

# Bacillus cereus 10072 Phytase - Detection, Purification, Characterization and Physiological Role

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**Abstract**— Phytase from *Bacillus cereus* MTCC 10072 was purified about 10.75 fold to apparent homogeneity with a recovery of 34% referred to the phytase activity in the crude extract. The monomeric enzyme displayed molecular weight of 45 KDa and showed maximum activity at temperature 60 °C and pH 6.5. Iso electric point of the purified enzyme was found to be 5.6. Substrate specificity studies showed it is highly specific to its substrate and maximum relative activity of 128% was obtained with calcium phytate. Activity was unaffected or moderately stimulated by a range of metal ions with only Ca<sup>2+</sup> exerting (118%) stimulatory effect. The enzyme is significantly thermo stable at 60 °C and retains a significantly greater proportion of maximal activity at physiological temperatures. This may render it of industrial interest. Further to check the applicability of the enzyme effect of different doses of crude enzyme (10, 25, 50 and 100 units) in dephosphorylation of animal feed was evaluated. Up to 66 h of incubation, the animal feed was monitored for the released inorganic phosphate content present in the feed. An enzyme dose of 100U and 50U of crude phytase enzyme per flask were found suitable to liberate enough amount of inorganic phosphorus in case of poultry and pig feed respectively.

**Key words:** Phytase, Iso electric focusing, *Bacillus cereus*, Enzyme characterization, Animal feed.

## I. INTRODUCTION

Phytases (myo-inositol hexakisphosphate hydrolases, EC 3.1.3.8 and EC 3.1.3.6) are histidine acid phosphatases that catalyze the stepwise release of phosphate from phytic acid [1]. Interest in phytase enzyme has increased in recent years due to their importance in several industrial processes. Mono-gastric animals like pigs and poultry have very low or no phytase in their digestive tracts so they cannot utilize the phytic acid present in plant based feed & the unutilized phytate is excreted in the feces. Moreover, phytic acid is an anti-nutrient, which complexes with protein and a variety of divalent metallic cations and decreases the availability of these nutrients. To overcome this difficulty the feed has to be supplemented with inorganic phosphate to meet the nutritional requirements of the animals [2,3]. The addition of phytase to feed improves phosphorus utilization in both ruminants [4] and monogastric animals [5], reducing the need for supplemental inorganic phosphate and the environmental problems that arise from organic phosphate excretion [6]. The present usage of phytase as feed enzymes by poultry producers are substantially greater than anticipated when they were first introduced. On the basis of pH profile, higher thermo stability, and strict substrate specificity as well as physiological nature of phytate in digestive tract, alkaline phytases from *Bacillus* sp. are considered ideal candidates for application in animal feed [7]. The aim of the current study was to purify and characterize a bacterial phytase which is thermo stable and

displays better thermal and pH stability and narrower substrate specificity while retaining high enzymatic activity at physiological temperatures and pH values associated with the feed processing of the animals.

## II. EXPERIMENTAL DETAILS

### A. Organism and Growth

Bacteria isolate used in this work was isolated from distillery waste in the laboratory and was identified from IMTECH Chandigarh as *Bacillus cereus* MTCC 10072. Culture was grown in a 250 ml flask containing 50 ml basal medium. The medium was inoculated with 1% of 24 h old inoculum.

### B. Enzyme assay

Crude and purified phytase was assayed by measuring the amount of inorganic phosphate released spectrophotometrically according to method described by Heinonen and Lahti [8]. One unit of phytase is defined as the amount of enzyme that liberates 1 μmol inorganic phosphate ml<sup>-1</sup> min<sup>-1</sup> under the standard assay conditions. Protein was estimated by Bradford's method [9] using BSA as standard.

### C. Enzyme Purification and Characterization

The culture was grown under optimized culture conditions and the enzyme extract was recovered by centrifugation (10,000g, 10 min). To the extract was added finely powdered AR grade ammonium sulfate to 80% saturation at 4 °C, followed by overnight incubation at -20 °C. The concentrated sample was purified by gel filtration chromatography using Sephadex G-100. Phytase was further purified by ion exchange chromatography using DEAE-Sephacel column (1.5 × 28 cm, bed volume 20 ml). Fractions containing phytase activity were pooled for biochemical characterization.

