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*Research Article*

# An Efficient *Bm Strain* JCT13 producing Nano-Phosphorus Particles from Phytin and Solubilizing Phosphates

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***Abstract***

*A unique bacteria Bacillus megaterium JCT13 was isolated purified and cultured from arid soils which are secreting a large amount of phytase along with phosphatases. The bacteria have the ability to breakdown P nanoparticles of 50 nm size from phytin salts (0.1mM concentration) within 24 hours. The characterization of nanoparticles were carried out with the help of Particle size analyzer (PSA), Transmission electron microscopy(TEM), X-ray diffraction spectroscopy(XRD) and Electron dispersive X-ray spectroscopy (EDX). A considerable increase in inorganic P ($P_i$) release was observed between 3 and 10 days with the inoculation of the bacteria to phytin, lecithin, Na-glycerophosphate and tri-calcium phosphate under solution culture. After inoculation, the net $P_i$ release in the soil was 1-3 times more at non sterile condition and 6-12 times more under sterile condition. The intensity of the $P_i$ release was also more under low organic matter than high organic matter soil. The results clearly demonstrated the potential of the isolated bacteria for biosynthesis of P nanoparticles from salt solution containing phytin and its role in mobilization of P in soil from different unavailable P sources after inoculation of bacteria to the soil.*

***Keywords***: *Biosynthesis, Characterization, P nanoparticles, Bacillus megaterium JCT13, Phytin, P mobilization*

## 1. Introduction

Phytic acid is the principal storage form of phosphorus in many plant tissues, especially bran and seeds. Phytate is not digestible to humans or no ruminant animals, so it is not a source of either inositol or phosphate if eaten directly. Moreover, phytic acid chelates and thus makes unabsorbable certain important minor minerals such as zinc and iron, and to a lesser extent, also macro minerals such as calcium and magnesium; Phytin which involve phytic acid and its salts has been shown to be the major component of phytic acid. Phytic acid itself is not resistant to hydrolysis in soils, but the iron and aluminum salts that present in acid soils and the calcium and magnesium salt that persist in alkaline soils form complexes with P, are resistant to hydrolysis (Rodriguez *et al*., 2006). Therefore, they accumulate to the soil which may be removed after treated with different enzymes and microorganisms. These approaches are further supported by the fact that the majority of the bacteria inhabit ambient conditions of varying temperature, pH, and pressure.

Nanoparticles are biosynthesized when the microorganisms release enzymes which breakdown the salts to the nano form (100 nm or less) by the action of enzymes released by the organisms. The importance of nano phosphorus is enormous as it may reduce the absorption of P by Ca, Fe, Al, clay, organic matter and may minimize the competition with Si in soil; therefore, it is expected to enhance P use efficiency.

The importance of soil organic P as a source of plant available P depends on its rate of solubilization and the rate of inorganic P (Pi) release. Phytase, a type of phosphatases are able to increase the rate of the dephosphorylation (hydrolysis) of organic P (Rodriguez *et al*., 2006). Microbial acid phosphatase was found to be more efficient in hydrolysis of organic P compounds than plant sources (Tarafdar *et al*., 2001). The addition of phytase increased the P content of maize seedlings when supplied with phytate, and it was concluded that the utilization of phytate by plant was limited by low rates of hydrolysis (Findenegg and Neiemans, 1993). To breakdown of organophosphorus compounds it was found necessasary to find a organism which have potential phosphatases and phytase activities to exploit the plant unavailable P into available form. Tarafdar and Gharu (2006) demonstrated a fungus *Chaetomium globosum* produces phosphatase and phytase, which mobilize P and enhance the production of wheat and pearl millet crops. The P use efficiency also reported to be enhanced (Tarafdar, 2013) in the nano form of Phosphorus.

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The present investigation intended to isolate and purify phosphate mobilizing organism from arid soils. The hypothesis was to prepare nano P by a bacterial strain, which has never done earlier, and to know whether the nano P producing bacteria is equally efficient in mobilizing the plant unavailable P.

