solubilization of inorganic P by PSBs whereas acid phosphatases play a major role in the mineralization of organic P in soil phosphate solubilization (Rodriguez et al. 2006). Along with the phosphate solubilizing trait, several other plant growth promoting activities performed by these microorganisms, like production of plant growth regulators (IAA, gibberellins, cytokinins) and siderophores, fixing atmospheric nitrogen, and antagonism against phytopathogenic microorganisms (Ahemad 2015). Microbial IAA can cause rapid development of roots and better uptake by the plant. Siderophore chelate available iron to make it available to plant. Also siderophore producing PSB can suppress growth of pathogens by depriving iron nutrient. Several studies performed under green house condition as well as field condition by inoculating the PSBs, as they increased P availability in the soil and influenced plant growth (Zaidi et al. 2003). The objectives of this study were to isolate a PSB from jute rhizosphere and to evaluate its phosphate solubilizing efficiency (in different phosphate concentrations and carbohydrate sources), to characterize its other *in vitro* PGP traits, following its molecular identification.

2. MATERIALS AND METHODS

2.1. Collection of soil sample and isolation of phosphate-solubilizing bacteria

Rhizospheric soil sample was collected from a jute plant grown in an agricultural field of Ghanashyampur village (22°53'60"N, 88°23'24"E) under Jangipara block of Hooghly District, West Bengal, India. The rhizospheric soil was collected in a sterilized

poly bag and brought to the laboratory for further study. The rhizospheric soil sample was subjected to serial dilution on Pikovskaya agar (PKVA) medium and incubated at 28±2ºC for 3-5 days for isolation of bacteria. The bacterial isolates showing a clear zone around their colonies were considered as positive.

2.2. Measurement of phosphate solubilization index

Qualitative estimation of the inorganic phosphate solubilization of the isolates was measured by determining phosphate solubilization index (PSI) which was calculated as the ratio of total diameter (colony + halo zone) to the colony diameter (Edi-Premono et al. 1996). Isolate having highest PSI was selected as promising isolate and used for further study.

2.3. Assay of phosphate solubilization in broth medium

The promising isolate was inoculated in the Pikovskaya (PKV) broth containing $Ca_3(PO_4)_2$ (0.5%), pH 7.2±0.2 and incubated at 28±2ºC on a rotator shaker at 140 rpm for 4 days. An aliquot was taken at one day interval. Growth was measured by observing optical density of the culture at 600 nm. Change in pH of the broth culture was detected using a pH meter. Amount of soluble phosphate in the culture supernatant was estimated using molybdate vanadate reagent (Jeon et al., 2003). For this, the culture was centrifuged at 10000 rpm for 10 min, 0.1 ml of supernatants from was taken and mixed with 3.9 ml of distilled water and to this 1 ml of molybdate vanadate reagent was added. After 25 min of incubation at room temperature, the absorbance at 470 nm was measured using a spectrophotometer. Amount of solubilized phosphate was determined using the standard curve of KH_2PO_4.

2.4. Study of effect of increasing concentrations of $Ca_3(PO_4)_2$ on phosphate solubilization

Four sets of phosphate-free PKV broth were prepared supplemented with four different concentrations (0.5%, 2%, 5% and 10%) of $Ca_3(PO_4)_2$. One set was kept as control without P source. After four days of incubation at 28±2ºC on a rotator shaker at 140 rpm the amount of phosphate solubilization was measured using molybdate vanadate reagent. Decrease in pH of the broth and growth of the culture were determined.

2.5. Study of effect of different inorganic phosphate salts on phosphate solubilization

Four sets of phosphate-free PKV broth were prepared supplemented with 0.5% of each four different phosphate salts commonly available in soil *viz*., $Mg_3(PO_4)_2$, $Zn_3(PO_4)_2$, $AlPO_4$, and $FePO_4$. One set was kept as control with $Ca_3(PO_4)_2$ as P source. After four days of incubation at 28±2ºC on a rotator shaker at 140 rpm the amount of phosphate solubilization was measured using molybdate vanadate reagent. Decrease in pH of the broth and growth of the culture were determined.

