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# Isolation and characterization of phytase producing *Bacillus* strains from mangrove ecosystem

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**Abstract**

Five aerobic endospore-forming bacilli, isolated from mangrove soil at Cochin, Kerala, India, which produce phytase enzyme, were taxonomically characterized. There were two strains of *Bacillus circulans* (MTCC 7635 and 7636), one strain each of *B. licheniformis* (MTCC 6824) and *B. pantothenicus* (MTCC 7638), and one was identified as *Bacillus* sp. (MTCC 7637). All strains were alkalophilic with *B. licheniformis* and *B. pantothenticus* tolerating pH up to 11, and other strains up to pH 9. All the strains were thermotolerant with *B. licheniformis* with good growth at 55°C. *B. pantothenicus* was found to be halotolerant species and tolerated 10% NaCl.

**Keywords**: Phytase, *Bacillus*, mangrove

## Introduction

Cereals, legumes, and oilseed crops are grown in over 90% of the world's harvested area. These crops serve as a major source of nutrients for humans and animals including fish. An important constituent in these crops is phytic acid (*myo*-inositol hexaphosphate). The salt form, phytate, is the major storage form of phosphorus and accounts for more than 80% of the total phosphorus in cereals and legumes (Reddy *et al.,* 1989). Phytases are enzymes capable of hydrolyzing phytic acid to less-phosphorylated *myo*-inositol derivates. Monogastric animals, such as pig, poultry and fish are not able to metabolize phytic acid and therefore inorganic phosphate is added to their diets to satisfy the phosphorus requirement. This consequently contributes to phosphorus pollution problems in areas of intensive livestock production (Common, 1989). Phytic acid also acts as an anti-nutritional agent in monogastric animals by chelating various metal ions needed by the animal, such as calcium, copper, and zinc (Graf, 1983; Lee *et al.,* 1988; Lei *et al*., 1993). Therefore, the enzymatic hydrolysis of phytic acid into less-phosphorylated myo-inositol derivatives in the intestine of monogastric animals is desirable. Many attempts to enzymatically hydrolyze phytic acid have been made to improve the nutritional value of feed and to decrease the amount of phosphorus excreted by animals (Simons *et al*., 1990; Pen *et al*., 1993). The phytase enzyme produced by bacteria is extracellular which are more appropriate than the intra cellular phytase produced by yeast in breaking down phytic acid (Konietzny and Greiner, 2004). Phytase has been isolated and characterized from a few Gram-positive and Gram-negative soil bacteria, e.g. *Bacillus subtilis* (Kerovuo *et al.*, 1998), *Bacillus amyloliquefaciens* (Kim *et al.*, 1998; Idriss *et al.,* 2002), *Klebsiella terrigena* (Greiner *et al.*, 1997), *Pseudomonas* spp. (Richardson and Hadobas, 1997) and *Enterobacter* sp. (Yoon *et al.*, 1996). Using phytase enzyme, improved phosphorus nutrition is achievable by mobilization of phosphorus fixed as insoluble inorganic polyphosphates and/or phytate, which accounts for 20–50% of the total soil organic phosphorus (Richardson *et al*., 2001). *Bacillus* strains belonging to the *B. subtilis*/*amyloliquefaciens* group isolated from soil are also reported to possess plant-growth-promoting activity (Krebs *et al.*, 1998; Idriss *et al*., 2002).

The objective of the present study is to isolate and characterize phytase producing bacterial strains from mangrove ecosystem.

## Materials and methods

***Isolation of phytase-producing bacteria:*** Isolation of the phytase-producing bacteria was carried out by sampling soil from Mangalavanam, a mangrove swamp at Cochin. Sediment samples (0-5 cm depth) were collected using a sterile stainless steel spatula into a sterile bottle. Three replicate samples were randomly collected from three sites (1 m apart) to make a composite sample and this was used for bacterial screening. The soil samples were subjected to HV medium (1 $gL^{-1}$ humic acid, 0.5 $gL^{-1}$ $Na_2$ $HPO_4$, 1.7 $gL^{-1}$ KCl, 0.05 $gL^{-1}$ $MgSO_4.7H_2O$, 0.02 $gL^{-1}$ $CaCO_3$, 0.03 $gL^{-1}$ B-vitamin, 18 $gL^{-1}$ agar), and cultivating the samples at 50°C to screen isolates which produce phytase extracellularly (Powar and Jagannathan, 1982).

