# Biochemical properties of extracellular phytases from *Bacillus* spp.

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*Key words*: enzyme, phytase, phytate, Bacillus, inorganic phosphate

## Abstract

Phytases are phosphohydrolases that catalyzes 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 P supplementation and fecal P excretion. Four species of *Bacillus*, *B. pumilus*, *B. megaterium*, *B. coagulans*, and *B. licheniformis* were used to study the biochemical characteristics of their phytases. The results showed that all the strains investigated were able to hydrolyze extracellular phytate. The activity of phytase increased markedly in late stationary phase in all the species tested. The highest enzyme activity was found in *B. megaterium* phytase after the 4th day of culture. The crude phytases from the different *Bacillus* strains were optimally active at pH values from 5.5 to 7.0 at 37°C and retained activity at assay temperatures up to 80°C. The enzymes exhibited thermostability, retaining ~50% activity at 70°C and were fairly stable up to pH 10. These properties indicate that the *Bacillus* phytases appear to be suitable for animal feed supplementation to improve the availability of P from phytates.

## INTRODUCTION

Phytic acid (myo-inositol hexakisphosphate) is the major storage form of phosphorus (P) present in seeds of higher plants, mainly cereal grains, legumes and oilseed crops.[1] In the context of human and animal nutrition, the following two aspects of phytic acid are critically important: (i) monogastric animals have low levels of phytic acid-degrading enzymes in their digestive tracts and since phytic acid is not absorbed, feeds for pigs, fish and poultry commonly are supplemented with inorganic phosphate in order to meet the phosphorus requirements of these animals; (ii) phytic acid is an antinutrient factor since it chelates positively charged nutrients and forms complexes with proteins and a variety of metal ions, therefore decreasing the dietary availability of these nutrients.[2] Consequently, when phytic acid is not assimilated by monogastric animals, the undigested phytate P is excreted in the manure which runs-off to lakes and sea, contributing to the eutrophication of surface waters in areas of intensive livestock culture.[3] Thus, enzymatic hydrolysis of phytic acid in the intestine of monogastric animals is desired.

Many attempts to hydrolyze phytic acid using phytase have been made to improve the nutritional value of feed and to decrease the amount of phosphate excreted by the animals. Numerous laboratory experiments and field trials have shown that addition of phytase can replace inorganic phosphorus supplementation and reduce total phosphorus excretion by 30-50% depending on the diet, species and level of phytase supplementation.[3),4),5)]

Several phytases have been cloned and characterized from microbial,[6),7)] animal[8)] and plant[9)] origin. Recent research has shown that microbial phytases are most promising for biotechnological applications because of the capacity of the microorganisms to produce and secrete large quantities of enzymes combined with the desired temperature and pH activities and stability properties. Commercial production currently focuses on the soil fungus *Aspergillus*.[10)] However, due to some properties, such as substrate specificity, resistance to proteolysis and catalytic efficiency, bacterial phytases are a real alternative to fungal enzymes.[11)] Generally, the phytases produced by fungi are extracellular, whereas the enzymes from bacteria are mostly cell associated. The only bacteria showing extracellular phytase activity are those of the genera *Ba-*

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*cillus*[4),12),13),14)] and *Enterobacter*.[15)]

The most practical aspect to look into is extracellular secretion of the enzyme because it is very difficult to shear open small organisms like bacteria and fungi just to extract the phytase. It will be a tremendous advantage if breaking open the cells is altogether avoided and the extraction would just entail purifying the enzyme from the culture media. Furthermore, other important aspects that should be considered for practical applications of phytase are: high specific activity, pH optima corresponding to various components of the animal digestive tract and thermal stability.[16)] Therefore there is an ongoing interest in screening microorganisms for novel and efficient phytases with an optimal combination of various properties.

In the present study, we describe the biochemical properties of crude extracellular phytases from different species of *Bacillus* that have not yet been studied. The aim of the current study is to characterize a bacterial phytase which display high enzymatic activity at physiological temperatures and pH values associated with the digestive tract of monogastric animals. Such a phytase is expected to be more suitable for feed applications.