2.6. Study of effect of different carbohydrate sources phosphate solubilization

Four sets of carbohydrate-free PKV broth were prepared supplemented with 1% of each four different carbohydrate sources (sucrose, maltose, mannitol, and sorbitol). One set each was kept as control with glucose or without any carbohydrate source. After four days of incubation at 28±2ºC on a rotator shaker at 140 rpm the amount of phosphate solubilization was measured using molybdate vanadate reagent. Decrease in pH of the broth and growth of the culture were determined.

2.7. Determination of other in vitro *PGP traits*

IAA production of the isolate was estimated using Salkowski's reagent. For this the isolate was grown in NB broth medium supplemented with L-tryptophan (500 mg/l) up to stationary phase and the cell free culture supernatant (1 ml) was mixed with Salkowski's reagent (2 ml) following addition of two drops of orthophosphoric acid, incubated at room temperature for 25 min and then the intensity of the pink colour developed by the reaction was measured immediately at 530 nm (Gordon and Weber 1951) and compared with the standard curve of IAA.

Siderophore production of the isolate was determined by using blue indicator dye, the Chrome-Azurol Sulfone (CAS) (Schwyn and Neilands 1987). CAS agar plate was inoculated with the bacterial isolate and incubated at 28±2ºC for 4 days. An orange halo around the colonies indicated siderophore positive. Quantitative estimation of siderophore production by the isolate was carried out using CAS reagent. The isolate was grown in a synthetic medium (Modi et al. 1985) and cell-free culture supernatant (or its dilution) was mixed with equal volume of CAS reagent. After incubation for 30 min at room temperature, OD of reaction mixture was measured at 630 nm and compared with the standard curve of desferal.

Hydrogen cyanide (HCN) production test was carried out through streak-inoculation of the bacterial isolate on NA medium supplement with glycine (4.4g/l). A sterile Whatman No.1 filter paper soaked with 0.5% picric acid and 2% Na_2CO_3 solution was placed in the upper lid of the petriplate and after placing the inoculated part of petriplate in an inverted position over the upper lid, the plate pair was sealed with parafilm and incubated at 28±2ºC for 4 days. Same treatment was also done for a non inoculated plate, regarded as control set. Change in colour of the filter paper from deep yellow to reddish-brown indicated as positive result (Bakka and Schippers 1987).

For the test of ammonia production, culture was grown in 10 ml of peptone water for 3 days and afterthat (0.5 ml) Nessler's reagent was added. Non inoculated peptone water treated with Nessler's reagent considered as control. The development of brown yellow colour was considered as positive result for ammonia production (Cappuccino and Sherman 1992).

2.8. Identification of PSB

The promising isolate was identified based on 16S rDNA sequence analysis. Genomic DNA of the isolate was extracted and was used as template for amplification of the 16S rDNA with bacterial specific forward and reverse primer set, 5'-

AGAGTTTGATCCTGGCTCAG-3' and 5'-TACGGTTACCTTGTTACGACTT-3'. The PCR product was purified and sequenced by ABI PRISM 377 automated DNA sequence (Perkin-Elmer, Applied Biosystem, Inc.). Nucleotide BLAST function at NCBI was used to find similarity of the sequence with nucleotide database (Altschul et al. 1990). Phylogenetic tree was constructed using the software MEGA6 (Tamura et al. 2013). Multiple sequences alignment was carried using CLUSTALW and the evolutionary history was inferred using the Neighbour-joining method (Saitou and Nei 1987).

