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Research Article

Screening, Optimization and Application of Extracellular Phytase from *Bacillus Megaterium* Isolated from Poultry Waste

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Abstract

Soil phosphatases play a major role in the mineralization processes of organic phosphorus. A phytase or myo-inositol hexakisphosphate phosphohydrolase (E.C. 3.1.3.8) effectively catalyzes the release of phosphate from phytate and phosphorylated compound. Phytase is derived from different sources such as bacteria and yeasts. The extracellular phytase producing bacteria was isolated from poultry and phytase production was screened. It was purified by ammonium sulphate fractionation of protein and dialysis followed by SDS-PAGE. The DNA from *Bacillus megaterium* was isolated using CTAB method. The maximum enzyme production was obtained in the 60th h at 37°C, pH 6.5 and 200 rpm shaking. The best carbon and nitrogen source was lactose 138 µg/mL and ammonium sulphate 124 µg/mL, respectively. The 16S rRNA gene sequencing was carried out for identification of positive bacteria. Pot experiment was carried out with *Zea mays* for phosphate utilization and accessed by root and maximum measurement was observed.

Keywords: Poultry waste, Phytase, *Bacillus megaterium*, Plant growth.

INTRODUCTION

Phytase (myo-inositol hexakisphosphate phosphohydrolase) effectively catalyzes the release of phosphate from phytate and phosphorylated compounds and is considered to be a unique type of phosphatase. In agriculture, phytase is considered to be one of the most important monogastric animal sources of nutrition because of its ability to improve phosphorus utilization efficiency significantly and its ability to lower anti-nutrients (Thomas *et al*., 2004). Phytase or myo-inosital hexaphosphate phosphohydrase (E.C. 3.1.3.8) was first discovered by (Suzuki *et al*., 2007). This enzyme can catalyze the hydrolysis of phytic acid to inositol and orthophosphoric acid. Phytic acid is the major storage form of phosphorous in cereal, oil and legume (Brian *et al*., 1999; Brinch-Pedersen *et al*., 2002) phytase, specific group of phosphatase hydrolysis phytic acid to myo-inositol and phosphoric acid.

Phosphorus is predominately stored in mature seeds as a mineral complex known as phytin. The molecule in its natural state is referred to as phytic acid. It is highly reactive and readily forms complexes with Ca, Fe, Mg, Cu, Zn, carbohydrates and proteins. These complexes are substantially less soluble in the small intestine and therefore, less likely to interact with phytase (Angel *et al*., 2000). In oilseeds and grain legumes phytin associated with protein and concentrated within sub-cellular inclusions called globoids that are present throughout the

kernel. The hydrolytic action of phytin –phosphorus has been known for some time (Dox and Golden, 1911); however, large-scale commercial production of phytases has occurred only in 1990's (Wodzinski and Ullah, 1996).

There are so many applications of phytase. Ruminants readily use phytate because of the phytase produced by rumen microorganisms. In most commercial agriculture, non ruminant livestock, such as swine, fowl, and fish, are fed mainly grains, such as maize, and legumes, such as soybeans. Because phytate from these grains and beans is unavailable for absorption, the unabsorbed phytate passes through the gastrointestinal tract, elevating the amount of phosphorus in the manure. Excess phosphorus excretion can lead to environmental problems, such as eutrophication. The bioavailability of phytate phosphorus can be increased by supplementation of the diet with the enzyme phytase. Ruminants digest phytate through the action of phytase produced by microbial flora in the rumen. The effect of feeding phytase to animals on pollution has been quantitatively determined the use of phytase as a feed enzyme sets certain demands on the properties of the enzyme (Nwanna *et al*., 2008).

Phytic acid is found within the hulls of nuts, seeds, and grains. Phytic acid has a strong binding affinity to important minerals, such as calcium, magnesium, iron, and zinc. When a mineral binds to phytic acid, it becomes insoluble, precipitates and will be non absorbable in the intestines. Enzyme phytase which catalyses the release of phosphate from phytate, hydrolyses the complexes formed by phytate and metal ions or other cations, rendering them more soluble, ultimately improving and facilitating their intestinal absorption (Wise, 1983).

MATERIALS AND METHODS

Isolation of phytase producing microorganisms

Poultry waste collected in Tambaram East, Chennai-73, Tamil Nadu, India. Serial dilution was done.10^{-9} sample was used for experiment and colonies were grown on using nutrient agar and wheat bran extract agar plates containing $(NH_4)_2SO_4$ -0.04%, $MgSO_4.7H_2O$ - 0.02%, casein - 0.1%, KH_2PO_4 - 0.05%, K_2HPO_4 - 0.04% and agar - 2%). The plates were incubated at 37°C for 24 h. The fundamental identification of the organism was done using colony morphology examination and gram staining. The pure cultures were obtained by streak plate method.

