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Role of Different Carbon Source on Phosphate Solubilization by Psychrotolerant Isolate

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A B S T R A C T

A lab experiment was conducted at the department of microbiology, G. B. Pant University of Agriculture & technology, Pantnagar. MPS activities were measured in liquid media (NBRIP broth) using Ammonium sulphate as N source, glucose and maltose as a carbon source at 10^{o}C. Glucose significantly increased MPS activity and this activity was three times (3610.12PPM) in comparison of maltose (1434.99PPM). The mineral phosphate solubilizing activity was strongly associated with the production of gluconic or citric acids. Moreover, isolate (*Enterobacter hormachei*), maximum 'P' solibilization was observed on 7th day of incubation at 10^{o}C as compared to 30^{o}C, when glucose is taken as a carbon source and Ammonium sulphate $(NH_4)_2SO_4$ as nitrogen source.

Introduction

Phosphorus is one of the major plant nutrients, second only to nitrogen in requirement. However, a greater part of soil phosphorus, approximately 95–99% is present in the form of insoluble phosphates and hence cannot be utilized by the plants. Biological Nitrogen Fixation depends appreciably on the available forms of phosphorus. 'P' is an important structural constituent of nucleic acids, phytin and phospholipids. Plants absorb inorganic form of 'P' and make about 0.2% of their dry weight. They take up inorganic phosphate in two soluble forms: the monobasic ($H_2PO_4^-$) and the dibasic (HPO_4^{2-}) ions (Vessey, 2004). 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 soil (Nopparat *et al.*, 2007). To increase the availability of phosphorus for plants, large amounts of fertilizer are being applied to soil. But a large proportion of phosphatic fertilizer applied is quickly transformed to the insoluble forms which decrease the efficiency of fertilizers. The unbalanced use of chemical fertilizer, has led to reduction in soil fertility and to environmental degradation (Gyaneshwar *et al.*, 2002). So, the need of those microbes arose which have the capacity to solubilize phosphorus. These microorganisms are called Phosphate solubilizing micro-organisms (PSMs). 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; Lucy *et al.,* 2004). Microbial mediated phosphorus management is an eco-friendly and cost effective approach for sustainable development of agricultural crop. Microorganisms are an integral component of the soil phosphorus cycle and are important for the transfer between different pools of soil phosphorus. Phosphate Solubilzing Microorganisms (PSM) through various mechanisms of solubilization and mineralisation are able to convert inorganic and organic soil P respectively into the bioavailable form facilitating uptake by plant roots. It is important to determine the actual mechanism of P solubilisation by PSM for optimal utilization of these microorganisms in varied field conditions. Hence it is imperative to better understand the plant-soil-microbial P cycle with the aim of reducing reliance on chemical P fertilizers. This has led to increased interest in the harnessing of microorganisms to support P cycling in agroecosystems. (Sharma *et al.,* 2013)

The mechanism of mineral phosphate solubilization by PSB strains is associated with the release of low molecular weight organic acids which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, thereby converting it into soluble forms. The phosphate-solubilizing activity is qualitatively assessed by the ability to form solubilization halos (light zones) around the microbial colonies (Mikánova and Nováková, 2002), when they are grown on plates of distinct culture media such as Pikovskaya agar, NBRIP medium (Nautiyal, 2000) and NBRIP-BPB medium (Mehta and Nautiyal, 2001).

Temperature is one of the important factors that immediately affect the interior of the cell. Low temperature habitats are colonized by psychrophiles and psychrotolerant microorganisms, Psychrophiles are those microorganisms whose cardinal growth temperatures are at or below 0, 15, and 20^{o}C, respectively. *Pseudomonas fluorescence, Pseudomonas putida, Pseudomonas striata, Acinetobacter, Enterobacter, Paecilomyces* etc. comes under higher psychrophilic 'P' solubilizer. 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, 2010). Many researchers have studied the effect of various carbon sources on phosphate solubilization (Narsian and Patel, 2000). Further, the glucose as a carbon source show higher levels of phosphorus solubilization as compared to glycerol, maltose, sucrose and galactose. The solubilization of rock phosphate using diverse types of C sources has been studied by several workers. Rose (1957) showed that glucose or xylose were the best energy sources for fungi in liquid medium, whereas Katznelson and Bose (1959) reported that either yeast extract or soil extract were essential for the proper growth of phosphate solubilizing organisms in liquid or solid medium. They observed that rock phosphate solubilizing bacteria, yeast and fungi utilized a variety of carbon compounds as energy sources, but the amount of phosphate solubilized varied significantly with different sources of energy. Under cultural conditions it has been observed that bacteria are more active in presence of hexoses and pentoses in the medium whereas fungi are equally effective in the presence of hexoses, pentoses as well as disaccharides. Carbon substrates, in soil are available in limited concentration for microbial growth; hence the organisms grow in close vicinity of roots in plant rhizosphere than in non- rhizosphere soil. The plant root exudates provide readily metabolizable carbon and nitrogen compounds