## 2. Materials and methods

### *2.1 Isolation of bacteria*

The soil bacteria were isolated from agricultural farm of Central Arid Zone Research Institute (CAZRI) located in Jodhpur, Rajasthan, India. The method employed for isolation of bacterial culture according to standard microbiological research steps including serial dilution method followed by spread plate using nutrient agar medium (Himedia laboratories Pvt.Ltd), which is specially used for bacterial growth. Inoculated plates were incubated at 25$^{o}$C for 48 hours in BOD (Biological Oxygen Demand) incubator, pure culture colony were picked and then again cultured on nutrient agar media (Peptone-10g, beef extract-10g, NaCl-5g, Agar-20g, Distilled water-1000mL). Stock culture was maintained by sub culturing at 15days interval. The soil microbial biomass was 72 µg/g.

### *2.2 Sample preparation*

Two hundred and 50 mL flasks was taken containing 1.5g nutrient agar media in 100mL double distilled water and the flasks were sterilized (121 °C at 15 psi for 15 minutes) on laminar air flow bench, the pure bacterial colonies was inoculated on it . These flasks were incubated at 25$^{o}$C for 7days on BOD incubator (Surana scientific, India) with intermittent mixing once a day.

### *2.3 Morphological and biochemical characterization of bacteria*

The bacterial strain was identified through colony morphology, microscopic examination, and biochemical characteristics. Gram staining, morphology and motility were observed by optical research microscope using phase contrast condenser at 100X oil immersion objective (Leica DM 5000B, Germany). Physiological and biochemical properties examine following methods suggested by Smibert and Krieg (1981).

### *2.4 Molecular characterization of Bacillus megatarium strain JCT13*

The molecular identification of isolated bacterial strain was carried out on the basis of 16S r RNA gene sequencing using universal primers viz ,EUB1and EUB2. Each amplification was performed using PCR (Corbett Research, San Francisco,USA) in a total volume of 50µL containing 0.2µL Taq DNA polymerase (5U/ µL of Promega), 5 µL of 10X PCR buffer (10mM Tries HCl, pH 8.3,500mM KCl,15mM $Mgcl_2$ sigma chem.),0.4µL dNTP mix 2.0mM each A,T,C,G (MBI Fermentas),1µL of each EUB1 and EUB2 PRIMERS, 2 µL of 5%(v/v) glycerol, 4 µL of genomic DNA and 36.4 µL d $H_2O$.The reaction were performed in a Thermal cycler (Corbett Research,USA) with the following condition i.e. 1min 20s elongation at 72$^{o}$C , repetition 34 times with a final elongation steps of 10 min at 72$^{o}$C. The PCR were visualized on 1% (w/v) Agarose gel in Tris-acetic acid EDTA (1X TAE) buffer at 60 V for 100 min. Agarose gel was stained with Ethidium bromide and photographed under UV light using gel documentation system (Syngene,USA).

Each amplified PCR product was subjected to direct sequencing using big dye terminator method on ABI prism DNA sequence. DNA amplified fragments were about 993bps. Nucleotide sequence comparisons were performed using Basic Local Alignment Search Tool (BLAST) Network services of the National Centre For Biotechnology information (NCBI) database. The bacterial strain designated to the sequence analysis based on similarity with the best aligned sequence of BLAST search. The complete sequence of *Bacillus megaterium* strain JCT13 was submitted to NCBI database and accession numbers obtained.

### *2.5 Synthesis and characterization of P nanoparticles*

Cell free filtrate of Bacillus megaterium JCT 13 was obtained using membrane filter. Using cell free filtrate, salt solution of phytin was prepared in concentration of 0.1mM in erlenmeyer flask which was found to be optimum salt concentration in our preliminary experiment. The entire mixture was put into shaker (150rpm) at 28°C. The reaction (1:1 ratio of cell free extract and salt solution) was carried out for 24 hours. Synthesis of nanoparticles was monitored using particle size analyzer (Bechman Delsa Nano C, USA) for size measurements and calculation of nanoparticles size distribution. The particle size analyzer was measured the rate of fluctuations in the laser light intensity scattered by particles as they diffuses through solvent. For confirmation of size and shape, TEM measurement were carried out using drop coating method in which a drop of solution containing nanoparticles was placed on the carbon-coated copper grids and kept under vacuum desiccation for overnight before loading them onto a specimen holder. The Transmission Electron Microscope (TEM) micrograph of the sample was taken using the TEM- 2100 F TEM instrument (Hitachi, Japan). The instrument was operated at an accelerating voltage of 200 kV. Electron Dispersive X-Ray spectroscopy (EDS) used particularly for elemental composition analysis. It does not give real quantities but the percentage of an element in a mixture of other element. Samples were prepared on carbon-coated copper grids and kept under vacuum desiccation for 3 h before loading them onto a specimen holder. Elemental analysis on single particles was carried out using Thermo-Noran (USA) EDS

attachment equipped for determination of the elemental composition and purity of the sample by atom % of metal.

