Appl Biochem Biotechnol (2008) 151:1–8
DOI 10.1007/s12010-008-8158-7

Bacillus Phytases: Present Scenario and Future Perspectives

Shijun Fu · Jianyi Sun · Lichun Qian · Zhiyu Li

Received: 7 November 2007 / Accepted: 23 January 2008 /
Published online: 20 February 2008

Abstract Phytases are a special class of phosphatases that catalyze the sequential hydrolysis of phytate to less-phosphorylated *myo*-inositol derivatives and inorganic phosphate. *Bacillus* phytases, which exhibit their desirable activity profile under neutral pH, higher thermal stability, and strict substrate specificity for the calcium–phytate complex, have considerable potential in commercial and environmental applications. This review describes recent findings concerning the production, biochemical properties, molecular characteristics, and expression of *Bacillus* phytases. Several potential applications of the *Bacillus* phytases in animal nutrition, human health, and synthesis of lower *myo*-inositol phosphates are also summarized.

Keywords Phytate · Phytase · *Bacillus* · Feed · Food · Lower inositol phosphates

Introduction

Phytate, the primary storage form of phosphate and inositol in many plants, serves as a strong chelator by binding positively charged proteins, amino acids, and minerals in insoluble complexes in the digestive tract [1]. The stepwise hydrolysis of phytate is catalyzed by phytase with minerals and orthophosphate being released and a series of lower isomers of *myo*-inositol phosphates and eventually *myo*-inositol being formed [2]. During the last few decades, phytases have been studied intensively because of the great interest in its application as feed additive [3, 4], processing of human food [5, 6], and synthesis of lower inositol phosphates [7, 8].

S. Fu · J. Sun (✉) · L. Qian · Z. Li
College of Animal Science, Zhejiang University, Hangzhou 310029, People's Republic China
e-mail: fsj729@yahoo.com.cn

S. Fu · J. Sun · Z. Li
The Key Laboratory of Molecular Animal Nutrition, Ministry of Education, Hangzhou 310029, People's Republic China

Phytases are widely distributed in nature and could be derived from a number of sources including plants, animals, and microorganisms. Based on their structural differences and various catalytic properties, phytases were categorized into three major classes including members of histidine acid phosphatases (HAPs), beta-propeller phytases (BPPs), and purple acid phosphatases (PAPs) [9]. Among these, BPPs are mainly isolated from *Bacillus* with higher thermal stability and optimum pH under neutral and exhibit unique Ca^{2+}-dependent catalytic property and highly strict substrate specificity for the calcium–phytate complex. This review focuses attention on the present finding of *Bacillus* phytases and the future prospects.

Bacillus Species with Phytase Activities

Production of *Bacillus* Phytases

Bacillus species producing thermally stable phytase was mainly isolated from soil [10, 11] and cooked rice [12]. Submerged fermentation by *B. amyloliquefaciens* DS11 in the medium consisting of wheat bran and casein hydrolysate produced thermostable, extracellular phytase at 37 °C [10]. The maximal phytase activity produced by *B. laevolacticus* was achieved under shake flask culture and optimized conditions (2.957 U/ml) [11]. *Bacillus* sp. KHU-10 produced the highest level of 0.2 U/ml phytase after 4 days of fermentation under the optimized conditions [12].

Characterization of *Bacillus* phytases

Purification and Properties

There have been reports of partially purified *Bacillus* phytase preparations. Power and Jagannathan [13] reported the isolation of a phytate preparation from *Bacillus subtilis* with a molecular weight of 36.5 kDa. This enzyme preparation was shown to have a pH optimum between 7.0 and 7.5. This phytase exhibited a maximum activity at a temperature of 60 °C and was stable up to a temperature of 70 °C. The specific activity of the purified enzyme was reported as 8.5 to 9.0 U/mg protein.

B. subtilis (natto) phytase described by Shimizu [14] was purified to homogeneity and shown to have a molecular weight of between 36 and 38 kDa. This enzyme had a pH optimum between pH 6.0 and 6.5 and an optimum temperature of 60 °C. The specific activity of this purified phytase was reported as 8.7 U/mg protein. Kerovuo et al. [15] isolated, purified, and characterized phytase from *B. subtilis* VTT E-68013 showing optimum temperature and pH at 55 °C and 7.0, respectively.