for the growth of the heterotrophic forms and because of that the bacteria and fungi form an associative symbiosis with the root system to get substrates from the plant roots and provide mineral which normally could not be absorbed by the roots including phosphorus. This experiment was conducted to evaluate the effect of different carbon sources on mineral phosphate solubilising ability of strain *Enterobacter hormachei.*

Materials and Methods

Isolation and Identification

The present experiment was conducted in the laboratory under the Department of microbiology, college of basic science and humanities, G.B. Pant University of Agriculture and Technology, Pantnagar. Strains were isolated from the soil of Almora district located between 29.62^{o}N Latitude and 79.67^{o}E longitude. The height of experimental site from sea level is 1651m and accession no.is HQ222364. Strain was isolate after serial dilution of soil solution on potato and dextrose agar (PDA) plates. Distinct colonies present on the plates were selected, purified by repeated culturing and maintained on PDA slants at 4ºC.

Solubilization index on solid media, growth condition

Strains were checked for phosphate solubilizing ability on Pikovskaya (PVK) agar medium (Pikovskaya, 1948) incorporated with tri-calcium phosphate ($Ca_3(PO_4)_2$) by observing the Solubilization index (S.I.). The medium contained l^{-1}: glucose, 10g; KCl, 0.20g; $Ca_3(PO_4)_2$, 5.00g; $(NH_4)_2SO_4.7H_2O$, 0.50g; $MgSO_4$,0.10g; $MnSO_4$, 0.0001g; $FeSO_4$, 0.0001g; Yeast extract, 0.50g; Agar, 15g. The pH of the media was adjusted to 7.0 before autoclaving. Sterilized PVK media was poured into sterilized Petri plates and after solidification of the media, a pinpoint inoculation of this strain was made onto the plates under aseptic conditions. They were incubated at 28±2ºC and 10^{o}C for 7 days with continuous observation for colony diameter. The formations of halo zone around the growing colony showing phosphate solubilization. Solubilization index was evaluated according to the ratio of the total diameter (colony + halo zone) and the colony diameter.

Growth promotory properties

Siderophore production

The chrome azurol sulfonate (CAS) assay–[universal assay – Schwyn and Neilands, 1987] was used since it is comprehensive, exceptionally responsive, and most convenient. The Chrome Azurol Sulfonate assay agar was used.

For the qualitative assay, cultures were spot inoculated onto the blue agar and incubated at 37^{0}C for 24- 48h.The results were interpreted based on the color change due to transfer of the ferric ion from its intense blue complex to the siderophore. The sizes of yellow orange haloes around the growth indicate total siderophore activity.

IAA production

For Qualitative estimation of IAA production Tryptone soy broth is used. Tryptone soy broth (5.0ml) tubes with and without tryptophan (200µl/ml) were inoculated with loopful of actively growing bacterial cultures aseptically and incubated for 48h at 28^{0}C under shaking condition. Cultures were centrifuged at 10,000rpm for 10min. 2ml of Salkowski reagent was added in 1ml supernatant. The mixture was incubated at 28^{0}C for 25min. Development of pink colour shows IAA production.

Solubilization of tri-calcium phosphate in liquid culture

Culture was grown overnight in NBRIP broth media. In addition, to see the effect of different carbon source on 'P' solubilization, the NBRIP broth was modified by replacing glucose by maltose as carbon source. The broth (50ml) was inoculated by this culture and incubated for 10 days at ambient as well as 10^{o}C. Aliquots were drawn out at 1st, 3rd 7th and 10th day of incubation, centrifuged at 4000rpm at room temperature for 10min to get clarified supernatant and then pH of supernatant was measured using pH meter. The 'P' solubilized was measured in culture supernatant using Ion chromatography (Dionex Model).