X-ray diffraction (XRD) measurements of the freeze-dried samples were carried out using a Rikagu MiniFlex II Benchtop XRD system ( Rigaku Company, Texas, USA) operated at a voltage of 20 kV and current of 15 mA with CuKx radiation. Phase analysis was carried out by comparing the calculated values of interplanner spacing and corresponding intensities of diffraction peaks with theoretical values from the Powder Diffraction File database (PCPDF-WIN,JCPDS-ICOD 2008).

*2.6 Enzyme activity assay*

Acid and alkaline phosphatases was assayed by adopting the standard procedure of Tabatabai and Bremner (1969) using acetate buffer (pH 5.4) and sodium tetraborate – NaOH buffer (pH 9.4), respectively. The enzyme substrate (4- nitrophenyl phosphate) mixture was incubated at $35^0$C for 1 h and the enzyme activity was expressed as enzyme unit (EU). One unit is the amount of enzyme, which hydrolysis 1.0µ mol of p-nitrophenyl phosphate $min^{-1}$ at pH5.4 (acetate buffer) for acid phosphatase or 9.4 (borax-NaOH buffer) at $35^o$C. Phytase activity was assayed by measuring the amount of inorganic phosphate (Pi) released by hydrolysis of sodium phytate (Yadav and Tarafdar, 2003). One unit of phytase activity was defined as the amount of enzyme, which liberated 1µ mol Pi $min^{-1}$.

*2.7 Efficiency of bacteria towards the hydrolysis of different plant unavailable P compounds*

To test the efficiency of bacteria towards the hydrolysis of different plant unavailable P compounds 1mL of bacterial culture with $10^{10}$ CFU in each triplicate was added to 9 mL of solution in 1mM of Lecithin, Phytin, Sodium Glycerophosphate, Tri Calcium Phosphate and incubated at 28°C for 3,7 and 10 days in a incubator . Each replicate contains lecthin P 251 mg, phytin P 1507 mg, sodium glycerophosphate P 251mg, tricalcium phosphate P 502mg. The Pi release was quantified calorimetrically as described by Jackson (1967) and expressed in µg /mL P (ppm).

*2.8 Native P mobilization from soil by the action of Bacillus megaterium JCT13.*

To test the efficiency of bacteria towards the hydrolysis of different P compounds two soils (low organic P and high organic P) were selected. Low organic matter soil (0.22%) was collected from Central Arid Zone Research Institute Jodhpur, Rajasthan, India and high organic matter (1.47%) soil was collected from Dehradun, Utttrakhand, India. There were four treatments (sterilized control, sterilized inoculated, non sterile-control, non sterile inoculated) with twelve replication each. For sterilized treatment the soil was sterilized in the autoclave for three alternate days at 15 lbs pressure at $121^0$C. Each replicate contain 50g soil in 100 mL Borosil beaker. Inoculated treatment received 10mL$10^{10}$ microbial population per gram of soil while control treatment received 10 mL of sterilized microbe's $50g^{-1}$ soil. The soil was incubated at $28^0$C. The release of inorganic P was estimated (Jackson, 1967) after harvesting four replicates each at 24, 48 and 72 hours.

*2.9 Statistical analysis*

Standard errors of the mean were calculated and where necessary we carried out analysis of variance of the data. The least significance was calculated (P=0.05) using t-method ( Sokal and Rohlf, 1981).

## 3. Results

*3.1 Isolation and identification of bacteria*

Identification of bacteria (*Bacillus megaterium* JCT13, NCBI GenBank Accession no. JX442240) on the basis of morphological character and molecular characterization of bacterial isolate was performed by sequencing of 16Sr RNA sequencing .The sequence was compared using Basic Local Alignment Search tool(BLAST) of NCBI and submitted sequence is available on public domain http:// www. ncbi.nlm.nih.gov .

