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A PSYCHROTOLERANT STRAIN *KLUYVERA INTERMEDIA* SOLUBILIZES INORGANIC PHOSPHATE AT DIFFERENT CARBON AND NITROGEN SOURCE

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ABSTRACT
A *Kluyvera intermedium is* a Gram negative psychrotolerant bacterium isolated from a high altitude rhizospheric soil from the Uttarakhand Himalayas where all over year temperature remains very low. This strain exhibited plant growth promoting traits both at 10°C and 30°C, under in-vitro conditions. The expression of plant growth promoting (PGP) traits like Indole acetic acid production by the bacterium was highest at 30°C, with a proportionate reduction in PGP activity at lower temperatures. Determination of phosphate solubilization by the bacterium at two incubation temperatures (10°C and 30°C) revealed a steady increase in the soluble phosphorus levels across the incubation temperatures, coupled with a concomitant drop in the pH levels of the culture supernatant, till the 10th day of incubation. The Uttrakhand soil has acidic in nature and the phosphorus applied to the soil get fixed and become unavailable to the plant. So, to provide phosphorous easily to the plant, this strain has been chosen because this strain have the capacity to solubilize unavailable phosphorus. The phosphorus solubilising ability has been increased while a different carbon and nitrogen source has been used. This strain show better result when glucose is taken as carbon source and ammonium sulphate as nitrogen source.

INTRODUCTION

Phosphorus (P) is an essential nutrient for biological growth. Absence of this element in the soil could limit the plant development (Prejambada *et al.,* 2009; Victoria *et al.,* 2009) and the productivity of plants in many terrestrial ecosystems (Plassard and Dell, 2010). Biological Nitrogen Fixation depends appreciably on the available forms of phosphorus. A large proportion of phosphatic fertilizer applied to enhance the availability of phosphorus is quickly transformed to the insoluble form which decreases the efficiency of fertilizers. So, the need of those microbes arose which have the capacity to solubilize phosphorus are called Phosphate solubilizing micro organisms (PSMs). Phosphate-solubilizing microbes can transform the insoluble phosphorus to soluble forms HPO_4^{2-} and $H_2PO_4^-$ by acidification, chelation, exchange reactions and polymeric substances formation (Delvasto *et al.,* 2006). Mineral phosphate solubilization is an essential plant growth-promoting ability via which PSM have been found to have extensive applications in agriculture as inoculants (Arcand and Schneider, 2006).

However, 'P' solubilization is a complex phenomenon, which depends on many factors such as nutritional, physiological and growth conditions of the culture (Chen *et al.*, 2006). To enhance phosphorus uptake efficiency, PSB play an important role in supplying phosphate to plants, which is environment friendly and sustainable approach. The significant genetic diversity in rhizobial strains exists with respect to their efficiency for solubilizing soil phosphate (Sajjad *et al.*, 2008). It has been reported that temperature has perplexing effects onto 'P' solubilizing abilities of naturally occurring psychrotolerant strains of *Pseudomonas fragi* (Selvakumar *et al.,* 2009) .Temperature is one of the important factors that immediately affect the interior of the cell. Bacteria not only respond to elevated temperature (heat shock), but also at downshifted temperatures (cold shock) by synthesizing a group of heat and cold shock proteins, respectively. These proteins are important for the survival of bacteria at higher or lower temperatures (Negi *et al.,* 2009). Low temperature habitats are colonized by psychrophiles and psychrotolerant microorganisms, which possess several adaptive characteristics to overcome such adverse conditions. Psychrophiles are those microorganisms whose cardinal growth temperatures are at or below 0, 15, and 20°C, respectively. *Pseudomonas fluorescence, Pseudomonas putida, Pseudomonas striata, Acinetobacter, Paecilomyces* etc. comes under higher psychrophilic 'P' solubilizer. However, the soils of Uttarakhand Himalayas are acidic in nature low in moisture content and organic matter. The applied water soluble 'P' fertilizers are rapidly fixed to unavailable forms which accounts for the low 'P' use efficiency of the crops grown in this region (Pal, 1998). Thus, the exploration of some potential psychrotolerant microorganisms is a need for sustainable agriculture in the sub-tropical regions of Uttarakhand.

