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Effect of varying doses of phytase enzyme from a novel strain of *Bacillus cereus* MTCC 10072 in animal feed

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Abstract
The present usage of phytase feed enzymes by poultry producers are substantially greater than anticipated when they were first introduced. Increasing ecological concerns in relation to P pollution, a better appreciation of the application of microbial phytases, and their decreasing inclusion costs, has contributed to this increasing acceptance. During the past 15 years, research on the evaluation of microbial phytases in diets for simple-stomached species has rapidly expanded, but much of the focus of this research has been on the evaluation of various phytases from different sources rather than the investigation of the underlying factors causing variability in phytase responses. Fundamental information in respect of phytate and phytase is lacking in many aspects, which needs to be generated and integrated for a more complete understanding of this subject. At the close of the 20th century, annual sales of phytase as an animal feed additive were estimated to be $500 million. Evolution of the market for this feed additive can be attributed to a chain of events during the late 20th century that created the demand for the enzyme, and thus, provided a means for its commercial development (Abelson 1999).
In the present study extra cellular phytase of *Bacillus cereus* was produced in nutrient broth medium in shake flasks at 37◦C for 72 h at 150 rev/min. 80% ammonium sulphate saturated and dialyzed enzyme was taken as crude phytase enzyme. Different enzyme doses viz 10, 25, 50 and 100 units were applied on animal feed (pig and poultry) and released phosphorus was estimated to check the efficacy of the enzyme.

Keywords: Incubation time, Phytase, *Bacillus cereus*, Animal feed, Inorganic phosphorus.

Introduction
Phytic acid, an organic form of phosphorus, myo-inositol hexakis-dihydrogen phosphate (IP6), is the primary source of inositol and storage form of phosphorus in plant seeds that are used as animal feed ingredients (oilseed meals, cereal grains and legumes) (Dahiya *et al* 2009). Most foods of plant origin contain 50–80% of their total phosphorus as phytates (Harland & Morris 1995). Phytic acid acts as an anti-nutritional factor by chelating various metal ions such as Ca^{+2}, Mg^{+2}, Zn^{+2}, and Fe^{+2}. Under gastrointestinal pH conditions, insoluble metal phytate complexes are formed which make the metals unavailable for absorption in the intestinal tract of animals and humans (Maga 1982). Phytates reduce digestibility of proteins, starch and lipids by forming insoluble complexes, and inhibits the action of certain enzymes such as amylase, trypsin, acid phosphatase and tyrosinase (Harland & Morris 1995). Phytic acid can be reduced by several methods like cooking, autoclaving, ion exchange etc. but with a loss of nutritional quality in the feed. So an efficient alternative to these methods is the enzymatic treatment of the feed with phytase enzyme which catalyses the stepwise release of inositol derivatives from the phytate making it available to the animals. Because of its multi-faceted properties, phytate is also a topic of great interest in human nutrition, medical science, food and feed technology, plant physiology and plant breeding (Feil, 2001). This has been fortuitous for poultry researchers, as this interest has generated a wealth of relevant information. During the last 10 years there has been an escalating usage of microbial phytase in pig and poultry diets, cascades of scientific publications, increasing field experience, and the introduction of new phytase feed enzymes (Zyta *et al*., 2001; Ciofalo *et al*. 2003; Chantasartrasamee *et al*. 2005; Vohra & Satyanarayana 2006). The enzyme phytase is a novel and cost effective tool in poultry and swine diets that improves phosphorus utilization from phytin, the storage form of phosphorus in feedstuffs. As phosphorus retention is still far below a hypothetical maximum of 100%, considerable room for improvement in phytin-phosphorus release and overall phosphorus retention by poultry and swine still exists. The main focus herein is to study the use of microbial phytase in animal nutrition in relation to phosphorus utilization and the extra-phosphoric effects of phytase, with an emphasis on pig and poultry feed. The purpose of this study was to investigate the efficiency of extra cellular bacterial phytase in the animal feed and the extent of the inorganic phosphate released at different time intervals with different doses of enzyme. Dephytinization of animal feed with increased level of released phosphorus would be beneficial and attractive for more instructive research in future on the animals.

Materials and methods
Bacterial strain
The bacterial strain was isolated from distillery effluent, Hisar and was identified as *Bacillus cereus* MTCC 10072 (Dahiya and Singh, 2014). The strain was grown on nutrient agar slants at 37 °C for 2 days, and preserved at 4C in a refrigerator. The culture is deposited at the Microbial Type Culture Collection

(MTCC), Institute of Microbial Technology (IMTECH), and Chandigarh, India.