***Cultivation of phytase-producing bacteria***: The bacterial isolates to be screened for phytase production were cultivated individually in TSB medium (15 $gL^{-1}$ tryptone, 5.0$gL^{-1}$ soyptone, 5.0 $gL^{-1}$ NaCl, 1. 0 L distilled water) at 45° C overnight. Cultivated bacteria (0.1 ml) were collected and inoculated into 50 ml phytase screen medium (PSM) (15 $gL^{-1}$ Glucose, 5.0 $gL^{-1}$ $NH_4$ $NO_3$, 0.5 $gL^{-1}$ KCl, 0.5 $gL^{-1}$ $MgSO_4$. $7H_2O$, 0.01 $gL^{-1}$ $FeSO_4$. $7H_2O$, 0.01 $gL^{-1}$ $MnSO_4.7H_2$ O, 0.5 % Ca-phytate, 20.0 $gL^{-1}$ Agar; pH adjusted to 5.5), allowing the bacteria to grow at 45° C for 4 days with agitation at a rate of 125 rpm. When individual isolates from TSB were seeded into the PSM plate, any developing colony which produced a clear zone was considered a potential phytase producer. All reagents used in the present study were obtained from Hi Media, Mumbai. Sub-culturing was done every 15 to 30 days in wheat bran agar by adapting the following procedure: for preparation of wheat bran extract of 100 ml, 100g wheat bran in 1000 ml distilled water was autoclaved for 1 h and filtered; 0.04 $gL^{-1}$ $(NH_4)_2$ $SO_4$, 0.02 $gL^{-1}$ $MgSO_4$. $7H_2O$, 1 $gL^{-1}$ Casein, 0.05 $gL^{-1}$ $KH_2PO_4$, 0.04 $gL^{-1}$ $K_2HPO_4$, 2 $gL^{-1}$ Agar, pH 6.0 to 6.2, autoclaved at 15 psi for 15 min; 0.2 ml $CaCl_2$ from a sterile 2% stock solution (Powar and Jagannathan, 1982).

***Characterization of phytase-producing bacteria***: Strains isolated in the present study were characterized by conventional microbiological methods (Farrow *et al.,* 1994; Ivanovo *et al*., 1998; Idriss *et al.,* 2002) and morphology of vegetative cells and sporangia and shape and position of spores. The other characteristics studied were: nitrate and nitrite reduction tests; indole, methyl red and voges proskauer tests, anaerobic growth; growth on Mac Conkey agar, gas production from glucose, degradation of starch, urea casein and gelatin, citrate utilization, oxidase and catalase tests, oxidation/ fermentation (O/F), production of arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase, acid production from adonitol, arabinose, cellobiose, dextrose, dulcitol, fructose, galactose, inositol, lactose, maltose, mannitol, melibiose, raffinose, rhamnose, salicin, sorbitol, sucrose, trehalose and xylose, growth at temperatures of 4°, 10°, 15°, 25°, 30°, 37°, 42°, 55°, 67 °C and NaCl requirement (2.5, 5, 7, 8, 10,%). Growth at different pH (5.0, 5.7, 6.8, 8.0, 9.0, 11.0) was also detected on the medium. The pH was adjusted with 10 M NaOH. Penicillin sensitivity was determined by using the routine diffusion plate technique. Cultures were grown overnight on the nutrient agar medium at 28°C with optical density of 0.5 ($1.5 \times 10^8$ cells per ml). A 0.1-ml portion of the suspension was plated onto agar, and disks containing antibiotics were placed onto the surface of the medium. After overnight incubation at 30°C the diameters of the zones of growth inhibition were measured.

