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Production and characterization of phytase from *Bacillus* spp. as feed additive in aquaculture

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Abstract. Phytases are phosphohydrolases that catalyze the release of phosphate from phytate (myo-inositol hexakisphosphate), the major phosphorus (P) form mostly occurring in animal feeds of plant origin. These enzymes can be supplemented in animal diets to reduce inorganic phosphorus supplementation and fecal phosphorus excretion. Four species of *Bacillus* namely, *B. pumilus*, *B. megaterium*, *B. coagulans*, and *B. licheniformis* were used to study the biochemical characteristics of their phytases. All the strains investigated were able to hydrolyze extracellular phytate. The activity of phytase increased markedly at the late stationary phase in all the species tested. Highest enzyme activity was found in phytase from *B. megaterium* after the 4th day of culture. The crude phytases from the different *Bacillus* strains were optimally active at pH values ranging 5.5 to 7.0 at 37 ^{0}C and retained their activity at temperatures up to 80 ^{0}C. The enzymes exhibited thermostability, retaining ~50 % activity at 70 ^{0}C and were fairly stable up to pH 10. These properties indicate that the *Bacillus* phytases appear to be suitable for animal feed supplementation in aquaculture to improve the bioavailability of phosphorus.
Key Words: phytic acid, phytase, phosphorus, enzyme, *Bacillus spp.*

Introduction. Phosphorus, similar to nitrogen, is essential for all forms of life. Phytate, (myo – inositol 1, 2, 3, 4, 5, 6 – hexakisphosphate, IP6) in its salt form, is the principal storage form of phosphorus in plants, particularly in cereals, grains and legumes, and it typically represents approximately 75% - 80% of the total phosphorus found in nature (Reddy et al 1982). It is also a storage form of myo – inositol – an important growth factor. It differs from other organo-phosphate molecules in having a high phosphate content, which results in a high negative charge over a wide pH range. Under normal physiological conditions phytic acid has strong chelating potential in the gut and complexes essential minerals such as calcium, magnesium, iron and zinc, thereby decreasing their bioavailability (Thomson & Yoon 1984; Nolan et al 1987). Phytic acid also binds to amino acids and proteins and inhibits digestive enzymes (Pallauf & Rimbach 1996). Thus, phytic acid is an antinutritive component in plant-derived food and feed, and therefore enzymatic hydrolysis of phytic acid is desirable. Phosphatases are a diverse class of enzymes catalyzing the cleavage of monophosphoester bonds in various organo-phosphate compounds.

The ruminants digest phytic acid through the action of phytases produced by the anaerobic gut fungi and bacteria present in their ruminal microflora. However, monogastric animals such as pig, poultry and fish utilize phytate phosphorus poorly because they are deficient in gastrointestinal tract phytases. Therefore, supplemental inorganic phosphate is added to their feed to meet the phosphate requirement and to ensure good growth. However, supplemental inorganic phosphate does not diminish the antinutritive effect of phytic acid. The antinutritive effect of phytic acid is especially

problematic in the feeding of fish (Richardson et al 1985), due to their short gastrointestinal tract. This hinders the use of plant-derived protein in fish feed. The problems mentioned above could be solved by hydrolysis of phytate using supplemental phytase (Simell et al 1989). Therefore, phytase has become an important industrial enzyme and is the object of extensive research. By working efficiently on the substrate in the prevailing conditions, supplemental phytase could diminish the antinutritive effects of phytic acid and reduce the cost of diets by removing or at least reducing the need for supplemental inorganic phosphate. In addition, phytase would be an environmentally friendly product, reducing the amount of phosphorus entering the environment (Kerovuo 2000).