Choi et al. [16] reported that an extracellular phytase from *Bacillus* sp. KHU-10 was purified and estimated to be 44 kDa. Its optimum pH and temperature were pH 6.5–8.5 and 40 °C without 10 mM $CaCl_2$ and pH 6.0–9.5 and 60 °C with 10 mM $CaCl_2$. About 50% of its original activity remained after incubation at 80 °C for 10 min in the presence of 10 mM $CaCl_2$. The enzyme activity was fairly stable from pH 6.5 to 10.0.

Gulati et al. [11] described the partially purified phytase from *B. laevolacticus* was optimally active at 70 °C and pH 7.0–8.0. This enzyme exhibited thermal stability with 80% activity at 70 °C and pH 8.0 for up to 3 h in the presence or absence of 5 mM $CaCl_2$.

Thermal Stability

The thermal stability of phytase is considered to be an important and useful criterion for application as an animal additive because of the process with high temperature and steam during pelleting [17, 18]. *Bacillus* phytases are quite stable at the high temperature range of 80–95 °C [10, 16, 19, 20]. Elkhalil et al. [21] revealed that *Bacillus* phytase is more resistant to heat treatments than *E. coli* and *Klebsiella* phytases. Considering this property, *Bacillus* phytases will no doubt be better candidates for feed supplements.

Effect of Calcium Ions

Bacillus phytases were found to be metal ion-dependent as they required calcium for activity and stability [10, 13, 14, 22]. Ca^{2+} binds two oxyanions from the phosphate groups of phytate to form an ideal positively charged distribution in the calcium–phytate complex. Because of the existence of three Ca^{2+}-binding sites at the active site cleft, Ca^{2+} serves as an essential activator to reduce the negative charge around the active site cleft, whereas excess amounts of Ca^{2+} acts as a competitive inhibitor [22]. In addition, Ca^{2+} has an important effect on the stability against temperature and pH [16].

Kerovuo et al. [23] studied the metal ion requirement of *B. subtilis* phytase and reported that the removal of metal ions from the enzyme by EDTA resulted in complete inactivation. The loss of enzymatic activity was most likely because of a conformational change, as the circular dichroism spectra of holoenzyme and metal-depleted enzyme were different. Metal-depleted enzyme was partially able to restore the active conformation when incubated in the presence of calcium. Only minor reactivation was detected with other divalent metal ions and their combinations.

Substrate Specificity

Bacillus phytases exhibit highly strict substrate specificity for calcium–phytate complex and have no enzymatic activity on other phosphate esters [10, 13–16, 24]. Oh et al. [22] reported that no detectable phytate hydrolysis was observed when the phytase was added to phytate in the Ca^{2+}-free environment at several different phytate concentrations. At fixed phytate concentration, increasing the concentration of Ca^{2+} enhanced the initial velocities of phytase in a saturating manner. This activation is mediated by a cooperative interaction of Ca^{2+} to the substrate and the Ca^{2+}-dependent increase of reaction rate followed a sigmoidal curve. Other divalent cations such as Co^{2+}, Cs^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, and Ni^{2+} have no effect on enzymatic activation. According to these results, the calcium–phytate complex is regarded as the true substrate for the BPPs, and Ca^{2+} plays an important role as a substrate activator in the formation of the true substrate.

The crystal structure of the phytase from *B. amyloliquefaciens* DS11 demonstrated that a negatively charged active site provides a favorable electrostatic environment for the positively charged calcium–phytate complex [20, 25].

Molecular Characteristics

Genes encoding *Bacillus* phytases from *B. amyloliquefaciens* [24, 26], *B. licheniformis* [19], and *B. subtilis* [15, 19] have been cloned and sequenced. They are composed of 383 amino acids and encoded an extracellular monomeric protein. The molecular mass is

approximately 42 kDa; and SDS-PAGE gives an apparent molecular mass of approximately 38–44 kDa. The amino acid sequences of phytases from *Bacillus* are highly homologous to each other with 90–98% sequence identity. However, these amino acid sequences do not align with any other previously well-characterized HAPs nor with PAPs. Most significantly, the active site heptapeptide motif RHGXRXP and the catalytically active dipeptide HD, which are highly conserved sequence among HAPs [27], are absent. Besides, *Bacillus* phytases do not carry disulfide bonds, which are necessary for the conformational stability and catalytic activity in several fungal phytases [28–30]. There are 11, 11, and 3 residues that are involved in the phosphate binding sites and high- and low-affinity calcium-binding sites, respectively.