*3.2 Biosynthesis and characterization of P nanoparticle*

The biosynthesis of P nanoparticles was carried out by exposure of a precursor salt as bulk phytin solution of 0.1 mM concentration to extracellular enzyme obtained by *Bacillus megaterium* JCT 13 in an aqueous solution. Synthesized nanoparticles were characterized for morphological analysis. Particle size distribution was analyzed by DLS. Histogram shows average particle size (based on intensity distribution) of 50.6 nm (Fig. 1). The polydispersity index was -0.904 reflects monodispersed nature of the particle. The size distribution of the particle was illustrated 1% particle of <14 nm, 5% particle of <17 nm , 20% particle of <25 nm, 50% particle of <42 nm and 90% particle of <91 nm size Since DLS measure hydrodynamic diameter, so it was further confirmed with TEM analysis. TEM measurements showed well distribution of P nanoparticles (Fig 2). XRD image (Fig. 3) also confirms that it was P nanoparticles. The EDX spectrum (full scan mode) of drop coated phosphorus nanoparticle shown in Figure 4, confirm the purity of phosphorus nanoparticles. The spectrum shows strong peak intensity of P (67 atom %).

**Table 1** Release of phosphatases (EU x $10^{-3}$ $mL^{-1}$)* by *Bacillus megatarium* JCT13 after incubation at different time interval

| Days after incubation | Acid phosphatase | Alkaline phosphatase | Phytase | LSD (p= 0.05) |
|---|---|---|---|---|
| 7 | 2.46 | 1.86 | 2.20 | 0.13 |
| 14 | 2.52 | 3.30 | 3.72 | 0.14 |
| 21 | 2.80 | 3.35 | 4.47 | 0.14 |
| 24 | 2.70 | 3.09 | 3.60 | 0.09 |
| LSD (p= 0.05) | 0.10 | 0.12 | 0.12 | |

*EU: Enzyme unit

**Table 2** Efficiency of *Bacillus megaterium* JCT13 towards hydrolysis of different P Compounds (± indicate the standard error of mean, Control - without inoculation,)

| | Release of P (mg $kg^{-1}$ from different P compounds) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| DAI* | Phytin | | Lecithin | | Na-glycerophosphate | | Tricalcium Phosphate | |
| | Control | inoculated | Control | inoculated | Control | inoculated | Control | inoculated |
| 3 | 4.1 | 10.1 (146.3)** | 3.6 | 9.7 (169.4) | 4.4 | 11.5 (161.4) | 5.9 | 13.5 (128.8) |
| 7 | 4.8 | 13.7 (185.4) | 4.9 | 10.9 (122.4) | 6.1 | 12.7 (108.2) | 6.2 | 15.1 (143.5) |
| 10 | 4.9 | 20.1 (310.2) | 5.4 | 13.0 (140.7) | 7.2 | 14.4 (100.0) | 7.3 | 15.8 (116.4) |
| LSD (P=0.05) | 0.08 | 0.08 | 0.03 | 0.07 | 0.09 | 0.09 | 0.07 | 0.09 |

*Days after incubation

** Figures under parenthesis indicate % improvement over control

**Table 3** Characteristic of the soil use for the study

| Parameters | Low P Soil | High P Soil |
|---|---|---|
| Soil group | Aridisol | Entisol |
| pH ( Soil:Water = 1:2) | 7.9 | 7.8 |
| EC (dS $m^{-1}$) | 0.2 | 0.2 |
| Organic matter (%) | 0.22 | 1.47 |
| Total P (mg $kg^{-1}$) | 526 | 1758 |
| Available P (mg $kg^{-1}$) | 5.5 | 37.3 |
| Minral P **(**mg $kg^{-1}$**)** | 421.3 | 127.2 |
| Organic P | 100.2 | 1593.5 |
| Microbial biomass (µg $g^{-1}$) | 72 | 810 |

*3.3 Release of P mobilizing enzymes by Bacillus megaterium JCT13*

The ability to release phosphatases (acid and alkaline) and phytase by the bacteria upto 24 days of incubation was presented as Table 1. Higher release of enzymes was observed between 14 and 21 days old stage. The activity was decline thereafter. In general more release of phytase than acid or alkaline phosphatases was observed. The release of alkaline phosphatase was more than acid phosphatase.

*3.4 P hydrolysis from plant unavailable P sources*

The P hydrolysis from unavailable sources was 1 to 3 fold more than control when the bacteria were inoculated with different organic P (phytin, lecithin, Na-glycerophosphate) and inorganic P (tri-calcium phosphate) salts for 3-10 days under solution culture (Table 2). In general, the inoculated bacterial contribution was much higher to release $P_i$ from phytin after 10 days (310.2%), $P_i$ from lecithin (169.4%) and Na-glycerophosphate (161.4%) after 3 days and $P_i$ from tri calcium phosphate (143.5%) after 7 days. In general, gradual release of $P_i$ with time was observed in all the cases.