Phytase catalyzes the hydrolysis of phytic acid to inositol phosphates and free orthophosphoric acid. These enzymes are widespread in nature that occurs in some animal tissues, microorganisms as well as in plants. Phytase – producing microorganisms comprise bacteria such as *Bacillus subtilis* (Power & Jagannathan 1982) *Pseudomonas* sp.*,* (Irving & Cosgrove 1971; Lazado et al 2010), *Psychrobacter* sp. (Lazado et al 2010) and *Escherichia coli* (Greiner et al 1993*);* yeast such as *Schwanniomyces castelli* (Segueilha et al 1992*);* and fungi such as *Aspergillus ficuum* (Howson & Davis 1983) and *Aspergillus terreus* (Yamada et al 1968*).*

In the present study, different *Bacillus* species were examined for phytase activity and crude phytase preparations that were obtained from these bacteria were examined of their activities in relation to exposure to fluctuations in temperature and pH levels.

Materials and Methods

Screening of phytase production in *Bacillus* spp. The following strains of *Bacillus* species namely: *B. pumilus* (Acc. No. 1513), *B. coagulans* (Acc. No. 1510), *B. megaterium* (Acc. No. 1643) and *B. licheniformis* (Acc. No. 1035) were taken from the Philippine National Collection of Microorganism, UP Los Baños, Laguna. Pure isolates of these bacterial strains were sub-cultured in phytase screening medium described by Kerovuo (1998) and screened for phytase production. Strain/s that produce clear zones on the screening medium were tested for phytase production in a medium containing 10g/L sodium phytate as a substitute for the inorganic phosphate. Positive strain/s was inoculated in LB (*Luria bertani* medium) agar plate. Single colony of the positive strain/s were re-inoculated in LB and incubated at 37^0C. Samples were withdrawn from the cultures at different time points up to 7 days. After incubation, the culture was centrifuged and the supernatant was assayed for phytase activity. Conditions for phytase assay were optimized such as linearity of the activity with the amount of enzyme and with the reaction time. The identified strains exhibiting the highest phytase specific activity were selected for quantitative production.

Assays on phytase activity. Phytase was routinely performed as described by Engelen et al (1994). Prior to the activity assay, the enzyme (*Bacillus sp.*) was centrifuged at 4,000 rpm for 15 min at 0 – 4^0 C. Briefly, 300µL enzyme supernatant (*Bacillus* sp) and 600 µL substrate solution (5.1 mmol/L of sodium phytate) in 100 mM sodium acetate buffer (pH 6.0) containing 2 mM $CaCl_2$ (as activator) were mixed and incubated in shaking water bath for 60 min at 37^0 C. Then, 900µL 5% TCA was added to stop the reaction. The release of inorganic phosphate was measured spectrophotometrically at 700 nm by adding 300µL of color reagent (4 volumes of 1.5% ammonium molybdate in 5.5% sulfuric acid and 1 volume 2.7% ferrous sulfate). Distilled water was used as blank. One unit of the phytase activity was defined as the amount of enzyme able to hydrolyze phytate to give 1 µmol of inorganic phosphate (P_i) per minute under the assay conditions. Specific activity was expressed in units of enzyme activity per mg protein. The assay was done as with the supernatant after centrifugation. Protein concentration was determined using the modified Lowry method (Lowry et al 1951).

Effects of pH on enzyme activity. The optimum pH for the activity of the crude enzymes was determined by carrying out above described standard assay using the following buffers (0.1M): Glycine – HCl (pH 2-3); NaOAc – HOAc (pH 4 -7); Tris – HCl

(pH 8); Glycine – NaOH (pH 9-11). The pH stabilities were examined by incubating the enzyme solution with these buffers at 25 ^{0}C for 1 h prior to performing the routine assay.
Effects of temperature on enzyme activity. The temperature profile of the crude enzymes was determined by performing the routine assay at different temperatures: 35 ^{0}C, 37 ^{0}C, 40 ^{0}C, 45 ^{0}C, 50 ^{0}C, 55 ^{0}C, and 60 ^{0}C to determine thermal stability, the crude enzymes were incubated at increasing temperatures from 25 – 80 ^{0}C for 1 h, cooled to 4 ^{0}C and assayed.
Statistical analysis. Differences among treatments were analyzed using one way analysis of variance (ANOVA) and Tukey's test was used to determine the significant differences at 5% level of significance. All statistical calculations were performed using a Statistical Package for Social Sciences (SPSS) version 13.0 windows software.