### D. Determination of molecular weight and isoelectric point of enzyme

Molecular weight was determined by using SDS-PAGE using 10% w/v polyacrylamide gel according to Laemmli [10] and Isoelectric point of the purified enzyme was determined using IPG phore III unit (GE healthcare) and SDS-PAGE (10% polyacrylamide gel), respectively.

### E. Characterization of purified phytase

The purified phytase enzyme was characterized with respect to its optimum pH (3-9), temperature (20-100°C), effect of different metal ions and salts such as K<sup>+</sup>, Co<sup>2+</sup>, Mn<sup>2+</sup>, Mg<sup>2+</sup>, Fe<sup>3+</sup>, Cu<sup>2+</sup>, Ba<sup>2+</sup>, Zn<sup>2+</sup>, Cd<sup>2+</sup>, Na<sup>+</sup> and Hg<sup>2+</sup> and ionic detergent such as sodium dodecylsulphate (SDS); non-ionic detergents like Triton X-100, Tween 80, Tween 20, inhibitors like EDTA, oxalate, citrate, sodium azide, β-mercaptoethanol and additives such as glycerol, urea and potassium iodide at three different concentrations i.e. 0.2,

0.5 and 1.0 mM on the enzyme activity was studied at 37 °C and pH 6.5. The substrate specificity of the enzyme was tested against different type of phosphate substrates (0.5% w/v), i.e. Na-phytate, Ca-phytate, AMP, ADP, ATP, *p*-nitrophenyl phosphate and glucose-6-phosphate.

#### F. Temperature and pH stability profile of the enzyme

The thermo stability of phytase activity was checked by incubating the enzyme at over two different temperatures 60° and 70 °C in the presence and absence of 5mM CaCl<sub>2</sub>. Similarly, for checking pH stability, the enzyme was subjected to different pH from 2.0 to 10.0 using glycine-HCl (pH 2.0 and 3.0), sodium acetate (pH 4.0–6.0), Tris-HCl (pH 7.0 and 8.0), and glycine-NaOH (pH 9.0 and 10.0) buffers (0.15M) in the presence and absence of CaCl<sub>2</sub> for 30 min at room temperature and then assayed at 60 °C (pH 6.5).

#### G. Dephytinization of animal feed

For application in dephytinization of animal feed (pig and poultry) culture of *Bacillus* MTCC 10072 grown in modified nutrient broth media containing phytate. Partially purified phytase by 80% ammonium sulphate cut and concentrated through dialysis was used for the experiment.

#### H. Preparation of feed sample for testing

Animal feed prepared in the laboratory as discussed above was dried in oven at 50-60 °C. For assessing the applicability of enzyme on the hydrolysis of insoluble phytate salts present in the feed, 10 g of the feed was suspended with different units of crude phytase enzyme (10, 25, 50 and 100) pH 6.5 and the mixture were incubated at 37 °C for 66 h at 150 rpm, and samples were withdrawn after every 3 h and centrifuged at 10,000 rpm for 20 min. Supernatant was then tested for the amount of inorganic phosphate liberated according to Fiske and Subbarow [11]. The control set were prepared by mixing the feed with distil water and were run simultaneously.

### III. RESULTS AND DISCUSSION

#### A. Purification of phytase

Conventional purification methods were effective to purify phytase, enzyme was purified using 80% ammonium sulphate cut followed by Sephadex G-100 and DEAE Sephadex (Fig. 1) followed by elution with NaCl gradient corresponding to enzyme purification of 10.75-fold over the culture supernatant with a yield of 34%. The phytase from *A. ficuum* NTG-23 was purified 71.5-fold from the crude extractive with 23.8% yield [12]. The purification factor and yield are related to elution procedure. A phytase from *Bacillus* sp. KHU-10 which had an estimated molecular weight of 44, 33.8, 36, and 44 KDa [14-16, 12] respectively. The purified enzyme from *Bacillus* sp. MD2 had a molecular weight of 47.5 KDa [17].

The purified enzyme aligned with SDS low molecular weight markers (Sigma, USA) gave molecular weight of approximately 45 KDa (Fig. 2). This is in correlation with earlier reports on the purified phytase from *B. subtilis*, *B. subtilis* (natto) N-77, *B. Amyloliquefaciens*, *Bacillus* sp. KHU-10 which had an estimated molecular weight of 44, 33.8, 36, and 44 KDa [14-16, 12] respectively. The purified enzyme from *Bacillus* sp. MD2 had a molecular weight of 47.5 KDa [17].

