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Purification and Properties of Extracellular Phytase from *Bacillus* sp. KHU-10

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Bacillus species producing a thermostable phytase was isolated from soil, boiled rice, and mezu (Korean traditinal koji). The activity of phytase increased markedly at the late stationary phase. An extracellular phytase from *Bacillus* sp. KHU-10 was purified to homogeneity by acetone precipitation and DEAE-Sepharose and phenyl-Sepharose column chromatographies. Its molecular weight was estimated to be 46 kDa on gel filtration and 44 kDa on SDS–polyacrylamide gel elctrophoresis. Its optimum pH and temperature for phytase activity 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 or 10 min in the presence of 10 mM $CaCl_2$. The enzyme activity was fairly stable from pH 6.5 to 10.0. The enzyme had an isoelectric point of 6.8. As for substrate specificity, it was very specific for sodium phytate and showed no activity on other phosphate esters. The K_m value for sodium phytate was 50 μM. Its activity was inhibited by EDTA and metal ions such as Ba^{2+}, Cd^{2+}, Co^{2+}, Cr^{3+}, Cu^{2+}, Hg^{2+}, and Mn^{2+} ions.

KEY WORDS: *Bacillus* sp.; phytase; calcium.

1. INTRODUCTION

Phytase (myo-inositol hexakisphopate phosphorhydrolase, EC 3.1.3.8) catalyzes the release of phosphate from phytate (myo-inositol hexakiphosphate), which is the main form of phosphorus predominantly occurring in cereal grains, legumes, and oilseeds (Irving, 1980; Nayini and Markakis, 1986). Hydrolysis of phytic acid (phytate) to myo-inositol and phosphoric acid is considered an important metabolic process in several biosystems. Increasing awareness of agricultural pollution and demand for regulations in particular to limit the phosphorus (P) content in manure has intensified phytase research. The focus has mainly been on its production and use as a means of reducing inorganic P supplementation in feed and consequent reduction in fecal P excretion. Environmental pollution due to high-phosphate manure has resulted in the accumulation of P at various locations, especially in water bodies. Phytase supplementation can reduce the amount of P in manure up to approximately 30%.

Phytase is significant for upgrading the nutritional quality of phytate-rich feed. Phytase also improves the bioavailability of phytate P in plant foods for humans (Martinez *et al.,* 1996). Two important groups of animals, pigs and poultry, lack the enzyme needed to digest phytate efficiently in their feed (Greiner *et al.,* 1993). As a result, they excrete large amounts of P into the environment. This results in pollution. However, for their proper skeletal growth, these animals need P at suitable concentration. This makes the situation complex. Supplementation of phytase to the feed provides an alter native to tackle both these conditions effectively.

Phytases can be derived from a number of sources including plants, animals, and microorganisms. Recent research has shown that microbial sources are more promising for the production of phytases on a commercial level. Although several strains of bacteria (Sreeramulu *et al.,* 1996; Choi *et al.,* 1999), yeasts (Mayer *et al.,* 1999),

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and fungi (Ahmad *et al.,* 2000; Kim *et al.,* 1999) have been used for production under different conditions, two stratins of *Aspergillus* sp., *A. niger* (Ahmad *et al.,* 2000), and *A. ficuum* (Kim *et al.,* 1999) have most commonly been employed for its commercial production.

Although phytase shows a potential to be utilized for phytate bioconversion, the enzyme activities and yields need to be increased to make them possible for industrial application. Therefore, it is important to isolate a variety of different microorganisms and their enzymes for phytate degradation.

We reported that *Bacillus* sp. KHU-10 produced a high level of an extracellular phytase in a maltose, peptone, and beef extract medium. Under optimized conditions, the production of the phytase reached a highest level of 0.2 unit/ml after 4 days of fermentation (Choi *et al.,* 1999). We report here the characterization of a novel bacterial phytase from the recently isolated heat-tolerant strain *Bacillus* sp. KHU-10.

2. MATERIALS AND METHODS

2.1. Bacterial Strain and Culture Conditions

Bacteria isolated from soil, boiled rice, and mezu (Korean soybean koji) were cultivated in liquid medium at 37°C by reciprocal shaking at 200 rpm. The supernatant of culture broth was tested for the best phytase activity. *Bacillus* sp. KHU-10 was isolated from boiled rice as a strain with the best phytase activity. *Bacillus* sp. KHU-10 was cultivated on a medium containing 1.0% maltose, 1.0% peptone, 0.5% beef extract, 0.1% $CaCl_2$, and 0.1% $MgSO_4 \cdot 7H_2O$ and the initial pH was adjusted to 7.0. The 100-ml sterile medium in 300-ml Erlenmeyer flasks was inoculated with 2% seed culture broth.