Results

Production of the enzyme. During the initial screening of the *Bacillus* spp for phytase production, we found that all the strains grew in the phytase screening medium. Clear zones formed around the colonies. Visual examination of clearing did not allow for the estimation of phytase activity thus the colonies were re-inoculated in Luria broth supplemented with sodium phytate as the sole phosphate source. Cultivation was carried out aerobically at 37^{0}C. Culture conditions for production of phytase under shake flask culture were optimized to obtain high levels of phytase (data not shown). Low levels of phytate-degrading activity were detectable during the first 3 days of culture period in all the species tested, but increased markedly after the cells reached the stationary phase or at the 4th day of culture (Figure 1). Highest phytase activity was found in *B. megaterium* but there was not significantly different observed between its catalytic properties and that of *B. pumilus* and *B. coagulans* under the specified assay conditions. After reaching the peak on the 4th day of culture, phytase activity gradually dropped with a subsequent decrease in cell density in longer incubation period (5-7 days).

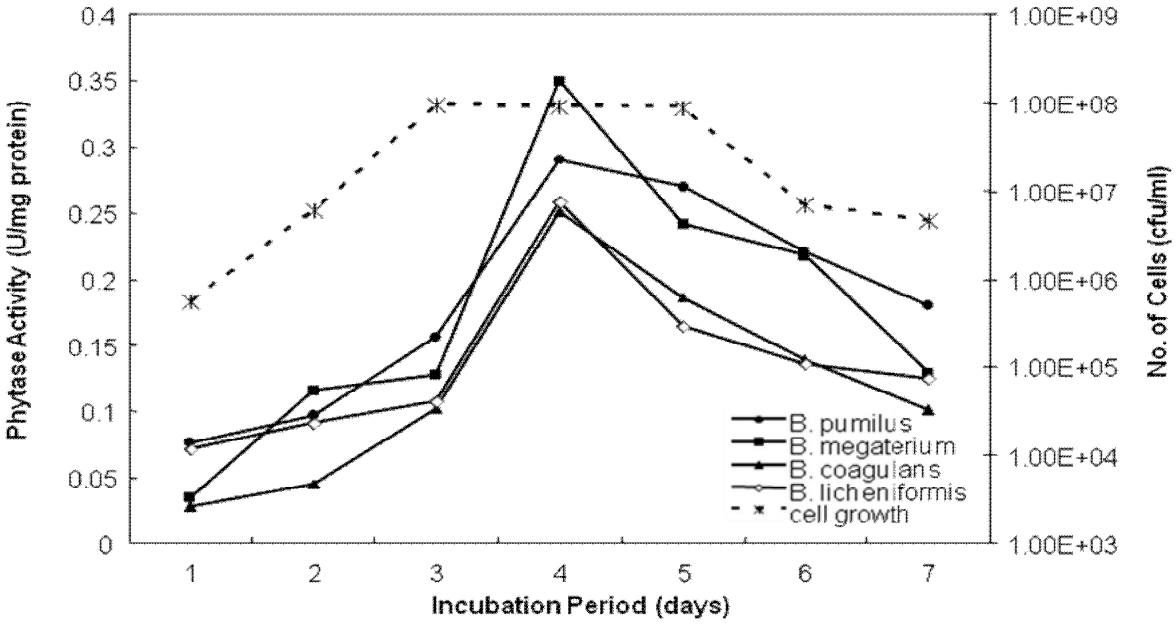

Figure 1. Profile of phytase production and cell growth of the *Bacillus* spp. cultivated on LB medium containing sodium phytate at the different incubation period.