Screening for the phytase activity

The isolated pure strains were screened for the production of extra cellular phytase using phytase specific medium (Chunshan *et al*., 2001) as a screening medium [KH_2PO_4 - 5g; $CaCl_2$ - 5g; phytic acid - 5 mL; agar - 2g in 100 mL of distilled water and pH 7]. The pure cultures were streaked at the centre of the plate and the plates were incubated at 37°C for 62 h. The observation was made to see the phytase solubilisation zone around the colony. Only positive and better zone formed strain was taken for further study. The positive and better zone formed strain was streaked on nutrient agar plates. The pure cultures were retrieved every month and stored at 4°C.

Enzyme production

The inoculum for further production of enzymes and other studies was prepared using following (g/L): $(NH_4)_2SO_4$ - 0.4g; $MgSO_4.7H_2O$ - 0.2g; casein - 1g; KH_2PO_4 - 0.5g; K_2HPO_4 - 0.4g was dissolved in 1000 mL of wheat bran extract pH should be maintained 6.7. The enzyme production was carried out by Shake Flask Fermentation using Phytase production media (Lan *et al*., 2002). The sterile production broth (500 mL) was prepared and 5% inoculum was transferred aseptically into the production medium. The inoculated medium was incubated at 37°C for 62 h. The medium was placed in a shaker with 200 rpm for better aeration and growth of the organism.

Phytase enzyme assay

The presence of phytase was assayed qualitatively by plate assay method and quantitatively by chemical assay method (Quan *et al. 2001).* The plate assay was performed using Phytase specific medium (Chunshan, 2001) supplemented with 2% of agar. The well was filled with culture filtrate (test) and another well was filled with non culture filtrate and incubated at 37°C for 62 h in humid chamber. The observation was made to see the phytase solubilizing zone around the well.

Crude culture filtrate was used as an enzyme sample for the chemical assay method using ammonium molybdate method. An aliquot of 150 µL of enzyme solution was incubated with 950 µL of substrate solution (sodium

phytate 4Mm, sodium acetate buffer 0.25M and 1000 mL of distilled water pH 4.5) at 37 °C for 30 min. The reaction was stopped by adding 1 mL of 10% TCA. The released inorganic phosphate was analyzed by adding 2 mL of a coloring reagent (Ammonium molybdate 1 gm, sulfuric acid 3.2 mL ferrous sulfate 7.2 gm and 100 mL of distilled water) and the absorbance was read at 700 nm in an ultrapro-1100 spectrophotometer. A standard curve was plotted with phytate standard which served as the reference for phytase activity. Heat killed enzyme with the substrate served as control.

Optimization studies

The optimum conditions for phytase production were standardized in this investigation (Choi *et al.,* 2001).

Effect of time on phytase production

A 250 mL of sterile production medium (pH 6.5) was prepared and 5 % inoculum was added aseptically. The inoculated medium was incubated at 37°C with shaking at 200 rpm. The culture was periodically grown for 12 h intervals up to 80 h. After incubation the culture filtrate was examined for the total protein content (Bradford method) and phytase activity (Bradford, 1976).

Effect of Temperature on phytase production

The sterile production medium (250 mL) was prepared in different Erlenmeyer flask and inoculated with 5% inoculum. Each flask was incubated at different temperatures such as 27°C, 32°C, 35°C, 37°C, and 45°C for 60 h at 150 rpm. The protein content and enzyme activity were estimated.

Effect of pH on phytase production

The sterile production medium (250 mL) was prepared in different Erlenmeyer flasks and was adjusted to different pH such as 5.5, 6, 6.5, 7, 7.5, and 8 using 0.1N NaOH and 0.1N HCl. After sterilization flasks were inoculated with 5% inoculum. The flasks were incubated at 37°C at shaker at 200 rpm for 60 h. The protein content and enzyme activity were estimated.

Carbon Source

The sterile production medium (250 mL) was prepared in different Erlenmeyer flasks. Each flask was amended with different carbon sources such as glucose, sucrose, lactose, sorbitol, dextrose. The flasks were inoculated with 5% inoculum and incubated at 37°C and kept on shaker at 200 rpm for 60 h. The culture filtrate was collected and protein content and enzyme activity was determined.