## MATERIALS AND METHODS

### Screening of phytase producers

The following strains of *Bacillus* spp. were obtained from the Philippine National Collection of Microorganism: *B. coagulans* Acc. no. 1510, *B. licheniformis* Acc. no. 1035, *B. megaterium* Acc. No. 1643 and *B. pumilus* Acc no.1513. Pure isolates of these bacterial strains were sub-cultured in phytase screening medium described by Kerovou (1998) and screened for phytase production. Strain/s that produced clear zones on the screening medium were tested for phytase production in a phytase production medium containing 10g/L sodium phytate as a substitute for the inorganic phosphate. Positive strains were inoculated in Luria-Bertani (LB) agar plate. Single colony of the positive strains were re-inoculated in LB and incubated at 37°C. Samples were withdrawn from the cultures at different time points up to 7days. After incubation the culture was centrifuged and the supernatant used as crude enzyme solution was assayed for phytase activity. The cell density was determined by Total Plate Count. For characterization studies, 4-day old cultures were used. Conditions for phytase assay such as linearity of the activity with amount of enzyme and with reaction time were optimized.

### Standard Phytase Activity Assay

Phytase activity was routinely assayed by mixing and incubating 300 $\mu$L enzyme solution and 600 $\mu$L substrate solution (5.1 mmol/L of sodium phytate) in 100 mmol sodium acetate buffer pH 6.0 containing 2 mmol $CaCl_2$ (as activator) at 37°C for 1h. After incubation, reactions were stopped by adding an equal volume of 5%TCA. The released inorganic phosphate was measured spectrophotometrically at 700 nm by adding an equal volume of color reagent (1.5% ammonium molybdate in 5.5% $H_2SO_4$ and 2.7% $FeSO_4$ solution). Blanks were run by addition of the color reagent solution prior to adding the enzyme to the assay mixture. One unit of enzyme activity is defined as the amount of enzyme hydrolyzing 1 $\mu$mol of $P_i$ per minute under the assay conditions. Specific activity will be expressed in units of enzyme activity per mg protein. Protein concentrations will be determined using the modified Lowry method.[17)]

### Effect of pH on Enzyme Activity

The optimum pH for the activity of the crude enzymes were 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°C for 1h prior to performing the routine assay.

### Effect of temperature on Enzyme Activity

The temperature profile of the crude enzymes was determined by performing the routine assay at different temperatures: 35°C, 37°C, 40°C, 45°C, 50°C, 55°C and 60°C. To determine thermal stability, the crude enzymes were by incubated at increasing temperatures from 25-80°C for 1h, cooled to 4°C and assayed.

## 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 were formed around the colonies. The 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°C. The 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 had reached the stationary phase or the 4th day of culture (Fig. 1). Highest phytase activity was found in *B. megaterium* but there were no significant differences observed between its catalytic properties and that of *B. pumilus* and *B. coagulans* at the specified assay conditions. After reaching the peak on the 4th day of culture, phytase activity gradually dropped with the subsequent decrease in cell density in longer incubation period (5-7 days).

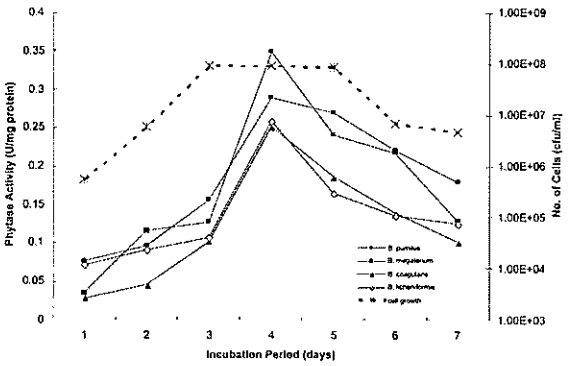


**Fig. 1.** Profile of phytase production and cell growth of the *Bacillus* spp. cultivated on LB medium containing sodium phytate at the different incubation period.

### Enzyme Properties

pH optimum and stability

Fig. 2 shows that the pH activity profile of all the *Bacillus* phytase displayed similar pattern 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.

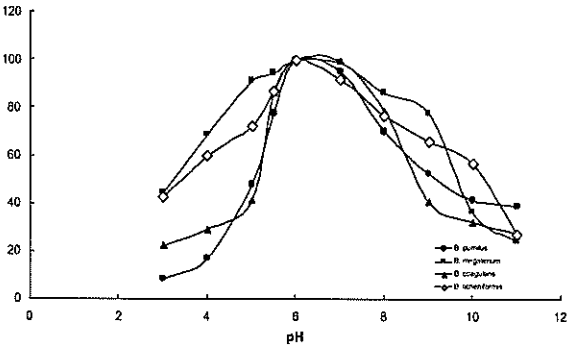


**Fig. 2.** Effect of pH on phytase activities of *Bacillus* spp. The enzyme activities were assayed at various pH buffers: Glycine-HCl (pH 2-3); NaOAc-HOAc (pH 4-7); Tris-HCl (pH 8); Glycine-NaOH (pH 9-11).