Optimum pH and stability. Figure 2 shows that the profile of all the *Bacillus* phytase displayed similar patterns of responses to varying pH. An increase in activity up to pH 6.0 and a gradual decline at succeeding pH levels were observed for the enzymes. These phytases exhibited broad pH optima, with the highest activities at slightly acidic (pH 6.0) to neutral pH range. At lower pH (3.0-5.0), less than 50% of the activity at optimal pH

was observed in *B. pumilus* and *B. coagulans* phytases. On the other hand, *B. licheniformis* and *B. megaterium* phytases exhibited relatively higher activities at acidic pH range retaining 40-90% of its maximum activity.

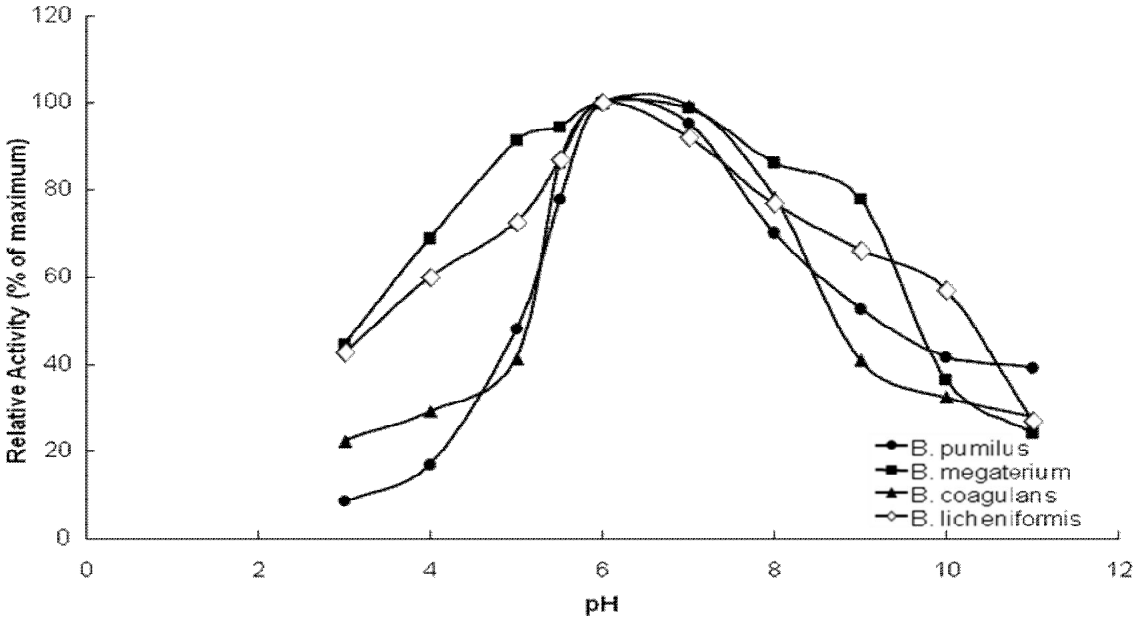

Figure 2. Effect of pH on phytase activities of *Bacillus* spp. The enzyme activities were assayed at various pH buffers: Glycine-HCl (pH 2-3); NaOAc-HOAc (pH 4-7); Tris-HCl (pH 8); Glycine-NaOH (pH 9-11).

All the bacillus phytases increased stability with increasing pH, peaking at pH 6.0, beyond which activity dropped gradually (Figure 3). Maximal stabilities of the *Bacillus* phytases were observed at pH range of 6.0 which is also the optimum pH for its activity. *B. megaterium* phytase has a wider pH stability range retaining 50-90% of its maximum activity at either lower (3-5) or higher (8-10) pH levels. At highly alkaline pH (11.0) a significant drop in phytate-degrading activities were observed in all species tested with *B. coagulans* exhibiting the lowest relative residual activity.