3. RESULTS

3.1. Isolation and screening of phosphate solubilizing isolates

In the present study, the collected soil sample was screened for isolation of phosphate solubilizing bacteria on Pikovskaya agar (PKVA) plate, containing $Ca_3(PO_4)_2$ as phosphate source. Based on the solubilization zone formation on the PKVA medium, six PSB were isolated from the rhizosphere of Jute plant. Out of the isolates, KUP312 was found to be highest phosphate solubilizer showing PSI of 2.143 (Table 1).

Table 1: *In vitro* PGP traits of the isolate KUP312

Phosphate solubilization index	2.143
IAA	110 mg/ml
Siderophore	131.69 nmole/ml
NH_3	+
HCN	-

3.2. Quantitative estimation of phosphate solubilization

In PKV broth containing $Ca_3(PO_4)_2$, the isolate KUP312 attained maximum growth within 24 hr of incubation, but its phosphate solubilization efficiency was gradually increased upto three days when concentration of solubilized phosphate in the broth reached 2015 mg/l and afterthat no further significant change of solubilized P concentration in the medium was observed (Fig. 1). Along with the solubilization of $Ca_3(PO_4)_2$, the initial pH in the medium (pH 7) was reduced to pH 4 within 24 hr of incubation and such acidic pH in the broth medium was maintained throughout the $Ca_3(PO_4)_2$ solubilization upto 4 days.

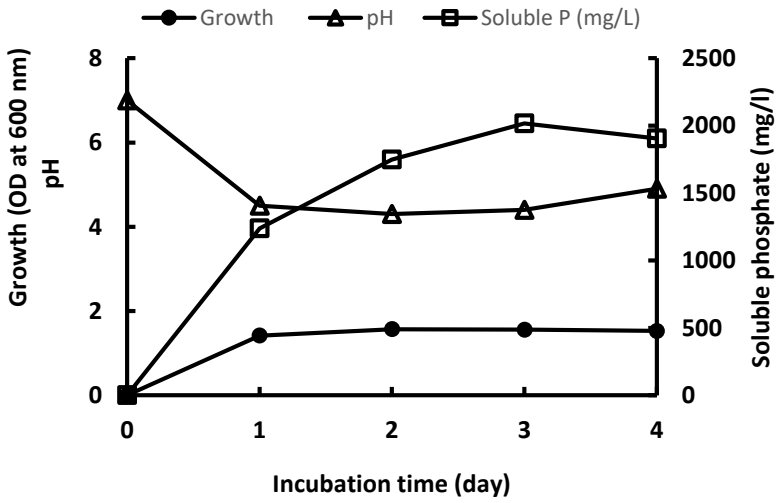

Fig 1: Growth (closed circle), inorganic P solubilization (open square) and decrease of pH in the PKV broth (open triangle) by the isolate KUP312 upto 4 days of incubation

3.3. Phosphate solubilization with increasing concentrations of $Ca_3(PO_4)_2$

In PKV broth containing increasing concentration of $Ca_3(PO_4)_2$, growth and acid production remained unaltered but phosphate solubilization was increased slightly and maximum solubilization was observed in presence of 10% of $Ca_3(PO_4)_2$ (Fig. 2).

3.4. Solubilization of different inorganic phosphate salts

P solubilization ability of the isolate KUP312 was observed in modified PKV medium containing each of $Zn_3(PO_4)_2$, $Mg_3(PO_4)_2$, $AlPO_4$ and $FePO_4$ as sole phosphate source (Fig. 3). The isolate was able solubilize all the four tested phosphate salt in a substantial level with sufficient growth and acid production in the medium.

3.5. Phosphate solubilization in presence of different carbohydrate sources

The isolate was tested for its growth and phosphate solubilization in modified PKV medium containing each of four carbohydrate sources by replacing glucose at a concentration of 10g/l (Fig. 4). From the result, it is clear that the isolate could utilize all the five carbohydrate sources and showed almost similar level of phosphate solubilization efficiency in presence of all the carbohydrate, although maximum phosphate solubilization was found in presence of glucose (positive control) and minimum in presence of sorbitol. In all cases, phosphate solubilization was directly related to growth and acid production of the organism.