Crystal Structure

Ha et al. [31] carried out preliminary x-ray crystallographic analysis of a novel phytase from a *B.amyloliquefaciens* strain using the hanging-drop vapor-diffusion method. The enzyme consists of five 4-stranded and one 5-stranded antiparallel β sheets aligned around a pseudo sixfold symmetry axis lying on the shaft of the propeller, which is a distinct central channel filled with many ordered water molecules.

Ha et al. [20] further determined the crystal structure of *B. amyloliquefacience* phytase at 2.1 Å resolution in partially and fully Ca^{2+}-loaded states. Two calcium ions (Ca1 and Ca2) form a biocalcium center where the Asp308 carboxylate serves as a bridging arm and tightens the "double clasp," stabilizing the circular arrangement of the propeller structure. The binding of Ca1 and Ca2 to high-affinity calcium-binding sites results in a dramatic increase in thermal stability by tightening of several peptide segments at the strategic location remote in the amino acid sequence. Three calcium ions (Ca4, Ca5, and Ca6) at the active site form a triadic calcium center and neutralize an otherwise negatively charged calcium cage surrounded by a total of six aspartate and glutamate residues. The top of the molecular structure forms a shallow cleft that is lined predominantly with negatively charged side chains. The calcium-occupied cleft turns into a favorable electrostatic environment for the binding of phytate together with nearby Lys76, Lys77, Arg122, and Lys179. In addition, Ca4, Ca5, and Ca6 participate in catalysis directly by binding the phosphate group(s) of the substrate and stabilizing the pentavalent transition-state intermediate. Ca^{2+} reduces the negative charge around the active site cleft such that phytate neutralized by Ca^{2+} can easily fit to the active site.

Furthermore, the enzyme has two low phosphate binding sites, the "cleavage site," which is responsible for the hydrolysis of a substrate, and the "affinity site," which increases the binding affinity for the atom of the substrates containing adjacent phosphate groups [25].

Pathways of Phytate Dephosphorylation

A bidentate (P_3-Ca^{2+}-P_4) of Ca^{2+}-InsP_6 initially binds to two phosphate-binding sites in the active site of *Bacillus* phytase, which preferentially hydrolyzes the phosphate group at the d-3 position of Ca^{2+}-InsP_6 to release Ins(1,2,4,5,6)P_5 as an initial product [24]. After the hydrolysis of the first phosphate group, the enzyme binds another bidentate (P_1-Ca^{2+}-P_2) of Ins(1,2,4,5,6)P_5, followed by the hydrolysis of the phosphate group at the d-1 position to release Ins(2,4,5,6)-P_4, as identified by 2-D NMR analysis of reaction intermediates. Finally, the enzyme binds a bidentate (P_5-Ca^{2+}-P_4) of Ins(2,4,5,6)P_4 and eventually

hydrolyzes the phosphate group at the d-5 position to yield *myo*-Ins(2,4,6)P_3 as a final product [32].

Greiner et al. [33] established the pathway of dephosphorylation of phytate by *B. subtils* 168, *B. amyloliquefaciens* ATCC15841, and *B. amyloliquefaciens* 45 using a combination of HPIC analysis and kinetic studies. The result demonstrated that these enzymes dephosphorylate phytate by sequential removal of phosphate groups via two independent routes: the routes via D-Ins(1,2,4,5,6)P_5 to Ins(2,4,5,6)P_4 and finally, to D-Ins(2,4,6)P_3. These enzymes prefer the hydrolysis of every second phosphate over that of adjacent ones. This finding does support previous phytate degradation models proposed by Kerovuo et al. [34] and Greiner et al. [35], but seems to fit with the structural model given by Shin et al. [25].

Expression of *Bacillus* Phytase

Expression of *Bacillus* Phytases in Microbial Hosts

Tye et al. [19] overexpressed the phytase gene (*phyL*) from *B. licheniformis* and phytase gene (*168phyA*) identified from *B. subtilis* strain 168 in *B. subtilis* using a φ105MU331 prophage vector system. Up to 35 U/ml were secreted into the culture media. Both phytases exhibited broad temperature and pH optima and showed high thermal stability. The phytase encoded by *phyL* exhibited higher thermal stability, even at a lower calcium concentration, as it was able to recover 80% of its original activity after denaturation at 95 °C for 10 min.