## Results and discussion

All the five bacterial strains obtained on screening for phytase production belonged to the genus *Bacillus* (Table 1). The genus *Bacillus* comprises a phylogenetically and phenotypically heterogeneous group of species. Due to their ubiquity and capability to survive under adverse conditions, heterotrophic *Bacillus* strains are hardly considered to be species of certain habitats (Claus and Berkeley, 1986). However, it is generally accepted that the primary habitat of the aerobic endospore-forming bacilli is the soil. Since most *Bacillus* species can effectively degrade a series of

biopolymers (proteins, starch, pectin, etc.), they are assumed to play a significant role in the biological cycles of carbon and nitrogen. All the five *Bacillus* strains isolated in the present investigation possessed typical cellular and colonial morphologies and physiological, biochemical and nutritional features. All the strains had typical morphological characteristics (configuration round except for *B. licheniformis* MTCC 6824 which alone was lobate), margin wavy (irregular for *B. licheniformis* MTCC 6824 alone), elevation convex, surface rough, density opaque and no pigment), gram positive rods, of moderate size, arranged singly. The organisms were motile and produced oval endospores located at terminal or central positions in the sporangia. The strains were found to utilize a wide range of organic compounds, were halo-tolerant and alkali tolerant, which may reflect their great metabolic flexibility. None of the strains grew at 10° C or below. While all the strains tolerated temperature up to 42° C, *B. licheniformis* MTCC 6824 tolerated and grew at 55°C also. *B. licheniformis* and *B. pantothenticus* were able to tolerate pH up to 11, while other strains tolerated pH up to 9.0. *B. pantothenticus* tolerated NaCl up to 10%, whereas, other strains tolerated up to 8% only. Other phenotypic characteristics of the strains studied are presented in Table 1.

The five strains have been typed as *B.*

Table 1. *Phenotypic comparison of phytase producing* Bacillus spp. *isolated from mangrove soil*

| Characteristics | *B. licheniformis* MTCC 6824 | *B. circulans* MTCC 7635 | *B. circulans* MTCC 7636 | *Bacillus* sp. MTCC 7637 | *B. pantothenticus* MTCC 7638 |
|---|---|---|---|---|---|
| **Spore** | | | | | |
| Endospore | + | + | + | + | + |
| Position | Terminal | Terminal | Central | Central | Terminal |
| Sporangia bulging | + ve | + ve | + ve | + ve | + ve |
| **Fluorescence** (UV) | - | + | + | + | + |
| **Growth at** | | | | | |
| 15 °C | + | + | + | - | - |
| 55 °C | + | - | - | - | - |
| 67 °C | - | - | - | - | - |
| **Growth at pH** | | | | | |
| 11. 0 | + | - | - | + | + |
| **Growth on NaCI (%)** | | | | | |
| 8.0 | - | ± | - | + | + |
| 10.0 | - | - | - | - | + |
| **Growth Under** | | | | | |
| **Anaerobic Condition** | + | + | + | + | + |
| Growth on Mac Conkey Agar | - | - | - | - | - |
| Indole Test | - | - | - | - | - |
| Methyl Red Test | - | - | - | - | - |
| Voges Proskauer Test | - | - | - | - | - |
| Citrate Utilization | - | + | - | + | - |
| Gas Production from Glucose | + | - | - | - | - |
| Casein Hydrolysis | - | + | + | - | - |
| Starch Hydrolysis | + | + | + | - | + |

*continued on next page*

| Characteristics | *B. licheniformis* MTCC 6824 | *B. circulans* MTCC 7635 | *B. circulans* MTCC 7636 | *Bacillus* sp. MTCC 7637 | *B. pantothenticus* MTCC 7638 |
|---|---|---|---|---|---|
| Urea Hydrolysis | - | - | - | - | - |
| Nitrate Reduction | + | + | + | + | + |
| Nitrite Reduction | + | + | + | + | + |
| $H_2S$ Production | - | - | - | - | - |
| Cytochrome Oxidase | + | + | + | + | + |
| Catalase Test | + | + | + | + | + |
| Oxidation/ Fermentation (O/F) | O | F | F | O | F |
| Gelatin Hydrolysis | - | + | + | + | + |
| Arginine dihydrolase | nd* | + | + | + | + |
| Lysine decarboxylase | nd | - | - | - | - |
| Ornithine decarboxylase | nd | - | - | - | - |
| **Acid production from carbohydrates** | | | | | |
| Adonitol | nd* | - | - | - | - |
| Arabinose | + | + | + | - | - |
| Cellobiose | nd | + | + | + | + |
| Dextrose | + | + | + | + | + |
| Dulcitol | nd | + | + | + | + |
| Fructose | nd | + | + | + | + |
| Galactose | + | - | - | - | - |
| Inositol | + | - | - | - | - |
| Lactose | + | - | - | - | - |
| Maltose | nd | + | + | + | + |
| Mannitol | + | + | + | + | + |
| Melibiose | nd | + | - | - | - |
| Raffinose | - | - | - | + | - |
| Rhamnose | - | - | - | - | - |
| Salicin | + | + | + | + | + |
| Sorbitol | nd | + | + | + | + |
| Sucrose | + | + | + | + | + |
| Trehalose | nd | + | + | + | + |
| Xylose | - | + | + | - | - |