*3.5. Contribution of bacteria to release plant available P*

The P status and microbial biomass observed in the experimental soil was shown Table 3. The total P under low P soil was 576 mg $kg^{-1}$ as compared to high P soil 1758 mg $kg^{-1}$. The microbial biomass was also 7 times less under low P soil. Two contrasting soils were chosen for the study where low P soil (0.27% organic matter) contains 80% mineral P, 1% available P and 19 % organic P and high P soil (1.47 % organic matter) contains 19% mineral P, 2%available P and 79 % organic P.

The effect of bacteria on hydrolysis of plant available P in low and high organic matter soil with time interval was presented as Table 4. The net $P_i$ release from soil was 1-3 times more under non sterile condition while 6-12 times more under sterile condition. The intensity of $P_i$ release was also more under low organic matter than high organic matter soil.

**Table 4** Contribution of *Bacillus megaterium* JCT13 to release plant available P in the soil with time interval

| Soil Type | Treatment | Release of Pi (mg $kg^{-1}$) | | | LSD (p=0.05) |
|---|---|---|---|---|---|
| | | 24h | 48h | 72h | |
| Low P Soil (Aridisol) | NS-C | 4.0 | 5.1 | 5.2 | 0.1 |
| | NS-I | 12.5 | 16.3 | 19.9 | 1.2 |
| | S-C | 2.7 | 2.2 | 2.2 | 0.1 |
| | S-I | 18.7 | 24.5 | 24.5 | 1.4 |
| High P Soil (Entisol) | NS-C | 15.7 | 17.9 | 18.4 | 0.3 |
| | NS-I | 31.4 | 35.2 | 37.1 | 1.6 |
| | S-C | 6.4 | 6.7 | 6.8 | 0.4 |
| | S-I | 47.5 | 52.3 | 58.4 | 2.3 |

NS- non sterile soil, S- Sterile Soil, C- Control, I- Inoculated

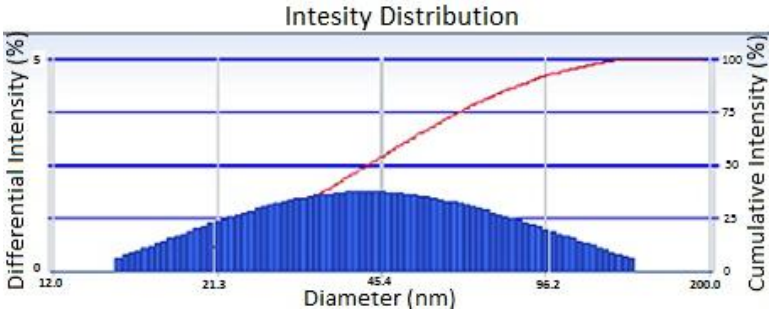


**Fig.1** Size distribution of synthesized P nanoparticles by *Bacillus megaterium* JCT13 (10% < 20nm, 50% <42nm, 90% <91 nm)

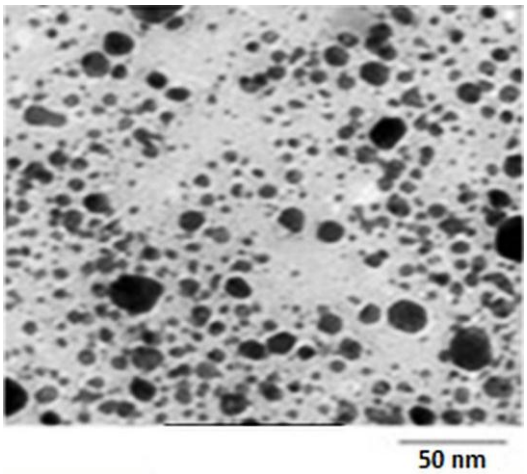


**Fig. 2** TEM micrograph of biologically synthesized P nanoparticles from phytin

The results also reflect that the microbial contribution on $P_i$ release from soil depends on the incubation time with the bacteria.

The entire results clearly reveal the role of *Bacillus megaterium* JCT13 for P nanoparticle production and P nutrition of higher plants through the breakdown of unavailable native P sources.