The gene-encoding phytase from *Bacillus* sp. DS11 was cloned in *E. coli* BL21 using the pET22b(+) vector with the inducible T7 promoter and produced up to 20% content of total soluble proteins [26]. The phytase gene with a native promoter derived from *B. amyloliquefaciens* was expressed in *B. subtilis*, which resulted in 100 times higher phytase yield in modified Luria broth medium [36].

Expression of *Bacillus* Phytases in Plants

Plants could be used as phytase producers and carriers for animal feeding. *Bacillus* phytases would be good candidates for producing transgenic plants because of their optimum pH under 7.0. The expression of a *Bacillus* phytase in transgenic plants may create a new biochemical pathway that mobilizes inorganic phosphate from phytate, so that more phosphorus is available for plant growth or physiology [19]. Engineering crop plants would produce heterologous phytase to reduce phosphate load on agricultural ecosystems with improving phosphate bioavailability [37].

Yip et al. [38] introduced a characterized phytase from *B. subtilis* into the cytoplasm of tobacco cells that resulted in equilibrium shift of inositol biosynthesis pathway, thereby making more phosphate available for primary metabolism. The transgenic line exhibited phenotypic changes like increased flowering, lower seed IP_6/IP_5 ratio, and enhanced growth under phosphate starvation conditions compared to wild type.

Lung et al. [39] reported that the phytase from *B. subtilis* (168phyA) was constitutively expressed in tobacco and *Arabidopsis* to generate transgenic plants capable of utilizing exogenous phytate. In tobacco, phytase activities in transgenic leaf and root extracts were seven to eight times higher than those in wild-type extracts; whereas, the extracellular phytase activities of transgenic plants were enhanced by four to six times. Similar results

were observed from the transgenic *Arabidopsis*. These results may offer a new perspective on mobilizing soil phytate into inorganic phosphate for plant uptake.

Chan et al. [40] studied the biochemical properties and kinetic parameters of the recombinant phytase (t168phyA) from transgenic tobacco. The t168phyA was glycosylated with a 4-kDa increase in molecular size. Its temperature optimum shifted from 60 to 45–50 °C and its pH optimum shifted from pH 5.5 to 6.0; whereas, the thermal stability remained unchanged. Kinetic data showed that the t168phyA had a lower K_{cat}, but a higher K_m than the native enzyme. Despite these changes, t168phyA remained catalytically active and has a specific activity of 2.3 U/mg protein. These results verify the activity of recombinant *Bacillus* phytase that is expressed in plants.

Commercial Prospects

Applications in Animal Nutrition

Because of the absence or insufficient amount of phytase in monogastric animal gastrointestinal tract, phytases are frequently added to their feed to facilitate optimal growth and decrease the supplementation of inorganic phosphate besides consequently reducing the phosphorus pollution in the areas of intensive livestock units. The *Bacillus* phytase is very suitable to be used in animal feed particularly in common carp feed because of its optimum pH with excellent 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].

Applications in Human Health

In addition to its major application in animal nutrition, phytase has also been found increasingly interesting for use in processing and manufacturing of food for human consumption. Supplemental phytases could play an important role in human nutrition, both for phytate degradation during food processing and in the gastrointestinal tracts [43]. Furthermore, phytase may find application in the production of functional foods or food supplements with health benefits [5, 44]. The effectiveness and limitations of food supplementation with phytases may also depend on their sensitivity to protease digestion [45]. The enzymes from *Bacillus* exhibit sensitivity to pancreatin, but a much higher susceptibility to pepsin [46]. Until now, *Bacillus* phytases are, however, not available for human consumption. With good thermal stability and the triphosphate as final product, future investigations into the application of *Bacillus* phytase in food are merited.

Application in Synthesis of Lower Inositol Phosphates

Lower inositol phosphates play crucial roles in energy metabolism, metabolic regulation, and signal transduction pathways in biological system [47]. The use of phytase has been shown to be very effective in producing different inositol phosphate species. Among the advantages of enzymatic hydrolysis over chemical synthesis are stereospecificity and mild

reaction conditions [8]. The *myo*-inositol trisphosphate isomer is the end product of phytate degradation by *Bacillus* phytases [25, 32, 33]. Therefore, these enzymes can be used for the preparation of *myo*-inositol triphosphates.