The basic idea behind the topic of this paper is that the strain *kluyvera intermedia* has a better ability to grow under low temperature region and show significant changes in phosphate solubilization under low temperature.

The phosphate solubilizing activity of PSMs is also affected by the presence of various carbon and nitrogen sources. Development of growth and activity of PSMs is very much affected by source of carbon, nature and concentration of salt and pH of soil (Yadav *et al.,* 2010).

Thus, keeping in view of above, the present study has been planned to study retrival and maintenance of psychrotolent bacteria at different carbon and nitrogen sources.

MATERIALS AND METHODS

The soil used for bacterial isolation was collected from high altitude location (31.01°N Latitude and 78.45°E Longitude) in Patalbhuwaneshwer district, of Uttarakhand state. Sampling were done during prevailing atmospheric temperature was 8°C. The samples were transported to the laboratory in temperature controlled conditions. The collected soil was serially diluted in sterile physiological saline, spread plated in triplicate on nutrient agar (Atlas, 1995), and incubated at 4°C for 48 h. A Yellowish, irregular, medium oval colony appeared to be predominant in the isolation plates was purified and maintained on nutrient agar slants and 20% glycerol at -80°C. The cell cultures of the bacterium have been deposited in the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh, India (Accession number HQ222366).

Quantitative estimation of phosphate solubilization

Cultures were grown overnight in NBRIP broth media. In addition, to see the effect of different carbon and nitrogen sources on 'P' solubilization, the NBRIP broth was modified by replacing glucose by maltose as carbon source and $(NH_4)_2SO_4$ by $NaNO_3$ as nitrogen source. Quantitative estimation of phosphate solubilization was done at two different incubation temperatures viz., 10 and 30°C, by inoculating 1 ml of bacterial suspension (3×10^7 cfu ml^{-1}) in 50 ml of phosphate growth (NBRIP) medium (Mehta and Nautiyal, 2001) in Erlenmeyer flasks (150 ml), and incubating for 10 days, at the respective temperature. At 1st, 3rd, 7th and 10th days, the culture suspension was centrifuged at 4,000rpm for 10 min, and the pH of the supernatant was determined with a pH meter (Merck 510, India). The 'P' solubilized was measured in culture supernatant using Ion chromatography (Dionex Model).

Qualitative estimation of 'P' solubilization

Strains were checked for phosphate solubilizing ability on Pikovskaya (PVK) agar medium (Pikovskaya, 1948) incorporated with tricalcium phosphate ($Ca_3(PO_4)_2$) by observing the Solubilization index (S.I.). Formation of a clear halo zone around the bacterial growth after 7 days of incubation at 10°C and 30°C respectively indicates phosphate solubilizing ability (Fig 1). S.I. (Premono *et al.,* 1996) was calculated on PVK plates by the formula:

Solubilization Index = Colony diameter + Halozone diameter/colony diameter.

Estimation of Siderophore, and IAA production

All the plant growth promotion traits were estimated at two different incubation temperatures viz., 10°C and 30°C. Siderophore production by the isolates was measured on Chrome Azurol-S (CAS) agar (Schwyn and Neilands, 1987), by measuring the diameter of the zone of the colour change from bluish green to orange.

Qualitative analysis of the Indole acetic acid (IAA) produced was carried out by inoculating a loopful bacterial culture in 5 ml tryptone soya broth containing L-tryptophan (17gml^{-1}), and incubating it in the dark for 48 h at 28°C under shaking condition. Cultures were centrifuged at 10,000 rpm for 10min. 2ml of Salkowski reagent was added in 1ml supernatant. The mixture was incubated at 28°C for 25min. Development of pink colour shows IAA production.