*nd= not determined

*licheniformis* MTCC 6824; *B. circulans* MTCC 7635; *B. circulans* MTCC 7636; *Bacillus* sp. MTCC 7637 and *B. pantothenticus* MTCC 7638. Several *Bacillus* strains from soils and mangrove sediments have already been reported as hydrocarbon degraders and emulsifier producers (Holguin *et al*., 2001; Macrae *et al*., 2001). Macrae *et al*. (2001) found bacilli as dominant rhizosphere organisms in mangroves and suggested that they should be targeted to provide microbial solutions which ameliorate polluted environments. In addition to phytase production as reported in the present study the strains have other characters of commercial importance. The extracellular products

of *B. licheniformis* include proteins from different functional classes, like enzymes for the degradation of various macromolecules, proteins involved in cell wall turnover, flagellum- and phage-related proteins and some proteins of yet unknown function (Voigt *et al*., 2005). It is also an industrial source of bacitracin, a medically useful antibiotic. *B. circulans* has been reported to produce starch degrading enzymes and streptomycin and it is also a plant growth promoting bacteria (PGPB) with cellulolytic properties (Hameeda *et al*., 2005) and the present finding of its phytase production is new to the strain. *B. pantothenticus* is also a halophilic bacterium capable of tolerating salt level as high as 10% and require pantothentic acid for growth, apparently unique to the genus *Bacillus.*

The characterization of different strains of *Bacillus* sp. capable of producing phytase from mangrove swamps as reported in this communication opens up an avenue for new and novel sources of the enzyme for future research and industrial application. The five strains have been typed as *B. licheniformis* MTCC 6824; *B. circulans* MTCC 7635; *B. circulans* MTCC 7636; *Bacillus* sp. MTCC 7637 and *B. pantothenticus* MTCC 7638 are deposited at IMTECH, Chandigarh, India, which is approved as a microbial repository in India (based on Berne convention, 1886).

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## References

Claus, D. and R. C. W. Berkeley. 1986. Genus *Bacillus,* Cohn 1872. *In*: Sneath P. H. A., N. S. Mair, M.E. Sharpe and J. G. Holt, (Eds.) *Bergey's Manual of Systematic Bacteriology*. Vol. 2. Baltimore: The Williams and Wilkins Co., p.1105–1139.

Common, F. H. 1989. Biological availability of phosphorus for pigs. *Nature*, 143: 370-380.

Farrow, J. A. E., S. Wallbanks and M. D. Collins. 1994. Phylogenetic interrelationship of round-spore-forming bacilli containing cell walls based on lysine and the non-spore-forming genera *Caryophanon, Exiguobacterium, Kurthia, and Planococcus*. *Int. J. Syst. Bacteriol*., 44: 74–82.

Graf, E. 1983. Calcium binding to phytic acid. *J. Agric. Food Chem*., 31: 851-855.

Greiner, R., E. Haller, U. Konietzny and K. D. Jany. 1997. Purification and characterization of a phytase from *Klebsiella terrigena*. *Arch Biochem. Biophys.* 341: 201-206.

Hameeda, B., O. P. Rupela, G. Reddy and K. Satyavani. 2005. Application of plant growth-promoting bacteria associated with composts and macrofauna for growth promotion of Pearl millet (*Pennisetum glaucum* L.). *Biol. Fertil. Soils*, 43(2): 221-227.

Holguin, G., P. Vazquez and Y. Bashan. 2001. The role of sediment microorganisms in the productivity, conservation and rehabilitation of mangrove ecosystems: an overview. *Biol. Fertil. Soils*, 33: 265-278.