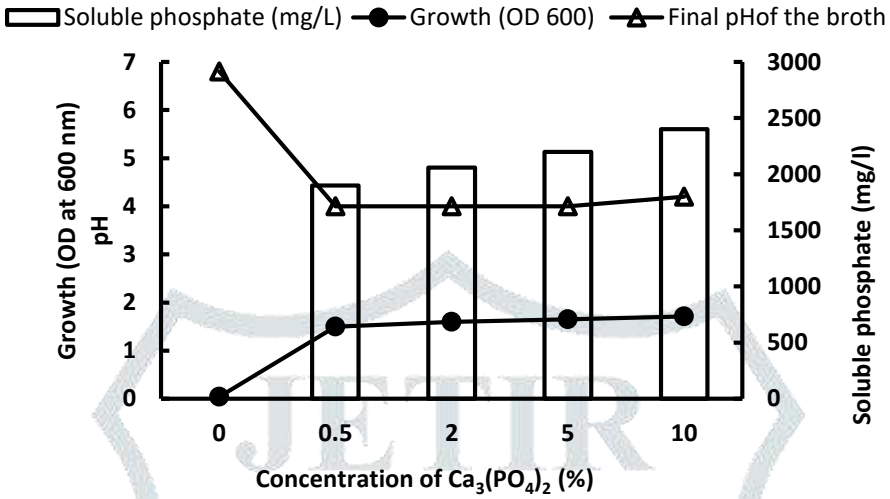

Fig 2: Effect of $Ca_3(PO_4)_2$ concentrations on growth (closed circle), inorganic P solubilization (open column) and decrease of pH in the PKV broth (open triangle) by the isolate KUP312 after 4 days of incubation

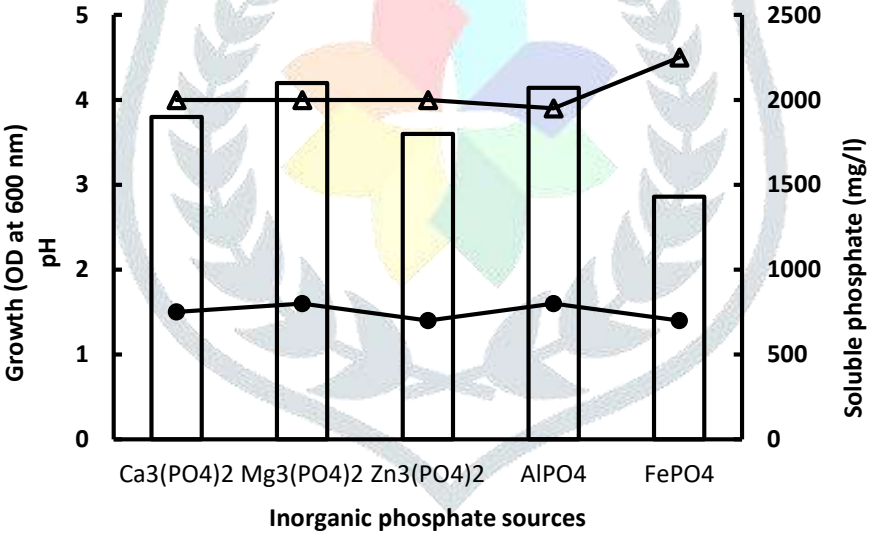

Fig 3: Effect of different inorganic P salts on growth (closed circle), inorganic P solubilization (open column) and decrease of pH in the PKV broth (open triangle) by the isolate KUP312 after 4 days of incubation