Conclusions and Future Perspectives

Along with the increasing concerns in relation to phosphorus pollution in the areas of intensive livestock, phytases have considerable potential in commercial and environmental applications. Potential applications of phytases in improving human health and in synthesis of lower inositol phosphates have increasingly gained attention. Under these conditions, significant progress has been made in phytase research during the last few decades. *Bacillus* phytases, which exhibit desirable activity profile under neutral pH, excellent thermal stability, and strict substrate specificity, are more promising for commercial exploitations. In addition, engineered transgenic plants harboring the phytase gene from *Bacillus* could also be used to improve soil fertilization and nutrient uptake by plants.

With the collaborative efforts of phytase scientists from different fields, effective solutions to the biotechnological development of *Bacillus* phytases for animal nutrition, human health, and environmental protection will be available in the near future.

Acknowledgments The authors gratefully acknowledge Miss Shirley for the critical reading of the manuscript. This study was financially supported by the Science and Technology Department of Zhejiang Province of China (2006C12036).

References

1. Cheryan, M. (1980). *Critical Reviews in Food Science and Nutrition*, *13*, 297–335.
2. Brinch-Pedersen, H., & Hatzack, F. (2006). *Current Analytical Chemistry*, *2*, 421–430.
3. Lei, X. G., & Porres, J. M. (2003). *Biotechnology Letters*, *25*, 1787–1794.
4. Cao, L., Wang, W. M., Yang, C. T., Yang, Y., Diana, J., Yakupitiyage, A., et al. (2007). *Enzyme and Microbial Technology*, *40*, 497–507.
5. Greiner, R., & Konietzny, U. (2006). *Food Technology and Biotechnology*, *44*, 125–140.
6. da Silva, L. G., Trugo, L. C., Terzi, S. C., & Couri, S. (2005). *Process Biochemistry*, *40*, 951–954.
7. Dvorakova, J., Kopecky, J., Havlicek, V., & Kren, V. (2000). *Folia Microbiologica*, *45*, 128–132.
8. Haefner, S., Knietsch, A., Scholten, E., Braun, J., Lohscheidt, M., & Zelder, O. (2005). *Applied Microbiology and Biotechnology*, *68*, 1–10.
9. Mullaney, E. J., & Ullah, A. H. J. (2003). *Biochemical and Biophysical Research Communications*, *312*, 179–184.
10. Kim, Y. O., Kim, H. K., Bae, K. S., Yu, J. H., & Oh, T. K. (1998). *Enzyme and Microbial Technology*, *22*, 2–7.
11. Gulati, H. K., Chadha, B. S., & Saini, H. S. (2007). *Journal of Industrial Microbiology & Biotechnology*, *34*, 91–98.
12. Choi, Y. M., Noh, D. O., Cho, S. H., Lee, H. K., Suh, H. J., & Chung, S. H. (1999). *Journal of Microbiology and Biotechnology*, *9*, 223–226.
13. Powar, V. K., & Jagannathan, V. (1982). *Journal of Bacteriology*, *151*, 1102–1108.
14. Shimizu, M. (1992). *Bioscience, Biotechnology, and Biochemistry*, *56*, 266–1269.
15. Kerovuo, J., Lauraeus, M., Nurminen, P., Kalkkinen, N., & Apajalahti, J. (1998). *Applied and Environmental Microbiology*, *64*, 2079–2085.
16. Choi, Y. M., Suh, H. J., & Kim, J. M. (2001). *Journal of Protein Chemistry*, *20*, 287–292.
17. Lei, X. G., & Stahl, C. H. (2000). *Journal of Applied Animal Research*, *17*, 97–112.
18. Oh, B. C., Choi, W. C., Park, S., Kim, Y., & Oh, T. K. (2004). *Applied Microbiology and Biotechnology*, *63*, 362–372.