Ivanova, E. P., D. V. Nicolau, N. Yumoto, T. Taguchi, K. Okamoto, Y. Tatsu and S. Yoshikawa. 1998. Impact of the conditions of cultivation and adsorption on antimicrobial activity of marine bacteria. *Mar. Biol*., 130: 545–551.

Idriss, E. E., O. Makarewicz, A. Farouk, K. Rosner, R. Greiner, H. Bochow, T. Richter and R. B. Idriss. 2002. Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth-promoting effect. *Microbiology*, 148: 2097-2109.

Kerovuo, J., J. Ruovinen, and F. Hatzack. 2000. Analysis of myo-inositol hexakisphosphate hydrolysis by *Bacillus* phytase: indication of a novel reaction mechanism. *Biochem. J.,* 352: 623-628.

Kim, Y. O., J. K. Lee, H. K. Kim, J. H. Yu and T. K. Oh. 1998. Cloning of the thermostable phytase (Phy) from *Bacillus* sp. DS11 and its overexpression in *Escherichia coli*. *FEMS Microbiol. Lett*., 162: 185-191.

Konietzny, U. and R. Greiner. 2004. Bacterial phytase: potential application, in vivo function and regulation of its synthesis. *Braz. J. Microbiol.,* [online] 2004, 35: 1-2 [cited 2007-09-15], p. 12-18. Available from: *http://www.scielo.br/scielo.php.*

Krebs, B., B. Höding, S. M. Kübart, A. Workie, H. Junge, G. Schmiedeknecht, R. Grosch, H. Bochow and M. Hevesi. 1998. Use of *Bacillus subtilis* as biocontrol agent. 1. Activities and characterization of *Bacillus subtilis* strains. *J Plant Dis. Prot.,* 105: 181-197.

Lee, D., J. Schroeder and D. T. Gordon. 1988. Enhancement of copper bioavailability in the rat by phytic acid. *J. Nutr*., 118: 712-717.

Lei, X., K. Pao, R. M. Elwyn, D. E. Ullrey and M. T. Yokoyama 1993. Supplemental microbial phytase improves bioavailability of dietary zinc to weanling pigs. *J. Nutr.*, 123: 1117-1123.

Macrae, A., C. M. M. Lucon, D.L. Rimmer and A.G. O'Donnell, 2001. Sampling DNA from the rhizosphere of *Brassica napus* to investigate rhizobacterial community structure. *Plant Soil*, 233: 223-230.

Pen, J., T. C. Verwoerd and A. Hoekema. 1993. Phytase-containing transgenic seeds as novel feed additive for improved phosphorus utilization. *Biotechnology*, 11: 811-814.

Powar, V. K. and V. Jagannathan. 1982. Purification and properties of phytase specific phosphatase from *bacillus subtilis*. *J. Bacteriol*., 151: 1102-1108.

Reddy, N. R., M. D. Pierson, S. K. Sathe and D. K. Salunkhe. 1989. *Phytates in cereals and legumes*. CRC Press, Inc., Boca Raton, Fla. 152pp.

Richardson, A. E. and P. A. Hadobas. 1997. Soil isolates of *Pseudomonas* spp. that utilize inositol phosphates. *Can. J. Microbio.,* 43: 509-516.

Richardson, A. E., P. A. Hadobas, and J. E. Hayes. 2001. Extracellular secretion of *Aspergillus* phytase from *Arabidopsis* roots enables plants to obtain phosphorus from phytate. *Plant J.,* 25: 641-649.

Simons, P. C. and H. A. J. Versteegh. 1990. Improvement of phosphorus availability by microbial phytase in broilers and pigs. *Br. J. Nutr*., 64: 525-540.

Voigt, B., T. Schweder, M. J. J. B. Sibbald, D. Albrecht, A. Ehrenreich, J. Bernhardt , J. Feesche, K. Maurer, G. Gottschalk, J. M. Dijl, M. Hecker. 2005. The extracellular proteome of *Bacillus licheniformis* grown in different media and under different nutrient starvation conditions. *Proteomics*, 6(1): 268- 281.

Yoon, S. J., Y. J. Choi, H. K. Min, K. K. Cho, J. W. Kim, S. C. Lee and Y. H. Jung. 1996. Isolation and identification of phytase-producing bacterium, *Enterobacter* sp. and enzymatic properties of phytase enzyme. *Enzyme Microb. Technol.,* 18, 449-454.

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