2.2. Phytase Activity Assay

The phytase activity was measured by incubating 0.1 ml of enzyme solution with 0.9 ml of 2 mM sodium phytate in 0.1 M Tris–HCl buffer (pH 7.0). The enzyme reaction was carried out at 37°C for 10 min and then the reaction was stopped by adding 0.75 ml of 5% trichloroacetic acid. The liberated phosphate was measured at 700 rpm after adding 1.5 ml of color reagent, which is prepared freshly before using by mixing four volumes of 2.5% ammounium molybdate solution in 5.5% sulfuric acid and one volume of 2.5% ferrous sulfate solution. One unit of phytase activity was defined as to liberate 1 μmol of phosphate per minute under the assay condition. Protein was determined according to Bradford using a protein assay kit (Bio-Rad Lab., Richmind, CA) with bovine serum albumin as the standard.

2.3. Phytase Purification

Without special comment, all buffers used for the enzyme purification contained 2 mM $CaCl_2$ because Ca^{2+} ion proved to stabilize the enzyme activity. Culture supernatant containing the enzyme was centrifuged to remove insoluble residue and then concentrated to 1/10 of original culture supernatant by ultrafiltration through an Amicon YM10 membrane (Amicon Corporation, Danvers). Concentrated enzyme solution was fractionationed with acetone (30–70%) and then resuspended in 10 mM Tris–HCl buffer (pH 7.0). After dialysis, the enzyme solution was loaded on a DEAE–Sepharose column. The phytase activity was found in the fraction eluted with 0.15 M NaCl. The eluted enzyme was loaded again on phenyl-Sepharose CL-4B column and then eluted with decreasing linear gradient of NaCl solution from 4.0 to 0 M. The active fraction was dialyzed against 10 mM Tris–HCl buffer without $CaCl_2$ and concentrated using an ultrafiltration cell with YM10 membrane.

2.4. Molecular Weight Determination

The native molecular weight was determined by gel filtration on a Sephadex G-100 column using standard proteins (Sigma Chemical Co., St. Louis, MO) and by 12% SDS–PAGE using a kit of SDS–PAGE molecular weight standards (Sigma Co.) according to the recommendations by the supplier.

3. RESULTS

3.1. Cultivation of *Bacillus* sp. KHU-10

The medium for the production of phytase was used as described in Section 2. No phosphate salts were included in the medium. Cultivation was carried aerobically out at 37°C in the culture medium for 5 days. The activity increased markedly after the cells had reached the late stationary phase (Fig. 1). Because the synthesis of the enzyme was started as soon as the growth rate began to fall, it was suspected that either a nutrient or an energy limitation known to occur in the stationary phase could be at the origin of phytase induction. This stationary phase induction suggests that the phytase is not required during balanced growth and may be synthesized in the response to a limitation in some nutrient.

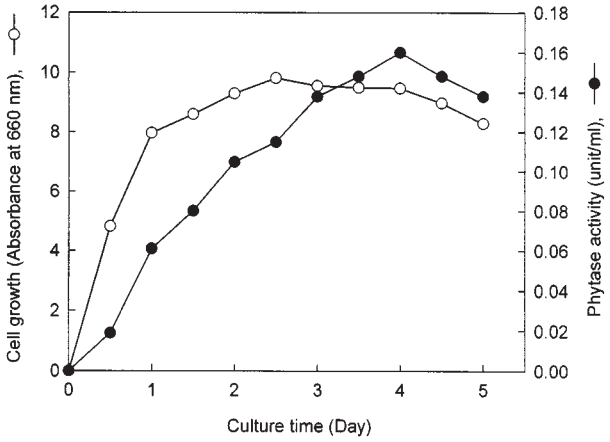

Fig. 1. Phytase activity during the growth of *Bacillus* sp. KHU-10 cultivated on medium containing 1.0% maltose, 1.0% peptone, 0.5% beef extract, 0.1% $CaCl_2$, and 0.1% $MgSO_4 \cdot 7H_2O$. The initial pH was adjusted to 7.0.

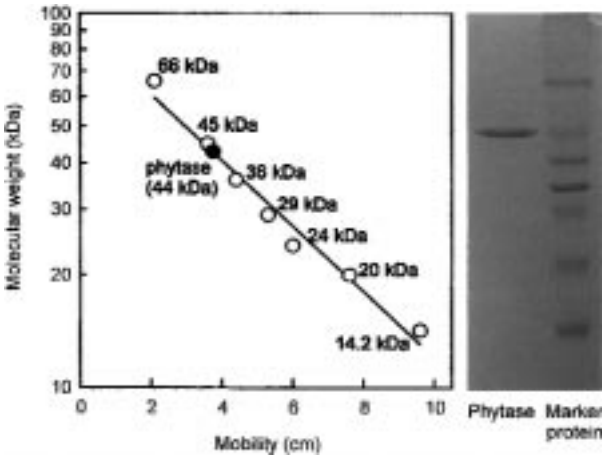

Fig. 2. SDS–polyacrylamide gel electrophoresis of the phytase from *Bacillus* sp. KHU-10. Lane 1: Purified phytase; lane 2: molecular weight standards including bovine albumin (66 kDa), oval albumin (45 kDa), glyceraldehyde-3-p-dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), and α-lactoalbumin (14.2 kDa).