Nitrogen source (organic and inorganic)

The sterile production medium (250 mL) was prepared in different Erlenmeyer flasks. Each flask was amended with 0.5% of different nitrogen sources such as peptone, beef extract, casein, urea, ammonium nitrate, and ammonium sulphate. The flasks were inoculated with 5% inoculum and incubated at 37°C and kept on shaker around 200 rpm for 60 h. The culture filtrate was collected and protein content and enzyme activity was determined.

Partial purification of phytase

The partial purification of phytase was done using Holman method (Holman *et al.,* 1943). The protein was precipitated using 70% w/v (65.8 gm in 150 mL of sample solution) ammonium sulphate. The mixture was then stored in cold room for 24 h to precipitate all the proteins. Then the precipitation was separated by centrifugation around 10,000 rpm for 10 min. The supernatant was carefully discarded and the remaining precipitate was dissolved with 2 mL of 50 mM Tris HCl (pH 8) buffer. The mixture was then subjected to dialysis.

SDS-PAGE analysis

The protein was separated using SDS-PAGE (Laemmli, 1970) to determine its molecular weight.

PCR amplification

The PCR amplification of 16S ribosomal RNA was carried out based on the methodology of Cortez-Herrera *et al.* (2008) in a Thermo cycler (PTC–100 TM Programmable Thermal Controller, USA). The following primers were used. Forward primer: 5'-AACGGCTCACCAAGGCGACG-3'; Reverse primer: 5'-GTACCGTCAAGGTGCCGCCC-3'. The PCR product was checked by agarose gel electrophoresis. The gel was visualized under UV transilluminator and photographed.

Effect of phytase on plant growth

The effect of phytase on the growth of maize plants was studied. The plants were germinated and grown for a period of 20 days in pots containing peat moss. They were

watered with 15 mL of tap water supplemented with dissolved phosphate (Test 2), phytase enzyme (Test 3) and a mixture of phosphate (7.5 mL) and phytase (7.5 mL), (Test 4). The control (Test 1) was maintained wherein the pots were watered only with 15 mL of tap water. The parameters like root length, shoot length, fresh and dry weight were measured after one month. The number of primary and secondary branching was also counted.

RESULTS

In this present investigation, a phytase producing bacterium was isolated from poultry waste. From the poultry waste around 12 bacterial species were isolated, of which four showed phytase activity.

Phytase enzyme assay

The enzyme activity was assayed using the plate and chemical assay method. Generally, during growth study, the biomass of the cells will be estimated in plate assay when the enzyme activity was identified by a clear zone (Figure 1). Further confirmation was brought out using the chemical assay technique when the resultant color change due to enzyme activity was measured spectrophotometrically at 700 nm.

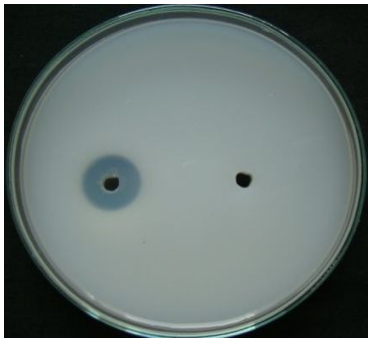

Figure 1: Enzyme activity using plate assay method

Optimization of phytase production using *Bacillus megaterium*

Effect physical parameters on phytase production

The effect of physical parameters like time, temperature and pH on phytase production was investigated. The culture filtrate was checked for enzyme activity every 12 hrs. The result revealed that the maximum production (126U/mL) of phytase was at the 60th h (Figure 2). It coincided with the peak protein content. The enzyme activity and the protein content were at its peak at 37°C (Figure 3). The optimum pH was found to be 6.5 (Figure 4).