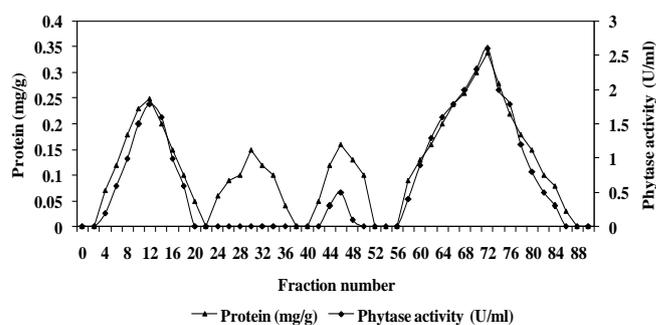


Fig. 1: Gel filtration chromatography of the purified enzyme.

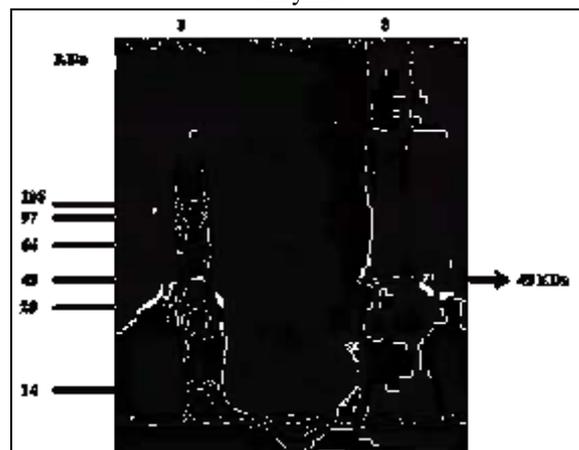


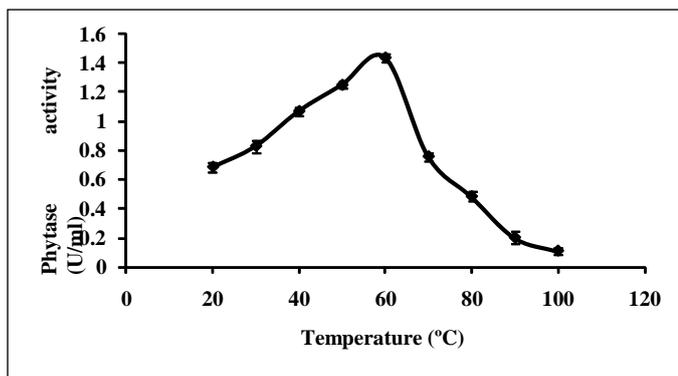
Fig. 2: 10 % SDS-PAGE analysis of purified protein. Lane 1: Molecular weight markers including β-galactosidase (116 KDa), phosphorylase b (97 KDa), serum albumin (66 KDa), Ovalbumin (45 KDa), Carbonic anhydrase (29 KDa), lysozyme (14 KDa). Lane 2: Purified phytase enzyme.

Isoelectric focusing gave one major band corresponding to a pI of about 5.6. These results indicate that the phytase could be regarded as homogeneous. All of the fungal, bacterial and plant phytases reported so far have an acidic pI values with the exception of *A. fumigatus* phytase, which has a pI of about 8.6 [18]. *B. subtilis* has a pI value between 6.3-6.5 [19]. The phytase of *Sporotrichum thermophile* has a pI value of 4.9 [20]. *Y. kristeensenii* phytase is a secreted protein with a pI of 6.17 [21].

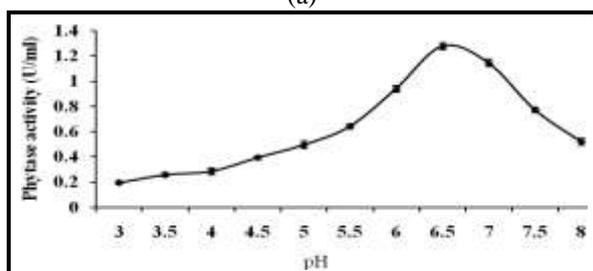
#### B. Characterization of the purified enzyme

Effect of pH, Temperature, Thermal and pH stability:

The effects of pH and temperature on the enzymatic activity of purified phytase were investigated (Fig. 3 a & b). Purified phytase showed maximum activity at 60 °C with 52% and 34% residual activity at 70° and 80 °C respectively. There was no activity at 100 °C (Fig. 3a). Phytase in general, shows high activity in the temperature range of 50–70 °C while optimum temperature is mostly between 45 and 60 °C. The optimum temperature of current phytase enzyme from *Bacillus cereus* isolate MTCC 10072 was exhibited at 60 °C, which is very similar to *A. ficuum* NTG-23 phytase (60 °C) [22] and *Kodamaea ohmeri* phytase (65 °C), [23] and higher than that of *A. niger* NRRL 3135 phyA (58 °C), [24] *A. niger* van Teighem phytase (52–55 °C) [25] and *S. cerevisiae* phytase (40 °C) [26]. Findings with other enzymes also correlate with the present study and it has been reported that the optimum temperatures of alkaline proteases from bacteria range from 50 to 70 °C [27].



(a)



(b)

Fig. 3: Effect of (a) temperature and (b) pH on the catalytic activity of phytase from *B. cereus*-NSD. Results are the average of experiments performed in triplicates. Vertical bars show standard deviation.

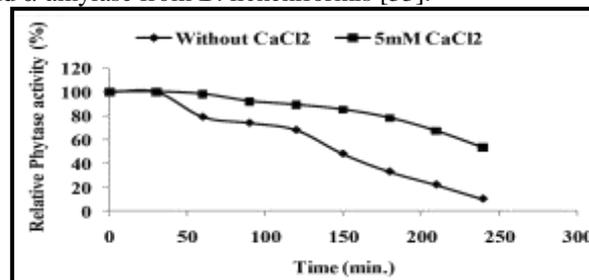
Maximum phytase activity was observed at pH 6.5 followed by pH 7.0 and 7.5. There was little activity at acidic pH (Fig. 3b). Generally, the phytases from the bacterial source have optimum pH in neutral to alkaline range while in fungi, optimum pH range is 2.5–6.0 and the stability of phytase decreased dramatically above pH 7.5 and below pH 3.0. This wide range of differences in pH optima could be due to the variation in molecular conformation or stereo-specificity of the protein from different sources. Phytase from *Bacillus* sp. KHU 10 showed maximum activity between pH 6.0 and 8.0 [13], *Bacillus licheniformis* (168 phyA, phyL) had an optimum pH between 4.5–6.0 [28], while enzyme from *Bacillus* sp. MD2 was optimally active between pH 6–7 [17].

### C. Effect of temperature and pH on enzyme stability

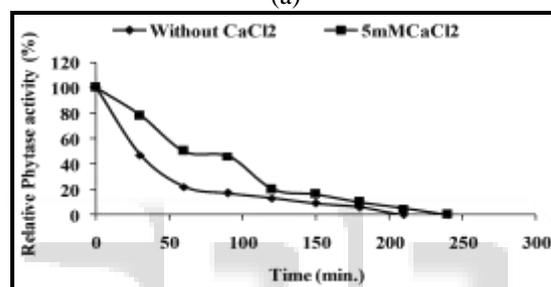
In order to study the thermo stability, the enzyme was exposed to different temperatures (60 and 70 °C) for varying time intervals and then assayed at 60 °C. The enzyme was found to be stable at 60 °C up to 98%, 89% and 78% of initial activity for 1, 2 and 3 h respectively in the presence of 5 mM CaCl<sub>2</sub> while in the absence of calcium ions enzyme activity starts degrading after 1h. In the absence of Ca<sup>2+</sup> ions enzyme activity was 79%, 68%, and 33% of initial activity after 1, 2 and 3 h of incubation (Fig. 4a).

At 70 °C, enzyme lost activity suddenly even in the presence of calcium ions. Enzyme activity remained 78%, 50% and 46% of initial activity after 30, 60 and 90 min respectively in the presence of Ca<sup>2+</sup> ions while it was 47%, 22% and 17% after 30, 60 and 90 min respectively in the absence of Ca<sup>2+</sup> ions (Fig. 4b). Thermo stability is prerequisite for the successful application of enzymes in animal feed which are exposed to 60–90 °C during pelleting process. Simon and Igbasan [29] have reported that the stability of *Bacillus* phytase is strongly dependent on the

presence of calcium ions. The polyols such as trehalose, sorbitol and glycerol and salts like CaCl<sub>2</sub> have the ability to maintain solvophobic interaction and have the capacity to form H-bonds that play a key role in supporting the native conformation of the protein, thereby, aiding in protein stability [30]. The observed stability of phytase from *B. cereus* was higher than the reported phytase activity between 50-60 °C for *Enterobacter* sp.4 [31], 60 °C for *B. subtilis* (natto) N-77 [15]. Calcium dependent thermo stability is also reported for other secreted *Bacillus* sp. strain EA.1 [32], and α-amylase from *B. licheniformis* [33].