RESULTS

The bacterium formed yellowish irregular colonies of 1-2 mm diameter with smooth irregular margins when incubated on nutrient agar at 30°C for 1-2 days. Microscopic examination revealed that the isolate was Gram (-ve), motile and the cells appeared as medium oval. (Fig. 2). Positive reactions were recorded for citrate utilization, ornithine decarboxylase, nitrate reduction and lysine decarboxylase activity. Negative reactions were recorded for deamination activity, urease activity and H_2S production.The bacterium was able to utilize glucose, adonitol, lactose, arabinose and sorbitol as sole carbon sources. The plant growth promotion traits of the isolate were determined at three different incubation temperatures. From the results presented in Table 1, it is evident that the temperature of incubation had a definite effect on the Siderophore production as measured by the diameter of zone of the color change of CAS agar, also reduced with the drop in the incubation temperature, expression of plant growth promotion traits of the bacterium. The incubation temperature exerted a definite influence on tri-calcium phosphate solubilization by the bacterium, the P release being highest at 10°C. After confirming the phosphorus solibilizing ability on solid media, the phosphorus solubilization in liquid media was carried out in NBRIP broth. Further the studies were done by replacing the carbon source by Maltose and the nitrogen source by $NaNO_3$, respectively till 10 days of incubation at

Table 1: Plant growth promotion attributes of *Kluvera intermedia*

Temperature	IAA production	Siderophore production (cm)
10°C	+ve	+ve (3.5 ± 0.2)
30°C	+ve	+ve (2.4 ± 0.2)

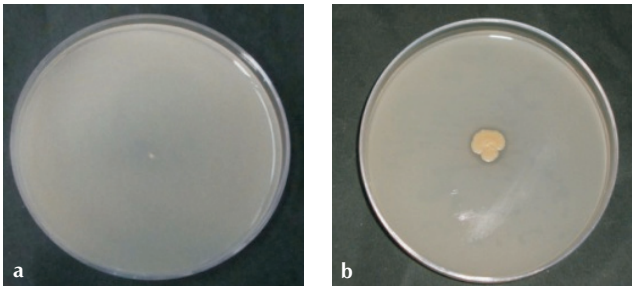

Figure 1: P Solubilization at (a) 10°C and (b) 30°C

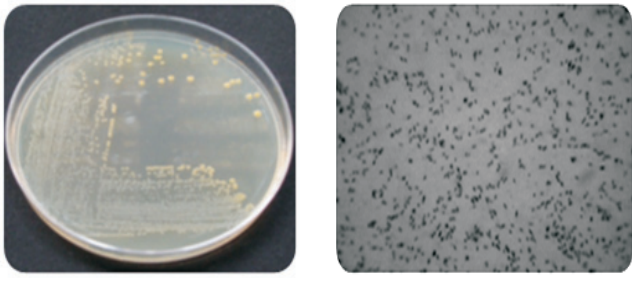

Figure 2: Microscopic examination of strain

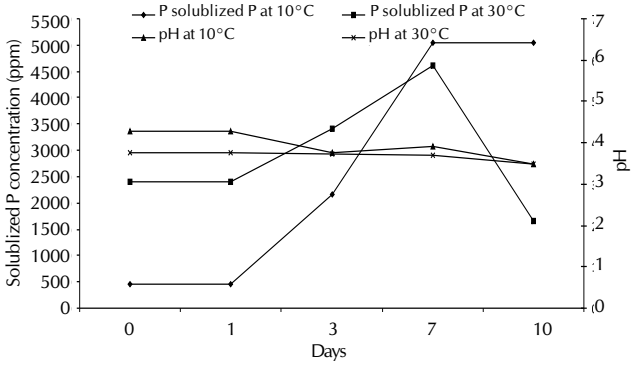

Figure 3a: pH and P Solubilized by the strain using glucose as carbon source & ammonium sulphate as nitrogen source at ambient as well as 10°C