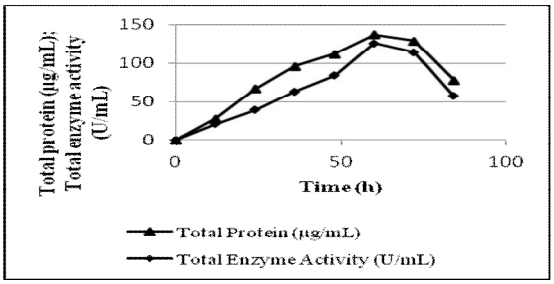

Figure 2: Effect of time on phytase production by *Bacillus megaterium*

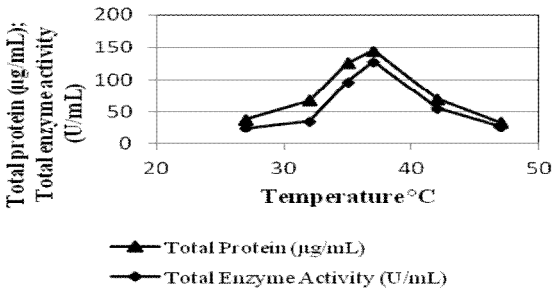

Figure 3: Effect of temperature on phytase production by *Bacillus megaterium*

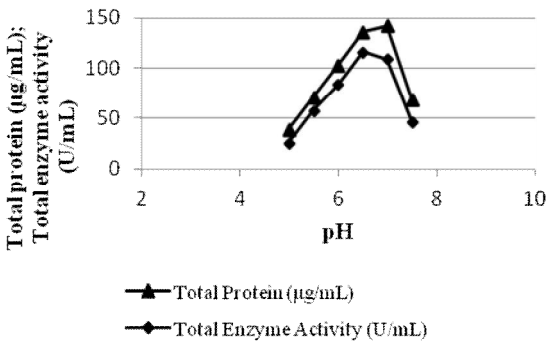

Figure 4: Effect of pH on phytase production by *Bacillus megaterium*

Effect chemical parameters on phytase production

The effect of chemical parameters like carbon and nitrogen source was also studied. The maximum enzyme activity was detected when fructose (116 U/mL) was used as carbon source. This was followed by lactose, dextrose, sorbitol, starch and sucrose (Figure 5). The best nitrogen source was found to be ammonium sulphate (102 U/ mL) (Figure 6).

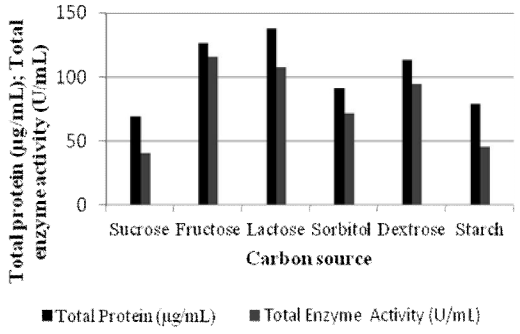

Figure 5: Effect of carbon source on phytase production by *Bacillus megaterium*

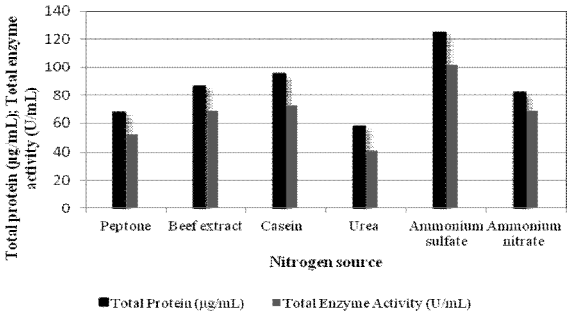

Figure 6: Effect of nitrogen source on phytase production by *Bacillus megaterium*

PCR amplification of the DNA

The genomic DNA from *Bacillus megaterium* was isolated by CTAB method and the sample was run in 0.7% agarose gel. The genomic DNA was found to be 1100 bp in size (Figure 7). The PCR product of the 16S rRNA of *Bacillus megaterium* was also analyzed.

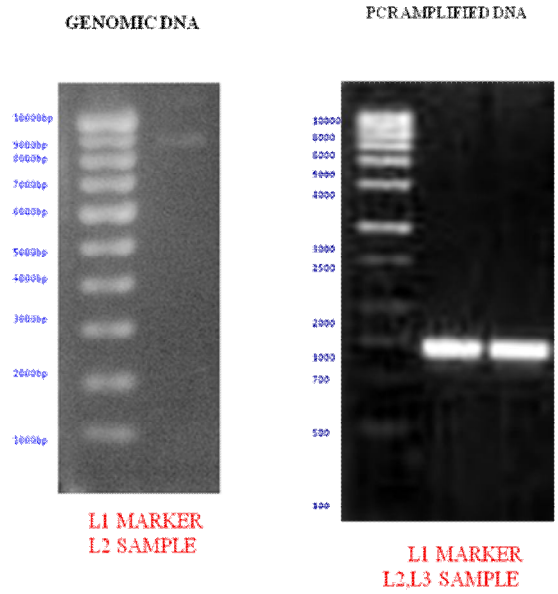

Figure 7: Genomic DNA and PCR product of 16S rRNA of *Bacillus megaterium*

Molecular identification of for *Bacillus megaterium*

The extracellular phytase produced for *Bacillus megaterium* was ascertained by its systematic position based on 16S rRNA sequence analysis and with the aid of computational programme. BLAST homology analysis was also carried out to compare with other 16S rRNA sequences available in the GenBank of NCBI. The sequence analysis of 16S rRNA gene for the isolate *Bacillus megaterium* shows the maximum homology (97%) with other *Bacillus megaterium* from the database.