(a)



(b)

Fig. 4 Thermal stability of *B. cereus*-NSD phytase at (a) 60 and (b) 70 °C.

The phytase was found to be fairly stable between pH 4.0 and 9.0 in the presence of 5mM CaCl<sub>2</sub> while in the absence of CaCl<sub>2</sub>, enzyme lost its stability with only 69% of residual activity at pH 6 (Fig. 5). Phytase from *Bacillus cereus* was found to be highly stable at pH 6.5 in the presence of CaCl<sub>2</sub> and was less stable at acidic pH. The purified enzyme from *A. niger* displayed activity over a wide range of pH values with maximum activity reported at pH 5.0 [22]. The major commercialized phytase preparations are essentially devoid of activity at or above pH 6.0, automatically rendering them inactive in the small intestine. In contrast, the *B. cereus* enzyme retains almost 89% of optimal activity at pH 6.0. This pH profile suggests that, unlike the commercial products, the enzyme would retain activity within the small intestine, particularly within its upper region. The pH of monogastric chime ranges from just under 6.0 to 7.8, with a mean of 6.8 by mid-duodenum.

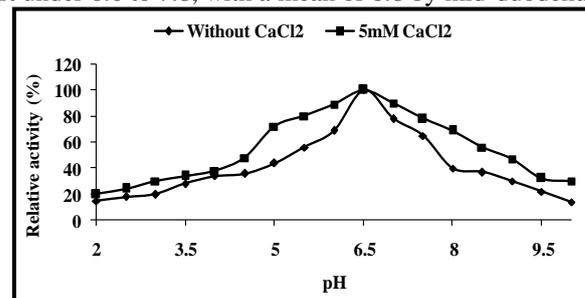


Fig. 5: pH stability of purified enzyme.

#### D. Effect of chemical agents and surfactants on purified phytase activity

Phytase activity of the purified enzyme was affected to different extent in the presence of different concentrations of additives. EDTA,  $\beta$ -mercaptoethanol, SDS, urea at a concentration of 1mM totally inhibits the enzyme activity. Citrate, sodium azide and potassium iodide also severely affects the enzyme activity and at a conc. of 1mM it remains 15% (0.21 U/ml), 22% (0.308 U/ml) and 28% (0.392 U/ml) of initial activity respectively. Phytase from *Eupenicillium parvum* BCC17694 [34] and *A. niger* van Teighem loses 92% of its activity at 1% SDS [25]. This inhibition may be due to the interactions between the negatively charged detergent and the positively charged active site of the enzyme, leading to the unavailability of active site for substrate. At 0.2 mM concentration, 15% of initial activity was remained in case of EDTA and sodium azide (47%),  $\beta$ -mercaptoethanol (51%), Toluene (52%) and TritonX-100 (58%) moderately inhibits the activity while in the presence of glycerol (70%), Tween-20 (85%), Tween-80 (65%) and oxalate (66%) enzyme activity was not very much affected. *B. cereus* isolate MTCC 10072 phytase was highly resistant to Tween-80, a type of polysorbate normally used as emulsifier in medicinal products. The chelating agent, EDTA, inhibit enzyme activity, suggesting that the enzyme require metal ions for its activity which is similar to most alkaline phytases which require  $\text{Ca}^{2+}$  [35]. The detergents not only facilitate oil drop dispersion but can also bind to protein and induce structural changes that appear to be stimulatory in case of non ionic (Tweens) and inhibitory in case of anionic (SDS) detergents. Chaotropic agents like DTT, urea and potassium iodide also significantly inhibited phytase activity and only 33%, 32% and 42% of initial activity remained at a conc. of 0.2mM, suggesting the role of non-covalent forces, such as H-bonds and van der waals interactions, in maintaining the active conformation of the enzyme [20,25,35]. Maximum stability was obtained in case of non-ionic detergent, Tween-20, Tween-80 and additive glycerol with 85% (1.2 U/ml), 65% (0.924 U/ml) and 70% (0.996 U/ml) of initial activity retained at a conc. of 0.2mM. The positive charge on these might be the cause of increased phytase activity. This effect could be due to the hydrophobic nature of the detergent that seems to promote an interaction between enzyme and substrate.