19. Tye, A. J., Siu, F. K., Leung, T. Y., & Lim, B. L. (2002). *Applied Microbiology and Biotechnology*, *59*, 190–197.
20. Ha, N. C., Oh, B. C., Shin, S., Kim, H. J., Oh, T. K., Kim, Y. O., et al. (2000). *Nature Structural Biology*, *7*, 147–153.
21. Elkhalil, E. A. I., Männer, K., Borriss, R., & Simon, O. (2007). *British Poultry Science*, *48*, 64–70.
22. Oh, B. C., Chang, B. S., Park, K. H., Ha, N. C., Kim, H. K., Oh, B. H., et al. (2001). *Biochemistry*, *40*, 9669–9676.
23. Kerovuo, J., Lappalainen, I., & Reinikainen, T. (2000). *Biochemical and Biophysical Research Communications*, *268*, 365–369.
24. Idriss, E. E., Makarewicz, O., Farouk, A., Rosner, K., Greiner, R., Bochow, H., et al. (2002). *Microbiology*, *148*, 2097–2109.
25. Shin, S., Ha, N. C., Oh, B. C., Oh, T. K., & Oh, B. H. (2001). *Structure*, *9*, 851–858.
26. Kim, Y. O., Lee, J. K., Kim, H. K., Yu, J. H., & Oh, T. K. (1998). *FEMS Microbiology Letters*, *162*, 185–191.
27. van Etten, R. L., Davidson, R., Stevis, P. E., MacArthur, H., & Moore, D. L. (1991). *Journal of Biological Chemistry*, *266*, 2313–2319.
28. Ullah, A. H. J., & Mullaney, E. J. (1996). *Biochemical and Biophysical Research Communications*, *227*, 311–317.
29. Song, G. Y., Wang, X. Y., & Wang, M. (2005). *Protein Peptide Letters*, *12*, 533–535.
30. Wang, X. Y., Meng, F. G., & Zhou, H. M. (2004). *Biochemistry and Cell Biology*, *82*, 329–334.
31. Ha, N. C., Kim, Y. O., Oh, T. K., & Oh, B. H. (1999). *Acta Crystallographica. Section D, Biological Crytallography*, *55*, 691–693.
32. Oh, B. C., Chang, B. S., Park, K. W., Ha, N. C., Kim, H. K., Oh, B. H., et al. (2006). *Biochemistry*, *40*, 9669–9676.
33. Greiner, R., Lim, B. L., Cheng, C., & Carlsson, N. G. (2007). *Canadian Journal of Microbiology*, *53*, 488–495.
34. Kerovuo, J., Rouvinen, J., & Hatzack, F. (2000). *Biochemical Journal*, *352*, 623–628.
35. Greiner, R., Farouk, A., Alminger, M. L., & Carlsson, N. G. (2002). *Canadian Journal of Microbiology*, *48*, 986–994.
36. Kim, Y. O., Lee, J. K., Oh, B. C., & Oh, T. K. (1999). *Bioscience, Biotechnology, and Biochemistry*, *63*, 2205–2207.
37. Brinch-Pedersen, H., Sorensen, L. D., & Holm, P. B. (2002). *Trends in Plant Science*, *7*, 118–125.
38. Yip, W., Wang, L., Cheng, C., Wu, W., Lung, S., & Lim, B. L. (2003). *Biochemical and Biophysical Research Communications*, *310*, 1148–1154.
39. Lung, S., Chan, W., Yip, W., Wang, L., Yeung, E. C., & Lim, B. L. (2005). *Plant Science*, *169*, 341–349.
40. Chan, W. L., Lung, S. C., & Lim, B. L. (2006). *Protein Expression and Purification*, *46*, 100–106.
41. Zeng, H., Yao, B., Zhou, W. H., & Fan, Z. Y. (2001). *Journal of Aquaculture*, *15*, 87 (in Chinese).
42. Park, S. C., Choi, Y. W., & Oh, T. K. (1999). *Journal of Veterinary Medical Science*, *61*, 1257–1259.
43. Kaur, P., Kunze, G., & Satyanarayana, T. (2007). *Critical Reviews in Biotechnology*, *27*, 93–109.
44. Konietzny, U., & Greiner, R. (2004). *Brazilian Journal of Microbiology*, *35*, 11–18.
45. Konietzny, U., & Greiner, R. (2002). *International Journal of Food Science & Technology*, *37*, 791–812.
46. Igbasan, F. A., Manner, K., Miksch, G., Borriss, R., Farouk, A., & Simon, O. (2000). *Archives of Animal Nutrition*, *53*, 353–373.
47. Vats, P., & Banerjee, U. C. (2004). *Enzyme and Microbial Technology*, *35*, 3–14.