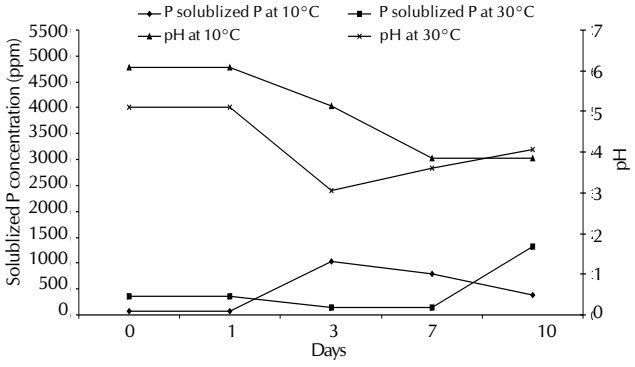

Figure 3b: pH and P Solubilized by the strain using maltose as carbon source & ammonium sulphate as nitrogen source at ambient as well as 10°C

10°C and 30°C, to find out suitable media formulation for better growth and solubilization of tricalcium phosphate by used strains. In presence of glucose as a carbon source and Ammonium sulphate $(NH_4)_2SO_4$ as nitrogen source, (Fig. 3a) maximum phosphorus solubilization was observed at 10°C on 7th day of incubation in comparison to 30°C. Strain showed maximum phosphorus solubilization (5054.22 ppm) on 7th day at 10°C with pH 3.9. At 30°C on 7th day of incubation, strain showed maximum phosphorus solubilization (4600.23 ppm) with pH 3.71. However, when maltose is taken as carbon source, Ammonium sulphate $(NH_4)_2SO_4$ as nitrogen source, strain showed comparatively very low phosphorus solubilization (222.85 ppm) at 10°C on 7th day of incubation and 1711.45 PPM phosphorus solubilize at 30°C on 10th day of incubation. (Fig. 3b). When Sodium nitrate ($NaNO_3$) was taken as a nitrogen source and glucose as a carbon source, Strain showed P solubilization (1312.20 ppm) at 30°C on 10th day of incubation with pH 4.06 and 1033.63 PPM at 10°C on 3rd day of incubation with pH 5.13. (Fig. 3c) An interesting feature that was noticed across the incubation temperatures (at 10°C) was the steady rise in the soluble P levels, till the 7th day of incubation, with a concomitant decrease in the pH of the medium. At all two incubation temperatures the maximum soluble P level was recorded on 7 days of incubation. Interestingly this also coincided with the lowest recorded pH.

DISCUSSION

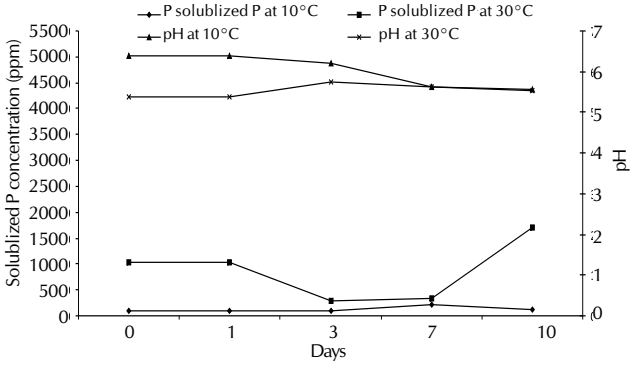

Figure 3b: pH and P Solubilized by the strain using maltose as carbon source & ammonium sulphate as nitrogen source at ambient as well as 10°C

Kluyvera intermedia form a considerable part of the microbial community of the plant rhizosphere and influence plant growth in a multitude manner. The present investigation gains significance, since to the best of our knowledge this an early report on the phosphate solubilization and plant growth promotion by a cold tolerant strain of *Kluyvera intermedia*, which opens up vistas for the agricultural importance of this hitherto lesser known bacterium. Earlier studies on phosphate solubilization/plant growth promotion by various Pseudomonads