The effect of pH on enzyme stability is shown in Fig. 3. All the bacillus phytases increased stability with increasing pH, peaking at pH 6.0, beyond which activity dropped gradually. 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 spp tested with *B. coagulans* exhibiting the lowest which is only 18% of the maximum.

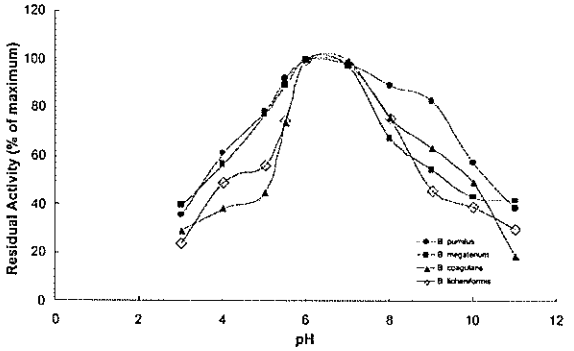


**Fig. 3.** Effect of pH on stability of *Bacillus* spp. phytases. The enzymes were incubated at various pH buffers: Glycine-HCl (pH 2-3); NaOAc-HOAc (pH 4-7); Tris-HCl (pH 8); Glycine-NaOH (pH 9-11) and the residual activities were measured.

Temperature optimum and thermal stability

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

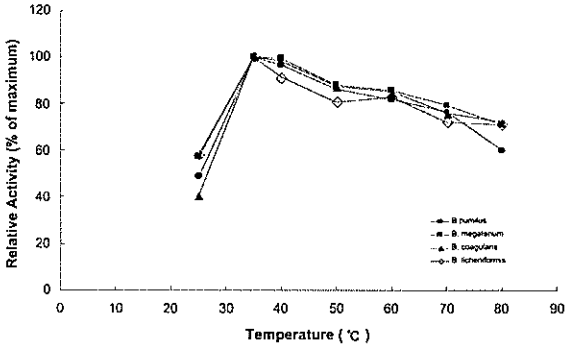


**Fig. 4.** Effect of temperature on phytase activities of *Bacillus* spp. The enzyme activities were assayed at various reaction temperatures.

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

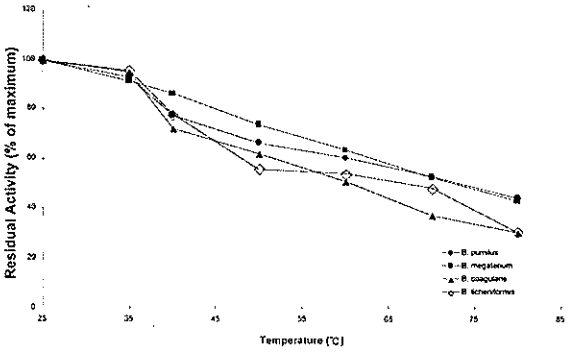


**Fig. 5.** Effect of temperature on stability of *Bacillus* spp. phytases. The enzymes were pre-incubated at different temperatures and the residual activities were measured.

## 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.[11] 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[18], 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*[12] 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. Maximal phytase activity in *Bacillus laevolacticus* under optimized conditions was also achieved after 60h of culture or during late exponential stage.[19] Phytase production and activity of the soil bacterium *Klebsiella pneumoniae*[20] 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*[21] 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 animal's digestive tract 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 op-

tima, 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.[11] Most of the studied microbial phytases belong to the acidic ones with their pH optima ranging from 4.0 to 5.5.[22] Phytase from the other bacterial species such as those belonging to Enterobacteriaceae family like *E. coli,*[22] Enterobacter sp.4 [23] and *Obesumbacterium proteus* [16] 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,*[24] a thermophillic fungus showed optimum activity at pH 6.0 while *Aspergillus niger* SK 57 [25] 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,[18] *B. subtilis* [12,13] and *B. amyloliquefaciens* [4,26] while *B. laevolacticus* [19] 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°C) and steam, enzyme thermal stability is very relevant in animal feed applications.[1] 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°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°C for *Bacillus* sp. KHU-10, [18] 55°C for *B. subtilis,*[12] 70°C for *B. laevolacticus,*[19] 60°C for *E. coli,*[22] 60°C for *Pantoea agglomerans*[21] and 50°C for *Citrobacter braaki*[27]. 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°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.[2] Several studies have shown that the extent of glycosylation of a protein may have an impact on its thermostability and on its refolding capacity.[28]

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°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°C and >40% at 80°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* [29] isolated from glacier soil exhibited optimal activity at 55°C and retained 54% of its activity after incubation at 80°C for 15 min, indicating good thermostability. *Bacillus* sp. strain DS11[4] had a temperature optimum at 70°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°C (in the presence of $CaCl_2$). The enzyme stability of *Bacillus* sp. strain DS11 phytase was drastically reduced above 50°C in the absence of $CaCl_2$, whereas it was rather stable up to 90°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.