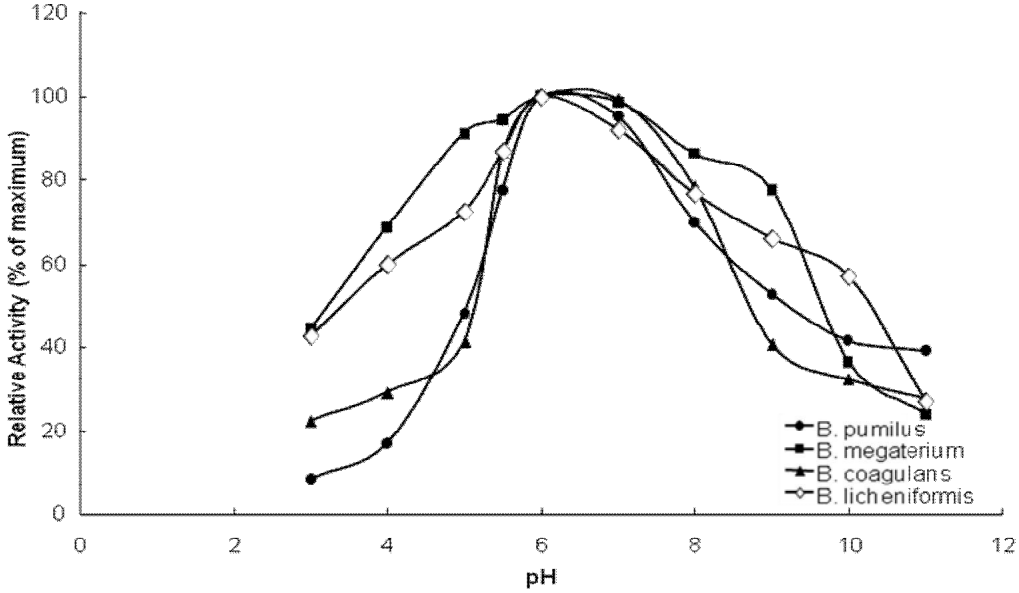

Figure 3. Effect of pH on stability of *Bacillus* spp. phytases. The enzymes were incubated at various pH buffers: Glycine-HCl (pH 2-3); NaOAc-HOAc (pH 4-7); Tris-HCl (pH 8); Glycine-NaOH (pH 9-11) and the residual activities were measured.

Optimum temperatura and thermal stability. Phytase activities of the *Bacillus* spp exhibited temperature optima at 35^{0}C (Figure 4). A sharp increase in phytase activity was observed when assay temperature was increased from 25 to 35^{0}C. Further increase in temperature however, caused gradual decline in phytate-degrading activities. *Bacillus* phytases exhibited very good tolerance to high assay temperatures retaining ~70% of the maximum activity at the highest temperature tested (80^{0}C).