## 4. Discussion

The present invention approaches for biosynthesis of phosphorus nanoparticles using soil bacteria *Bacillus megaterium* JCT 13 (NCBI GenBank accession No. JX442240). Selection of bacteria was based on synthesis of phosphorus nanoparticle using extracellular secrets from total isolate of 23 in phytin salts. The morphology of nanoparticles was monitored by particle size analyzer (Fig 1) and XRD (Fig 3).

A TEM micrograph (Fig 2) showed homogenous distribution of P nanoparticle at the measurement scale bar of 50 nm. The purity of biosynthesized P nanoparticles was confirmed by EDX, where 67% nano particles were found as P (Fig 4). Besides this a Ca and Mg peak present due to chemical composition of salt or impurities. C and O peaks are found which indicates the presence of bacterial protein as encapsulated material surrounds the phosphorus nanoparticles.

In the microbial synthesis of metal nanoparticles extracellular secreting enzymes are produced which reduces the metal salt of macro and micro scale into nanoscale diameter through catalytic effect (Jain *et al*., 2011).

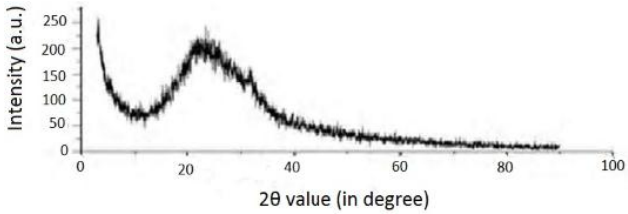


**Fig. 3** XRD Spectrum of biologically synthesized P nanoparticles

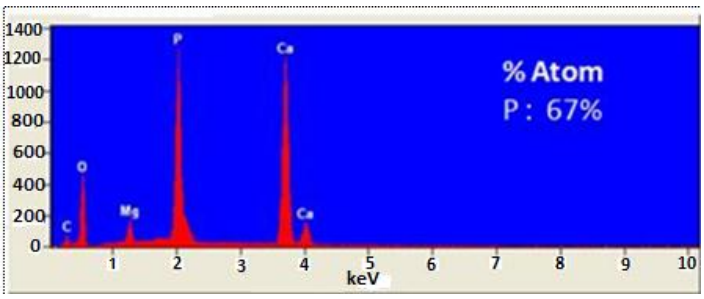


**Fig. 4** EDX Spectrum of biologically synthesized P nanoparticles by *Bacillus megaterium* from phytin

Extracellular secretion of enzymes offers the advantage to obtain pure, mono disperse nanoparticles, associated with downstream processing. The microbial synthesis of P nanoparticles involves an enzyme mediated process which is present in extracellular secrets and another protein act as capping protein play role in the further encapsulation of P nanoparticles and increase stability. Such biologically synthesized, very tiny functional nanoparticles are economically chief, relatively stable, easy downstream processing and environmentally safe as they are encapsulated by mother protein which is water soluble.

The ability of isolated bacteria for enhanced secretion of phytase along with phosphatases (Table 2) may help to enhance more plant available P though the hydrolysis of organic esters (Tarafdar and Marschner, 1994) or mobilizing tri- calcium phosphate (Table 4). The importance of soil microorganisms for increasing the availability of P from phytate through the provision phytase activity has similarly been suggested by Richardson *et al*. (2001). Besides the cleavage of C-O-P ester bond by microbial phosphatase or phytase, the microorganisms may also produced different organic acids (Jones, 1998), which may possibly help in greater release of Pi.

Microbial activity is a central factor in the soil organic P cycle (Tarafdar and Gharu, 2006) and affects the transformation of inorganic P (Kucey *et al*., 1989).

In our study, maximum enzyme release was observed after 3 weeks (21 days) of growth. Higher enzyme release by the organisms indicated the potential of soil to affect the biochemical transformations necessary for the maintenance of soil fertility (Rao *et al*., 1990). A higher biomass accumulation would be expected under sterile condition which to be associated with greater release of phytase and phosphatases and the mobilization of more unavailable P. Enhanced secretion of phytase by rhizosphere microorganisms (Tarafdar and Marschner, 1994) may contribute to Pi acquisition through the hydrolysis of organic esters in the rhizosphere. The results presented here clearly demonstrate the importance of isolated bacteria *Bacillus megaterium* JCT13 for biosynthesis of P nanoparticle and P mobilization from native soils for plant nutrition.

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