For the fungal phytases, Wyss[2] and co-workers investigated the thermostability properties of three phytases of fungal orgin (*A. fumigatus* and *A. niger* phytase, and *A. niger* pH 2.5 acid phosphatase).The phytases of *A. fumigatus* and *A. niger* were both denatured at temperatures between 50 and 70°C. They concluded that *A. niger* phytase

was not thermostable, neither did it have the capacity to refold after heat denaturation. In contrast to the two phytases, *A. niger* pH 2.5 acid phosphatase displayed higher thermostability with irreversible inactivation observed only at temperatures of ≥ 80°C.

In summary, the *Bacillus* phytases tested were able to release Pi from sodium phytate. Two of these enzymes (*B. pumilus* and *B. megaterium*) have high thermal stability as well as broad active and stable pH range. These results indicate that these phytases have the potential for use as feed additive 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 P from feed and feed ingredients.

## REFERENCES


1 ) Lei, X.G. and J.M. Porres (2003). Phytase enzymology, applications and biotechnology. *Biotechnol. Lett.*, **25**:1767-1794.

2 ) Wyss, M., L. Pasamontes, R. Remy, J. Kohler, E. Kusznir, M. Gadient, F. Muller and A.P.G.M van Loon (1998). Comparison of the thermostability properties of three acid phosphatases from molds: *Aspergillus fumigatus* phytase, *A. niger* phytase, and *A. niger* pH 2.5 acid phosphatase. *Appl. Environ. Microbiol.*, **64**:4446-4451.

3 ) Adeola, O., J. S. Sands, P.H. Simmins and H, Schulzer (2004). The efficacy of an *Escherichiacoli*-derived phytase preparation. *J. Anim. Sci*, **82**:2657-2666.

4 ) Kim, Y. O., H. K. Kim, K.S. Bae, J.H. Yu, and T.K. Oh (1998). Purification and properties of a thermostable phytase from *Bacillus* sp. DS11. *Enzyme Microbiol. Technol,*. **22**:2-7.

5 ) Matsui, T., Y. Nakagawa, A. Tamura, C. Watanabe, K. Fujita, T. Nakajima, and H. Yano (2000). Efficacy of yeast phytase in improving phosphorus bioavailability in a corn-soybean meal-based diet for growing pigs. *J. Anim. Sci.*, **78**:94-99.

6 ) Greiner, R., U. Konietzny and K.D. Jany (1993). Purification and characterization of two phytases from *Escherichia coli. Arch. Biochem. Biophys.*, **303**:107-113.

7 ) Ullah, A. H. J. (1988). Production, rapid purification and catalytic characterization of extracellular phytase from *Aspergillus ficuum. Prep. Biochem.*,.:**18**:443-45.

8 ) Craxton, A, J.J. Caffrey, W. Burkhart, S.T. Safrany, and S.B. Shears (1997). Molecular cloning and expression of a rat hepatic multiple inositol polyphosphate phosphatase. *Biochem. J.*, **328**:75-81.

9 ) Greiner, R. and M. Larsson Alminger (2001). Stereospecificity of myo-inositol hexakisphosphate dephosphorylation by phytate-degrading enzymes of cereals. *J. Food Biochem,*. **25**:229-248.

10) Cho, J.S., C.W. Lee, S.H. Kang, J.C. Lee, J.D. Bok, Y.S. Moon, H.G. Lee, S.C. Kim, and Y.J. Choi (2003). Purification and characterization of a phytase from *Pseudomonas syringae* MOK1. *Curr. Microbiol.*, **47**:290-294.

11) Konietzny, U. and R. Greiner (2004). Bacterial phytase: potential application, *in vivo* function and regulation of its synthesis. *Braz. J. Microbiol.*, **35**:11-18.