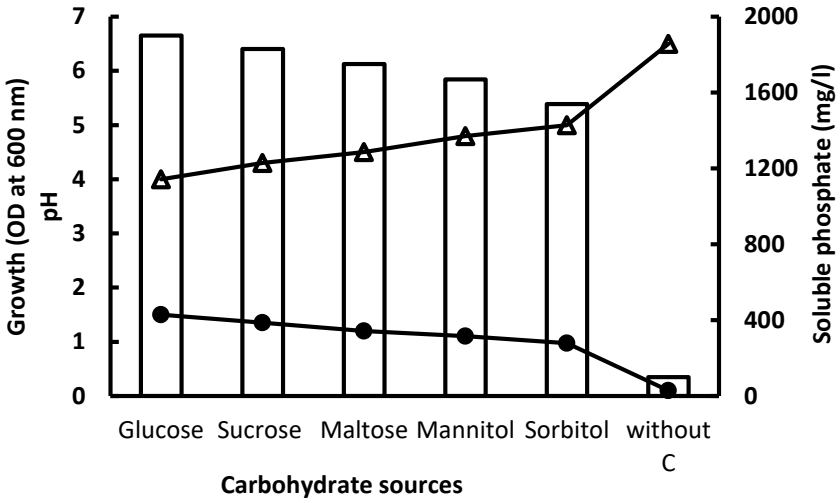

Fig 4: Effect of different carbohydrate sources on growth (closed circle), inorganic P solubilization (open column) and decrease of pH in the PKV broth (open triangle) by the isolate KUP312 after 4 days of incubation

3.6. Production of other in vitro *PGP traits*

The PSB isolate KUP312 also showed various plant growth promoting traits, like production of IAA, siderophore and NH_3 (Table 1). The isolate KUP312 produce 110mg/ml of IAA and 131.69 nmole/ml of siderophore.

3.7. Molecular identification of KUP312

Bacterial isolate KUP312 was identified based on 16S rDNA sequence analysis. 16S rDNA sequence has been considered as a suitable molecular marker for bacteria. About 1.5 kb amplicon of 16S rDNA region of the isolate was amplified and sequenced. Search for sequence homology through nucleotide BLAST function in NCBI database was performed and maximum identity (98%) of KUS312 was found with the 16S rDNA sequence of *Pantoea dispersa* LMG2603. The 16S rDNA gene sequence of KUP312 was submitted to Genebank under the accession number KY593922.

When a phylogenetic tree was constructed by Neighbour-joining method based on 16S rDNA sequence of KUP312 and other members of *Pantoea* and one outgroup as *Pseudomonas putida*, relatedness of KUP312 with other *Pantoea* spp. was observed as KUP312 positioned within the genus *Pantoea* and it belonged to the same evolutionary branch with *P. dispersa* LMG2603 (Fig. 5).