include *P. fluorescens* (Di Simine *et al.,* 1998), *P. corrugata* (Pandey and Palni, 1998), *P. Aeruginosa* (Musarrat *et al.,* 2000), *P. stutzeri* (Vazquez et al., 2000), *P. putida* (Pandey et al., 2006), *P. trivialis* and *P. Poae* (Gulati *et al.*, 2007). Soil bacteria and fungi play a predominant role in the recycling of soil phosphorus reserves. Bacterial mineral phosphate solubilization has been attributed to the activity of glucose dehydrogenase; a membrane bound enzyme that is involved in the direct oxidation of glucose to gluconic acid, which subsequently gets converted to 2-ketogluconic acid and 2, 5-diketogluconic acid (Goldstein, 1995). The 2-ketogluconic acid is more effective than gluconic acid solubilizing phosphate (Kim *et al.,* 2003). In a dramatic shift from the past, there has been a surge of interest on P solubilization at cold temperatures by natural and mutant psychrotolerant strains in the present decade (Katiyar and Goel, 2003; Trivedi and Sa, 2007). On analyzing the comparative profile of 'P' solubilized by the strains using different media, it was observed that after 7th day of incubation i.e. on 10th day at 10°C and 30°C, a considerable drop in the values were observed. Acidification of the medium was less when glucose was replaced by maltose as carbon source. Relwani *et al.,* (2008). Narsian and Patel, (2000) showed that $(NH4)_2SO_4$ was the best nitrogen source for growth of PSM. The reduction in pH in the case of $(NH4)_2SO_4$ indicates the possibility of the operation of a NH_4/H^+ exchange mechanism acidifying the medium as reported by Roos and Luckener, (1994). Hence acidification due to NH_4^+ is more evident rather than NO_3^-. The extent of soluble phosphate was positively correlated with drop in pH of the culture filtrate. Phosphorus solubilizing microorganisms are reported to dissolve insoluble phosphates by the production of inorganic or organic acids (tartaric, oxalic acid, lactic, citric and gluconic acids) and/or by the decrease of the pH (Whitelaw, 2000). Oxalic acid showed high 'P' solubilization but did not liberate

Fe into the soil solution. Acetic, lactic and succinic acids could bring about a release of 'P' only at a concentration of 100 mM. Solubilization of 'P' increased when there is a sufficient amount of energy available to the organisms to result in the formation of organic acids. All the strains showed much higher drop in pH and simultaneous higher 'P' solubilization when glucose was taken as carbon source as compared to Maltose. These results are similar to Pradhan and Sukla, (2005). Significant differences were observed among the carbon sources and isolates in relation to the solubilization of phosphates in the culture medium, reduction in pH of the culture filtrate. Glucose and xylose were associated with the greatest range of solubilization. Of the phosphates tested, the order of microbial solubilization is $CaHP0_4^-$ > $Ca_3(P0_4)_2$ > $FePO_4$ > $Mg_3(P0_4)_2$ > $Al_3(PO_4)_2$. Glucose produced the greatest increase in total soluble phosphate. Starch, xylose and mannose taken as carbon source, when substituted for glucose showed better the phosphate-solubilization. The effect of different carbon sources (glucose, galactose, fructose) has been determined on the production of enzyme (Qureshi *et al.*, 2010). Mannitol and glucose were also reported to be the best sources for *A. niger* to solubilize phosphorus. Reyes *et al.*, (1999) showed that sucrose was the best carbon source for *P. rugulosum* for solubilization of hydroxyl-apatite and $FeSO_4$. Narsian and Patel, (2000) reported maximum P solubilization by *Aspergillus aculeatus* with arabinose and glucose. According to, Nautial *et al.,* (1999) when glucose was used as carbon source, microorganisms produced higher amount of organic acids. Organic acids may play important role in phosphate solubilization but are not the only possible mechanism for 'P' solubilization. (Illmer and Schinner, 1992). These results suggest that phosphate solubilization was affected by various carbon and nitrogen sources (Reyes *et al.*, 1999). The ability of the given strains to solubilize mineral phosphate in glucose as a carbon source was increased at a high level up to 7 days. These results are consistent with the earlier report where P-solubilizing ability increased with increasing concentration of glucose in *Pseudomonas* sp. (Banik, 1983) and *P. agglomerans* R-42 (Son *et al.*, 2006). Acid phosphatases and phytases secreted by these microorganisms also have an important role in phosphate solubilization (Achal *et al.*, 2007; Rechardson *et al.*, 2000). These observations indicate that 'P' solubilization is a complex phenomenon which depends on many factors such as the nutritional, physiological, and growth conditions of the cultures (Cunningham and Kuiack, 1992).

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