#### E. Effect of metal ions and salts on catalytic activity of enzyme

Metal ions play an important role in the catalytic activity of the enzymes and most of the enzymes are dependent on metal ions for maximum catalytic activity. In the present work, phytase activity was completely inhibited in the presence of cations  $\text{Co}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{Hg}^{2+}$  (1mM) in the reaction mixture. None of the cations except calcium ions had a stimulatory effect on phytase activity. Phytase of *Bacillus* sp. DS11 was strongly inhibited by  $\text{Cd}^{2+}$  and  $\text{Mn}^{2+}$  and moderately inhibited by  $\text{Hg}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Ba}^{2+}$  and  $\text{Cu}^{2+}$  at 5 mM concentrations [16]. The phytases from *B. subtilis* [15, 19] and *B. amyloliquefaciens* [16] were also reported to be  $\text{Ca}^{2+}$  dependent which under alkaline conditions formed metal-phytate complex with divalent metal cations. Zinc and copper ions are reported to inhibit phytase activity of several fungi such as *A. japonicus*, *A. niger* [36] and *Sporotrichum*

thermophile [20]. The inhibition could be due to the formation of insoluble complex between phytate and metal ions, leading to a decrease of available substrates for the enzyme [37]. Results of the present study are also in correlation with finding by others where alkaline proteases from bacteria require a divalent cation such as  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Mn}^{2+}$  or a combination of these cations for maximum activity [27].

#### F. Application in Animal Feed

The phytic acid/phytate present in the pig and poultry feed was hydrolyzed by phytase of *B. cereus* isolate MTCC 10072 efficiently that led to the liberation of inorganic phosphate and dephytinization of the animal feed. The hydrolysis rate of insoluble phytate was different with different enzyme units and in each case released inorganic phosphate was tested. There was a linear response in inorganic phosphate release in case of flasks with 10 and 25 enzyme units with maximum release of 5.93 and 7.57 mg/g respectively in case of poultry feed. Likewise poultry feed same trend was observed in case of pig feed regarding 10 and 25 enzyme units with maximum value of 7.35 and 8.08 mg/g respectively. But the results were totally different with 100 enzyme units when a linear response in inorganic phosphate release was observed with a maximum 9.22 mg/g of inorganic phosphate release was observed after 54 h of incubation. This may be due to the saturation of enzyme-substrate complex after a particular enzyme dose and higher amount doesn't show any marked difference. Difference could be due to difference in composition of pig feed and poultry feed.

These results are in correlation with the others where the results confirmed that the relationship between phytase additions and improvement in digestibility was not linear [38-40]. The *Bacillus* phytase is very suitable to be used in animal feed particularly in pig and poultry feed because of its excellent pH and thermal stability. Zeng et al. [41] reported that *Bacillus* phytase supplementation of 300 U/kg could gain the same result as that of 1,000 U/kg supplementation of acidic phytase and neutral phytase supplementation of 1,000 U/kg could replace the inorganic phosphorus supplement. Moreover, a combination of *Bacillus* phytases and other acidic phytases might induce a more effective hydrolysis of phytate in both the stomach and small intestine of animals in terms of the pH of the animal gastrointestinal tract [42].

#### IV. CONCLUSION

The biochemical properties of the *B. cereus* enzyme, particularly its thermo stability, stability under simulated digestive tract pH and likely ability to retain activity at pH present in the upper part of the small intestine as well as in the stomach, suggests it could potentially be a particularly suitable enzyme to include in second generation phytase cocktails for application in animal feed. Phytase from *B. cereus* isolate MTCC 10072 has been found to be efficient in utilizing phytate present in the animal feed and in dephytinizing of different substrates and agro-wastes. An enzyme dose of 100U and 50U of crude phytase enzyme per flask were found suitable to liberate enough amount of inorganic phosphorus in case of poultry and pig feed respectively. Ultimately however this could only be

confirmed by direct animal feed trials.

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