12) Kerovou, J., M. Lauraeus, P. Nurminen, N. Kalkkinen, J. Apajalahti, (1998). Isolation, characterization, molecular gene cloning and sequencing of a novel phytase from *Bacillus subtilis. App. and Environment. Microbiol.*, **64**:2079-2085.

13) Powar, V. K. and V. Jagannathan (1982). Purification and properties of phytate-specific phosphatase from *Bacillus subtilis. J. Bacterio,l.*:**151**:1102-1108.

14) Shimizu, M. (1992). Purification and characterization of phytase from *Bacillus subtilis* (natto) N-77. *Biosci. Biotechnol. Biochem.*, **56**:1266-1269.

15) 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. 4,and enzymatic properties of phytase enzyme. *Enzyme Microbiol. Technol.*, **18**:449-454.

16) Zinin, N.V., A.V. Serkina, M.S. Gelfand, A.B. Shevelev and S.P. Sineoky (2004). Gene cloning, expression and characterization of novel phytase from *Obesumbacterium proteus. Microbiol. Lett.*, **236**:283-290.

17) Lowry, O.H., N.J. Rosebrough, A.L.Farr, and R.J.Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J.Biol.Chem.*:**193**:265-275.

18) Choi, Y.M., H.J.Suh and J.M. Kim (2001). Purification and properties of Extracellular phytase from *Bacillus* sp. KHU-10. J. Prot. Chem., **20**: 287-292.

19) Gulati, H.K., B.S. Chadha, and H.S. Saini (2006). Production and characterization of thermostable alkaline phytase from *Bacillus laevolacticus* isolated from rhizosphere soil. *J. of Indust. Microbiol. Biotechnol.*, **34**:91-98.

20) Wang, X., S. Upatham, W. Panbangred, D. Isarangkul, P. Summpunn, S. Wiyakrutta and V. Meevotisum (2004). Purification, characterization, gene cloning and sequence analysis of a phytase from *Klebsiella pneumoniae* subsp. *Pneumoniae XY-5. Science Asia.* **30**:383-390.

21) Greiner, R. (2004). Purification and properties of a phytate degrading enzyme from *Pantoea agglomerans. The Protein Journal.*,**23**:567-576.

22) Yin, Q.Q., Q.H. Zheng, and X.T. Kang (2006). Biochemical characteristics of phytases from fungi and the transformed microorganism. *Anim. Feed Sci. and Technol.*, **132**:341-350.

23) Kang, S.H., K.K. Cho, J.D. Bok, S.C. Kim, J.S. Cho, P.C. Lee, S.K. Kang, H.G. Lee, J.H. Woo, H.J. Lee, S.C. Lee and Y.J. Choi (2005). Cloning, Sequencing and Characterization of a novel phosphatase gene, phoI, from soil bacterium *Enterobacter* sp. 4. *Curr. Microbiol.*, **52**: 243-248.

24) Berka, R.M., M.W. Rey, K.M. Brown, T. Byun and A.V. Klotz (1998). Molecular characterization and expression of a phytase gene from the thermophilic fungus *Thermomyces lanuginosus. Appl. Environ. Micriobiol.*, **64**:4423-4427.

25) Nagashima, T., T. Tange and H. Anazawa (1999). Dephosphorylation of phytate by using the *Aspergillus niger* phytase with a high affinity for phytate. *Appl. Environ. Microbiol.*, **65**:4682-4684.

26) Idriss, E.E., A. Farouk, R. Greiner, H. Bochow, and R. Borris (2002). Extracellular phytase activity of several *Bacillus amyloliquefaciens FZB45* contributes to its lant-growth-promoting effects. *Microbiol.*,**148**:2097-2109.

27) Kim, H.W., Y.O. Kim, J.H. Lee, K.K. Kim and Y.J. Kim (2003). Isolation and characterization of a phytase with improved properties from *Citrobacter braakii. Biotechnol. Lett.*, **25**:1231-1234.

28) Brugger, R., C.S. Nunes, D. Hug, K. Vogel, P. Guggenbuhl, F. Mascarello, S. Augem, M. Wyss, A.G.P.M. van Loon, and L. Pasamontes (2004). Characteristics of fungal phytases from *Aspergillus fumigatus* and *Sartorya fumigata. Appl. Microbiol. Biotechnol.*, **63**:383-389.

29) Huang, H., H. Luo, P. Yang, K. Meng, Y. Wang, T. Yuan, Y. Bai, and B. Yao (2006). A novel phytase with preferable characteristics from *Yersinia intermedia. Biochem. Biophys. Res. Commun.*, **350**:884-889.