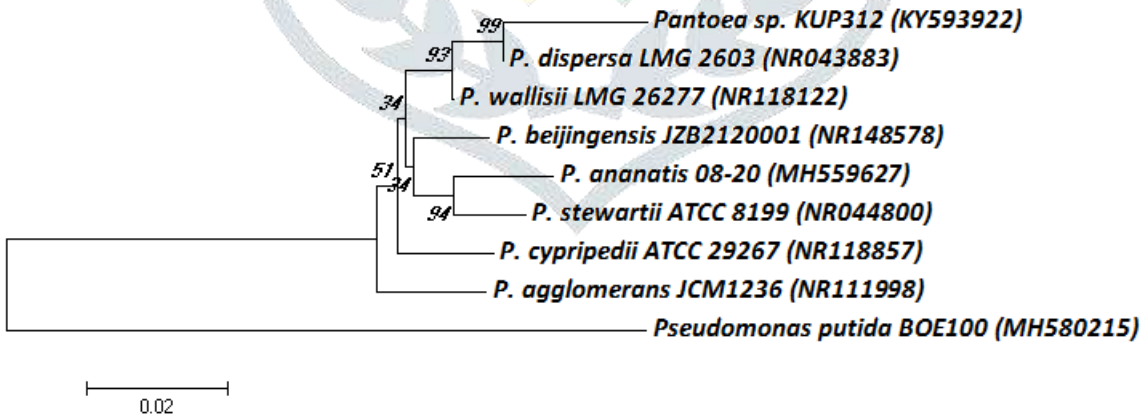

Fig 5: Phylogenetic tree of *Pantoea* sp. KUP312 showing relatedness with other *Pantoea* spp. based on 16S rDNA sequence comparison, where *Pseudomonas putida* was used as an outgroup. The topology of the tree was estimated by bootstraps based on 1000 replications. The numbers at the branch points are the percentage support by bootstraps. Bar represents 1% sequence divergence. Except for the sequence determined in this study, all 16S rDNA sequences were retrieved from GenBank. GenBank accession numbers are included in parentheses.

4. DISCUSSION

Plant growth promoting rhizobacteria (PGPR) are free-living soil bacteria that actively colonize rhizosphere and rhizoplane and promote plant growth through supply of nutrients, production of growth stimulants and protection against pathogens. PSBs may contribute to plant nutrition by liberating P from insoluble phosphates compounds. The beneficial effects of PSBs on plant growth varied significantly depending on environmental conditions, bacterial strain, host plant and soil conditions (Sahin et al. 2004;

Cakmakci et al. 2006). The most common mechanism used by PSBs for solubilization of inorganic phosphates, such as tricalcium phosphate was found to be involved with acidification of the medium via biosynthesis and release of a wide variety of organic acids, such as gluconic acid, 2-ketogluconic acid, acetic acids, glycolic acid, oxalic acid, malonic acid, succinic acid, citric acid and propionic acid (Hayat et al. 2010).

In the present study, *Pantoea* sp. KUP312 was isolated from jute rhizosphere and it showed a halo around its colony on Pikovskaya agar medium with PSI of 2.143. Based on 16S rRNA sequence analysis, the isolate showed 98% similarity with *Pantoea dispersa* LMG2603. The *Pantoea* spp. has been isolated from diverse ecological niches, including plants (as endophytes or epiphytes), water, soil, humans and animals (as opportunistic pathogens) and currently seven spp. have been distinguished (Deletoile et al. 2009). *Pantoea* sp. KUP312 also produced IAA (110 mg/ml), siderophore (131.69 nmole/ml) and NH_3 (Table 1). PGPR belonging to *Pantoea agglomerans* with P solubilizing activities have been reported by Khalimi et al. 2012 and Silili-Cherif et al. 2012.

Solubilization of phosphate is a complicated process, which depends upon various factors like nutritional richness and growing status of the bacterium. Solubilization of inorganic phosphate by *Pantoea* sp. KUP312 was associated with the reduction of pH of the medium. The maximum concentration of solubilized P was measured as 2015 mg/l after three days of incubation during stationary phase of growth whereas pH of PKV broth was reduced to 4 due to extracellular synthesis of organic acids within 24 hr after attaining maximum growth (Fig. 1). With the increase of $Ca_3(PO_4)_2$ concentration in the PKV broth P solubilization was increased but growth and acid production remained unchanged (Fig. 2). The isolate was able solubilize P from all the four inorganic phosphate salts [$Zn_3(PO_4)_2$, $Mg_3(PO_4)_2$, $AlPO_4$ and $FePO_4$] with sufficient growth and acid production in the medium (Fig. 3). Since P solubilization is directly linked to the metabolism of the organisms, its P solubilization efficacy was tested in presence of five carbohydrate sources and found that it could utilize all the carbohydrate and solubilized substantial amount of P (Fig. 4). *Pseudomonas lurida* showed maximum solubilization of $Ca_3(PO_4)_2$ with glucose followed by maltose>galactose>sucrose> xylose (Pallavi and Gupta 2013).

Based on this study, *Pantoea* sp. KUP312 could be considered as a good candidate for improving soil fertility and increasing phosphate availability to the crop plants. However, its extent of plant growth promotion under *in vivo* condition requires further investigation before its formulation.

5. ACKNOWLEDGMENT

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