Effect of phytase on plant growth

The maize plants were subjected to different treatments to study the effect of phytase on its growth parameters. The results in Table 1 clearly show that phytase promotes growth in the treated plants. The combination of phytase and phosphate (Test 4) increased all the growth parameter analyzed. All together, the test 4 showed exponential growth when compared to the control and all the other tests.

The phosphate solubilizing bacteria are widely distributed and in this present investigation phytase was isolated from *Bacillus megaterium* which was isolated from poultry waste. These organisms help in the translocation of

Table 1: Effect of phytase on the growth of maize (*Zea mays*)

Test no	SHOOT SYSTEM				ROOTS SYSTEM			
	Total length (cm)	Shoot Length (cm)	Shoot weight (gm)	No of leaves	Roots length (cm)	Roots weight (gm)	No of lateral roots	Total weight (gm)
Test 1	51.6	35.4	1.95	5	16.2	1.57	8	3.52
Test 2	55.9	37.2	2.47	5	18.7	1.72	11	4.19
Test 3	62.4	42.6	3.37	6	19.8	2.37	16	5.74
Test 4	92.5	63.8	6.08	7	28.7	6.12	21	12.20

nutrients to the plants. The current isolate *Bacillus megaterium* produced maximum phytase activity when incubated at 37°C. The temperature and pH requirement of the organism is based on the nature of environment where they grown. The genus of *Bacillus megaterium* isolated from the poultry sample has shown higher production of extra cellular phytase enzyme at pH 6.5.

DISCUSSION

Naturally microorganisms are having ability to produce the variety of industrial enzymes like amylase, protease, cellulase, lipase, pectinase, phosphatase, etc., and most of the enzymes are industrially important and human welfare. *et al.*, 2008) and Maize (Ponmurugan and Gopi, 2006). In higher plants, phytases occur predominantly in grains, seeds and pollen (Konietzny and Greiner, 2002), where they are responsible for phytate degradation during germination to make phosphate, minerals, and *myo*-inositol available for plant growth. In this present investigation about the phytase bacterial species were isolated from poultry waste (Pai et al., 2003); the sample was collected from Tambaram East Chennai-73 Tamil Nadu India.

Bacillus megaterium is a rod-shaped, Gram-positive, endospore forming; species of bacteria used as soil inoculants in agriculture, horticulture and also produces penicillin amidase used for making penicillin. It is effective and economical bio inoculants to use in the integrated nutrient and pest control system. It is considered aerobic, but, it is also capable of growing under anaerobic conditions when necessary. The chemical assay method was followed as per the method of Quan *et al, .2001* where the phytate concentration was analyzed spectrophotometrically at 700 nm.

After 60^{th} hrs, the production level of enzyme has reduced significantly reduced. These results clearly indicated that the isolate has maintained its lag phase before 12^{th} hrs of its growth and should have maintained its log phase from around 12^{th} hrs to 60^{th} hrs, because the considerable and peak production has occurred in this duration. The current isolate *Bacillus megaterium* produced maximum phytase activity when incubated at 37°C. The temperature requirement of the organism is based on the nature of environment where they grown. The pH is the important parameter which determines the growth of the organism and phytase production. The genus of *Bacillus megaterium* isolated from coastal region has produced the extra cellular phytase at pH 9 (Marion hulett *et al.*, 1990) and in contrast, but the *Bacillus megaterium* isolated from the soil sample has shown higher production at pH 6.5.

Phytase is one of the important enzymes, which can be produced from various microorganisms like fungi, actinomycetes and bacteria. Phytate solubilizing microbes have been routinely isolated from poultry waste soil of various plants such as wheat (Ahmad

The carbohydrates are essence energy source for most of heterotrophic organisms. In many of the other enzyme, the production will be carried out by medium amended with glucose as a general carbon source for better growth and production. But the isolate has produced higher quantity of phytase from Fructose. The nitrogen sources are of secondary energy sources for the organisms, which play an important role in the growth of the organism and the

production. The nature of the compound and the concentration that we are using may stimulate or down modulate the production of enzymes. The inorganic nitrogen source Ammonium sulphate found to be a better nitrogen source for this isolated bacterial culture (Choi et al., 2001).

The sequence analysis of 16S rRNA gene for the isolate *Bacillus megaterium* showed the maximum homology (97%) with more than 10 other *Bacillus megaterium* from the database. A pot experiment for phytate utilization in maize (*Zea mays* .L) was made in surface sterilized pots.

The control pot plant growth showed normal whereas compared with experimental group Test 4 (phosphate + phytase). All together, the test 4 showed exponential growth than the all other group (Adekayode *et al*., 2010).

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