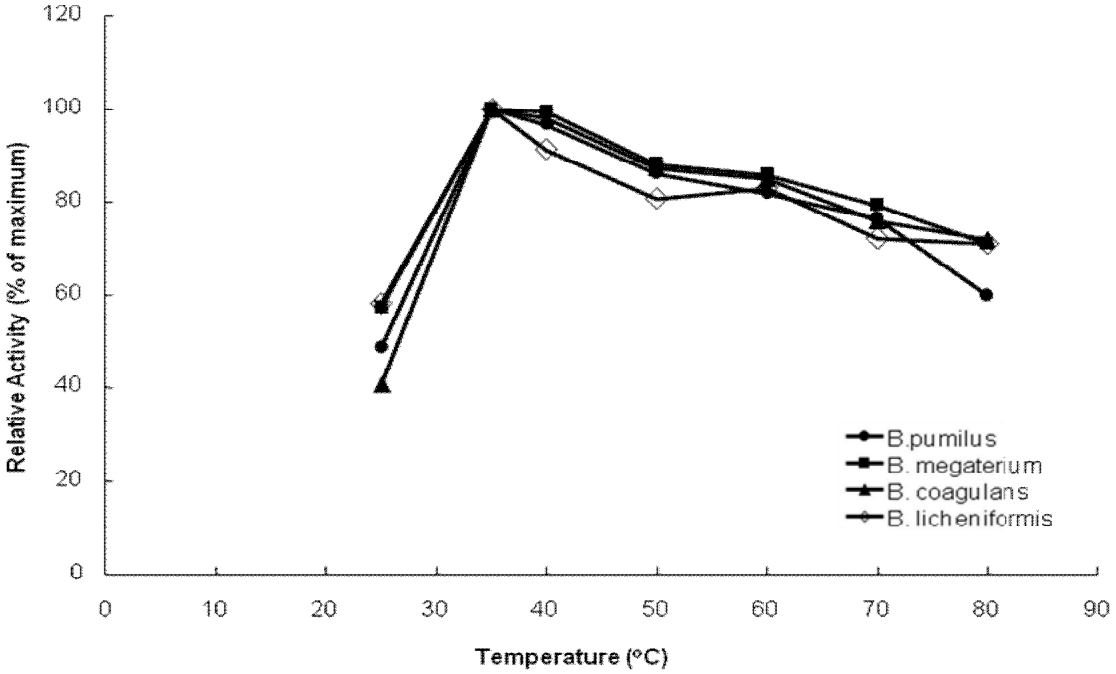

Figure 4. Effect of temperature on phytase activities of *Bacillus* spp. The enzyme activities were assayed at various reaction temperatures.

Thermal stabilities of the *Bacillus* phytases tested were maximal at incubating temperature of 25^{0}C (Figure 5). At temperatures higher than 25^{0}C, enzyme activities start to decrease with further temperature increments. *B. pumilus* and *B. megaterium* showed higher thermal stability retaining ~50% of the maximum activity with incubating temperatures up to 70^{0}C. Enzyme activities decreased significantly at 80^{0}C retaining only ~30% of the maximum.

Discussion. The different *Bacillus* spp. produced phytase when grown in minimal medium containing sodium phytate as the sole phosphate source. These phytases are synthesized in the post-exponential phase of growth as shown in the results of the current study. The stationary phase induction observed suggests that phytase is not required for growth of the organism and may be produced only as a response to some nutrient limitation, (Konietzny & Greiner 2004). Phytase formation however is not controlled uniformly among different bacterial species. Bacterial phytases are found to be inducible enzymes with its expression subjected to a complex regulation. The *Bacillus* phytases in the present study share the same enzyme induction property with other *Bacillus* spp. In *Bacillus* sp. KHU-10 (Choi et al 2001), phytase activity increased markedly after the cells had reached the late stationary phase. When phosphate became rate limiting, growth rate began to fall and the synthesis of the enzyme started. The same mode of induction was observed in *Bacillus subtilis* (Kerovuo et al 1998) in which phytase production is induced by the presence of phytate as the sole source of phosphate in the culture medium. This suggests that production of phytase is induced only when inorganic phosphate is a limiting factor.