Cultivation of bacteria

The bacteria was cultivated on the nutrient broth media, [(%w/v): nutrient broth, 1.3; glucose, 15.0; NH_4NO_3, 5.0; $MgSO_4.7H_2O$, 0.5; $MnSO_4.4H_2O$, 0.001; $FeSO_4.7H_2O$, 0.001 pH 7.0] at 37 °C. The culture filtrate containing phytase activity was subjected to ammonium sulphate precipitation (up to 80% saturation) and the concentrated enzyme was dialyzed and used in the animal feed experiments.

Preparation of feed sample for testing

Animal feed like pig feed and poultry feed were brought from local animal feed shop. Feed was thoroughly washed under tap water and then with distill water to remove excess of phosphorus present in the feed. The feed was dried in oven till no moisture content remained. Four flasks of each feed and one control were taken for experiment. For assessing the applicability of enzyme on the hydrolysis of insoluble phytate salts present in the feed, 10 gm of the feed was suspended with different units of crude phytase enzyme (10, 25, 50 & 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 hr and centrifuged at 10,000 rpm for 20 min. Supernatant was then tested for the amount of inorganic phosphate liberated. The controls were prepared by mixing the feed with distil water and were run simultaneously.

Determination of Inorganic phosphate released

Inorganic phosphate liberated was estimated according to a method described by Fiske and Subbarow (1925). To 1ml of the sample, 1 ml of distilled water was added followed by the addition of 0.5 ml 9N H_2SO_4. The mixture was allowed to stand for 15 min, and then 0.5 ml ammonium molybdate was added, vortexed and incubated for 15 min in dark. This was followed by the addition of 0.5 ml ferrous sulfate solution. The contents were thoroughly mixed, incubated in dark for 30 min and absorbance was recorded at 660 nm. One unit (U) of phytase activity was defined as the amount of enzyme required to liberate 1 µmol of inorganic phosphate per min under the assay conditions.

Results

The phytic acid/phytate present in the pig & poultry feed was hydrolyzed by phytase of *B. cereus* 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.

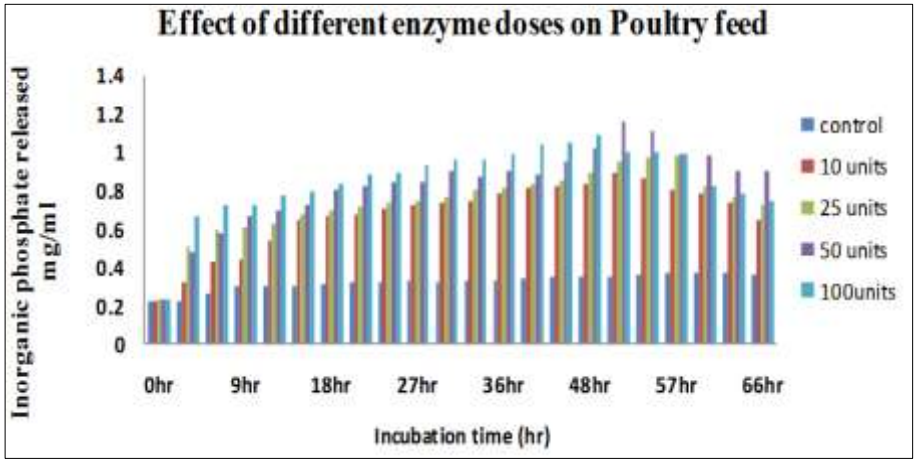

Fig 1: Effect of different enzyme doses on Poultry feed

In case of poultry feed there was a gradual increase in the liberation of inorganic phosphate up to 50-55 hr followed by stabilization & decline, which could be due to denaturation of the enzyme and/or end product inhibition. Maximum inorganic phosphate released was in case of flasks with 50 units of enzyme.