Results and Discussion

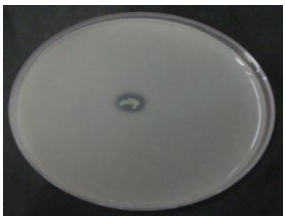
The bacterium formed yellowish irregular colonies when incubated on nutrient agar at 30^{o}C for 1days. Microscopic examination revealed that the isolate was Gram (-ve), and the cells appeared as thick small rods (Fig. 1). Positive reactions were recorded for citrate utilization, Lysine decarboxylase, Ornithrine decarboxylase, Nitrate reduction, glucose, lactose and arabinose utilization (Table 1). Negative reactions were recorded for Urease, Deamination, H_2S production, Adonitol and Sorbitol utilization. The plant growth promotion traits of the isolate were determined on incubation temperature at 30^{o}C. Siderophore production as measured by the diameter of zone of the colour change of CAS agar. This bacterium shows positive reaction for both Siderophore and Indole acetic acid (IAA) production. Bacterial plant growth promotion is a complex phenomenon which usually observed quantifiable effects includes increase in the plant biomass (Hameeda *et al.*, 2006). Those strains which showed better result for 'P' solubilization also has high ability to produce auxin have been reported earlier (Asea *et al.*, 1988). IAA production in the presence of a suitable precursor such as tryptophan has been reported for several other PGPR belonging to *Azospirillum, Azotobacter, Bacillus, Burkholderia, Enterobacter, Erwinia, Pantoea, Pseudomonas and Serratia* (Gulati *et al.*, 2008; Kumar *et al.*, 2009). This strains could indirectly augmenting the availability of phosphorus, as the siderophore due to their high affinity for iron are also involved in the release of iron bound phosphorus (Pratibha *et al.*, 2010).

The incubation temperature exerted a definite influence on inorganic phosphate (tri-calcium phosphate) solubilization by the bacterium, the P release being highest at 30^{o}C in comparison of 10^{o}C (Fig. 2).

Fig.1 Morphological and colony characterization on agar plates (a and b)

Fig.2 P solubilisation potential of strain on Pikovskaya agar at (a) 10°C and (b) 30°C

a.



b.

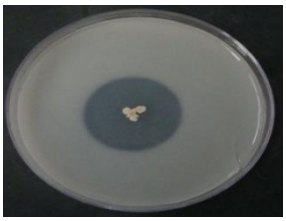


Table.1 Biochemical characteristics of *Enterobacter hormachei*

No.	Biochemical tests	Characteristics
1.	Shape	Thick small rod
2.	Gram staining	-ve
3.	Citrate Utilization	+ve
4.	Lysine decarboxylase	+ve
5.	Ornithrine decarboxylase	+ve
6.	Urease	-ve
7.	Deamination	-ve
8.	Nitrate reduction	+ve
9.	H_2S	-ve
10.	Glucose	+ve
11.	Adonitol	-ve
12.	Lactose	+ve
13.	Arabinose	+ve
14.	Sorbitol	-ve

However, isolate showed some strange response when NBRIP were supplemented with different carbon source. When glucose is taken as a carbon source, isolate showed maximum P solubilization (3610.12PPM) at 10°C on 7th day of incubation with pH 4.15 and 2888.74 PPM at 30°C with increase in pH 5.00. In presence of glucose the organism (*Citrobacter freundii*) shows maximum solubilization of P, when TCP is used, followed by galactose and then sucrose. Complete order from maximum to lowest is as follows: Glucose> galactose> maltose> sucrose> fructose> lactose> mannose> xylose> mannitol> glycerol. Thus, simple sugars are preferred more as compared to sugar alcohols. Glucose > sucrose > fructose > galactose > mannitol > mannose > maltose > glycerol > xylose > lactose. (Rathore. P, 2014). Glucose is the most favoured carbon source for max solubilization. Similar results have been reported by Joshi *et al.*, (2012) for Aspergillus. However, when maltose is taken as carbon source isolate solubilized 1776PPM and 1434.99PPM 'P' with pH 4.41and 4.5 at 30°C and 10°C incubation temperature. 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).

Organic acids may play important role in phosphate solubilization but are not the only possible mechanism for 'P' solubilization (Illmer and Schinner, 1992). 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 *et al.*, (2005). 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.

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