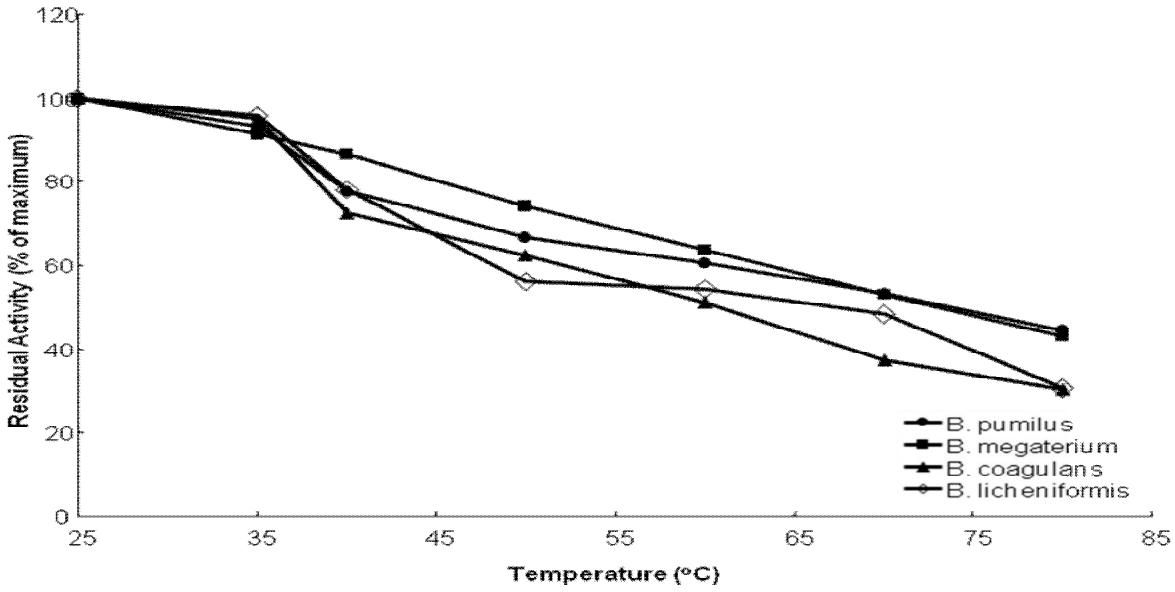

Figure 5. Effect of temperature on stability of *Bacillus* spp. phytases. The enzymes were pre-incubated at different temperatures and the residual activities were measured.

Maximal phytase activity in *Bacillus laevolacticus* under optimized conditions was also achieved after 60h of culture or during late exponential stage, (Gulati et al 2006). Phytase production and activity of the soil bacterium *Klebsiella pneumonia* (Wang et al 2004) followed the same trend in phytase production, reaching a plateau around days 4-5 of culture and dropped drastically thereafter. In contrast, synthesis of phytase from *Pantoea agglomerans* (Greiner 2004) is not triggered by a nutrient or energy limitation. Low phytate-degrading activity was detected in all stages even after the cells have reached the stationary phase. Neither starvation in inorganic phosphate or carbon or oxygen limitation increased phytase production significantly.

As the effectiveness of phytase to degrade phytate in the digestive tract of the animal is described by its biochemical characteristics, it is important to determine the effect of pH and temperature on the enzyme's activity and stability. The low pH in the stomach which is the main functional site of feed phytases makes an enzyme with an acidic pH optimum certainly desirable. In terms of pH optima, there are two main types of phytases identified: acid phytases with an optimum pH around pH 5.0 and alkaline phytases with an optimum pH around pH 8.0, (Koniettzny & Greiner 2004). Most of the studied microbial phytases belong to the acidic ones with their pH optima ranging from 4.0 to 5.5, (Yin et al 2006). Phytase from the other bacterial species such as those belonging to Enterobacteriaceae family like *E. coli,* (Yin et al 2006) Enterobacter sp.4 (Kang et al 2005) and *Obesumbacterium proteus* (Zinin et al 2004) exhibited much lower pH point (pH 3.0-4.5) and larger pH range (pH 2.0 to 5.5). Some fungal phytases also exhibit similar trend in activity when assayed at different pH conditions. Phytase from *Thermomyces lanuginosus,* (Berka et al 1998) a thermophillic fungus showed optimum activity at pH 6.0 while *Aspergillus nige*r SK 57 (Nagashima et al 1999) had a double pH optimum of pH 5.5 and pH 2.5.