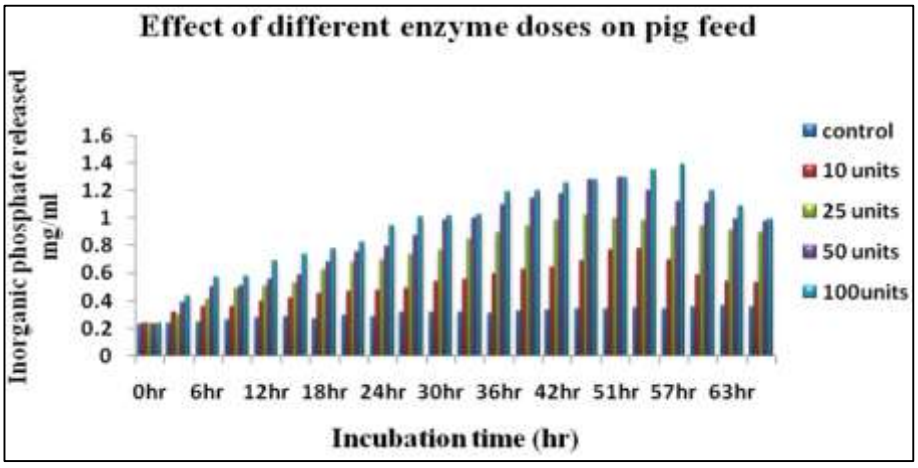

Fig 2: Effect of different enzyme doses on Pig feed

In case of pig feed 100 units of crude enzyme showed maximum release of inorganic phosphate followed by 50 units. There was an increase in the liberation of inorganic phosphate for all the enzyme units with the incubation time followed by stabilization & decline, which could be due to saturation of the enzyme substrate complex with time and/or end product inhibition.

Similarly, *A. carbonarius* phytase reduced the phytic acid content of canola meal (Al-Asesh & Duvnjak 1995). Supplementation of the animal feed with *A. niger* & *A. Fumigatus* phytases resulted in the liberation of inorganic phosphate (Wyss *et al*., 1999). The yeast *Schwanniomyces occidentalis* could be used to produce protein-rich feedstuff free of phytic acid, and phytates were removed from wheat bran and glandless cotton flour using the phytase of *S. castellii* at pH 4.4 and 70◦C (Simons *et al*. 1990). When microbial phytase was fed to broilers, the availability of phosphorus increased to 60% and the amount of phosphorus in the droppings declined by 50% (Nair & Duvnjak 1990). Canola meal, used as a feedstuff for livestock and fowl, was successfully dephytinized by *A. niger* NRRL 3135 in solid state fermentation (Segueilha *et al*. 1993; Ebune *et al*. 1995). Various researchers have shown that phytase supplementation improved bone ash content, body weight gain, and tibia length in the broiler diets (Sebastian *et al*., 1996; Qian *et al*., 1997; Azcona *et al*., 2000). Nelson *et al*. (1971) first showed that supplementing broiler diets with phytase from *Aspergillus ficuum* improved phytate phosphorus utilization based on bone ash content. More recently, Zyla *et al*. (2005) found that supplementing broiler feed with phytase significantly increased bone ash content when using 600 and 1,000 phytase units (FTU)/ kg and 0.15% nonphytate phosphorus when compared to no phytase supplementation and the same level of available phosphorus. Azcona *et al*. (2000) also observed an increase in body weight when Natuphos 5000 was supplemented to broiler diets. Qian *et al*. (1997) observed that adding phytase at 300, 600, and 900 FTU/ kg increased body weight gain and bone ash content linearly when diets contained 0.27% nonphytate phosphorus. It can be concluded that one of the most common methods of reducing phosphorus excretion in poultry is the supplementation of feed with microbial-derived phytase.

Discussion

Phytase from *B.cereus* has been found to be efficient in utilizing phytate present in the animal feed. The effect of different enzyme doses was evaluated. Up to 66 hr of incubation, the animal feed was monitored for the released inorganic phosphate content of the feed. An enzyme dose of 100U & 50U of crude phytase per flask were found suitable to liberate enough amount of inorganic phosphorus in case of pig feed and poultry feed respectively (Fig.1&2). The ability of *B. cereus* phytase to hydrolyze not only chemically pure soluble calcium and sodium phytate, but also natural phytate in feeds, is practically important, because phytate in plant based feeds exists in the form of an insoluble phytic acid salt. Our preliminary results clearly indicate that *B.cereus* phytase may improve the nutritional quality of animal feed; however, animal testing is now needed to confirm the ability of the enzyme to increase nutrient consumption and decrease phosphate excretion. Although the phytase productivity was relatively low when compared to phytase production by fungi, it might be improved by cloning to increase gene copy number or other manipulation. In addition, an efficient fermentation process would have to be developed. This might lead to an alternative process for phytase production from a bacterial source. Addition of phytase to the animal feed would result in higher growth due to higher digestion efficiency. The phytase reaction also provides phosphorus usable by the animals and leads to less phosphorus excretion to the environment.

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