In the present study, the *Bacillus* phytases share pH activity profiles that are in agreement with phytases from other *Bacillus* species. Optimal activities of the four phytases were observed at the range of pH 5.5-7.0. The same pH optima for activity were found in *Bacillus* sp KHU-10, (Choi 2001) *B. subtilis* (Kerovuo et al 1998; Powar & Jagannathan 1982) and *B. amyloliquefaciens* (Kim et al 1998; Idriss et al 2002) while *B. laevolacticus* (Gulati et al 2006) exhibited optimum activity at neutral to slightly alkaline pH (7.0-8.0). This activity at low pH values makes these *Bacillus* phytases suitable as feed additives for monogastric animals having stomach pH values of 2-6.

Because commercial feeds are often pelleted, a process which uses high temperatures (60-80^{0}C) and steam, enzyme thermal stability is very relevant in animal feed applications (Lei & Porres 2003). It is therefore imperative to examine the optimum temperature for reaction and thermal stability of any given phytase in order to determine its suitability for feed incorporation. The *Bacillus* phytases in the present study exhibited highest phytate-degrading activities at lower temperature (35^{0}C) but maintained activities even in high reaction temperatures. Optimal temperature for reaction of the phytases in the present study is relatively lower compared to those of other microbial origin that ranged from 40-60^{0}C for *Bacillus* sp KHU-10, (Choi et al 2001) 55^{0}C for *B. subtilis*, (Kerovuo et al 1998) 70^{0}C for *B. laevolacticus*, (Gulati et al 2006) 60^{0}C for *E. coli*, (Yin et al 2006) 60^{0}C for *Pantoea agglomerans*, (Greiner 2004) and 50^{0}C for *Citrobacter braaki*, (Kim et al 2003). Given these findings, it may be safe to speculate that the *Bacillus* enzymes in this study may be able to perform optimal phytate-degrading activities in the stomach temperatures.

During the pelleting process the enzyme is exposed to high temperatures (80-85^{0}C) for a particular time. The thermostability of a phytase is determined by its ability to resist heat denaturation and/or its ability to refold into fully active conformation after heat denaturation, (Wyss et al 1998). Several studies have shown that the extent of glycosylation of a protein may have an impact on its thermostability and on its refolding capacity (Idriss et al 2002)

In order to determine the residual activity of the enzyme that might be lost during pelleting, the *Bacillus* phytases in the current study were exposed to increasing temperatures up to 80^{0}C and assayed. The enzymes may have been partially denatured at the highest tested temperature because the phytate-degrading activities were significantly reduced by 60-70%. Among the Bacillus species tested, only *B. megaterium* and *B. pumilus* exhibited relatively higher thermostability retaining activity of >50% at 70^{0}C and >40% at 80^{0}C.

Bacterial phytases in general have a relatively higher temperature optima and thermostability compared to those of fungal origin. A novel phytase from *Yersinia intermedia* (Huang et al 2006) isolated from glacier soil exhibited optimal activity at 55^{0}C and retained 54% of its activity after incubation at 80^{0}C for 15 min, indicating good thermostability. *Bacillus* sp. strain DS11 (Kim et al 1998) had a temperature optimum at 70^{o}C, which is higher than the temperature optimum of phytases in general. It was also very thermostable with 100% residual activity after 10 min incubation at 70^{o}C (in the presence of $CaCl_2$). The enzyme stability of *Bacillus sp.* strain DS11 phytase was drastically reduced above 50^{o}C in the absence of $CaCl_2$, whereas it was rather stable up to 90^{o}C in the presence of $CaCl_2$. In the present study, the enzymes were exposed to high temperatures for 1 h which is considerably longer than the 10min incubation time used in Bacillus sp DS11. This might have caused the denaturation of the enzyme thus the lower thermostability.

Conlusions. In summary, the *Bacillus* phytases tested were able to release inorganic phosphorus from sodium phytate. Two of these enzymes (derived from *B. pumilus* and *B. megaterium*) have high thermal stability as well as broad activity at different pH levels. These results indicate that these phytases have the potential for use as feed additives for monogastric animals. Further work is underway for the purification and characterization of the enzyme as well as studies on its ability to release inorganic phosphorus from feed and feed ingredients of aquatic animals.

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