3.2. Purification of the Phytase

Cells harvested in the late stationary phase were used as the source of enzyme. The phytase was purified using ion-exchange chromatography and hydrophobic column chromatography. The purification procedures for the phytase are summarized in Table I. The phytase activity was eluted as a single sharp activity peak from each column used. The phytase was purified 124-fold from the culture broth with 15.4% yield. The enzyme exhibits an activity of 36.0 units/mg.

3.3. Molecular Properties

The molecular mass and homogeneity of the purified enzyme were estimated by SDS–PAGE and gel filtration. Polyacrylamide gel electrophoresis under denaturing conditions revealed only one single protein band after Coomassie staining of the gels (Fig. 2). Isoelectric focusing gave one major band corresponding to a pI of about 6.8. These results indicate that the phytase could be regard as homogeneous. The apparent subunit molecular mass of the purified phytase was approximately 44 kDa as estimated by SDS–polyacrylamide gel electrophoresis (Fig. 2). The molecular mass of the native enzyme was determined as 46 kDa on a calibrated Sephadex G-100 gel chromatography.

3.4. Substrate Specificity

The substrate specificity of KHU-10 phytase on several phosphate esters was tested in 0.1 M Tris–HCl buffer (pH 7.0). Controls were run for determining initial phosphorus in each substrate. As summarized in Table II, the enzyme had high activity for sodium phytate, but no activity on other phosphorylated compounds including *p*-nitrophenylphosphate, a general substrate for acid phosphatase.

KHU-10 phytase was followed by Michaelis–Menten kinetics in the hydrolysis of sodium phytate. This is confirmed by the linear Lineweaver–Burk plot with the

Table I. Purification Procedures for the Phytase from *Bacillus* sp. KHU-10

Purification step	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Purification fold	Yield (%)
Culture broth	1500	510	0.34	1.0	100.0
Acetone fractionation	325	265	0.82	2.4	51.9
DEAE-Sepharose	18	165	9.17	26.9	32.4
Phenyl-Sepharose	3	108	36.00	105.8	21.1

Table II. Substrate Specificity of the Phytase from *Bacillus* sp. KHU-10

Substrate (2 mM)	Relative activity (%)
Sodium phytate	100
p-Nitrophenylphosphate	0
b-Glycerophosphate	0
Glucose-6-phosphate	0
Sodium glycerophosphate	0
AMP	0
ADP	0
ATP	0

substrate. The Michaelis constant (K_m) of KHU-10 phytase for sodium phytate was 50 μM. The corresponding calculated k_{cat} was 26.6 sec^{-1}.

3.5. Effect of pH and Temperature on the Enzyme Activity

The phytase activity was measured from pH 3.0 to 10.0 using *trans* aconitate-NaOH (pH 3.0–5.5), Tris–malate (pH 6.0–7.0), Tris–HCl (pH 7.5–8.5), and glycine–HCl (pH 9.0–10.0) buffer. The phytase showed high activity at relatively broad pH range between pH 6.5 and 8.5 in the reaction mixture without $CaCl_2$ (Fig. 3). The addition of 10 mM $CaCl_2$ to the reaction mixture enhanced the enzyme activity and showed higher activity in the wide pH range 6.0–9.5. However, the enzyme was fairly stable at pH 6.5–8.5 with the incubated buffers without $CaCl_2$ (Fig. 3). The addition of 10 mM $CaCl_2$ to the incubated buffers made the enzyme more stable in the pH range 6.5–10.0. Without $CaCl_2$, the optimum temperature for enzyme activity was 40°C and the enzyme was stable up to 40°C in 0.1 M Tris–HCl buffer (pH 7.0) for 10 min (Fig. 4). In the presence of 10 mM $CaCl_2$, the enzyme activity was the highest at 60°C and the remaining enzyme activity was above 95% after incubation at 60°C for 10 min. These results indicated that Ca^{2+} ion has an effect not only on the enzyme stability, but on enzyme activity against temperature and pH.

3.6. Effect of Metal Ions on the Phytase Activity

The effects of metal ions on phytase activity were examined with sodium phytate as a substrate after removal of Ca^{2+} ion from enzyme solution by ultrafiltration (Table III). The reduction (90%) of phytase activity by EDTA (1 mM) could be caused by an influence on the interfacial area between substrate and enzyme (Sztajer, 1992). The inhibition by EDTA was reversed by the addition of Ca^{2+} ion at double the concentration. Ba^{2+}, Cd^{2+}, Co^{2+}, Cr^{2+}, Cu^{2+}, Hg^{2+}, and Mn^{2+} ions reduced the phytase activity to 65%, 100%, 40%, 55%, 41%, 57%, and 83%, respectively. However, the reduction of the phytase activity by these metal ions was fairly recovered by the addition at the same concentration of Ca^{2+} ion, except for Cd^{2+} ion.

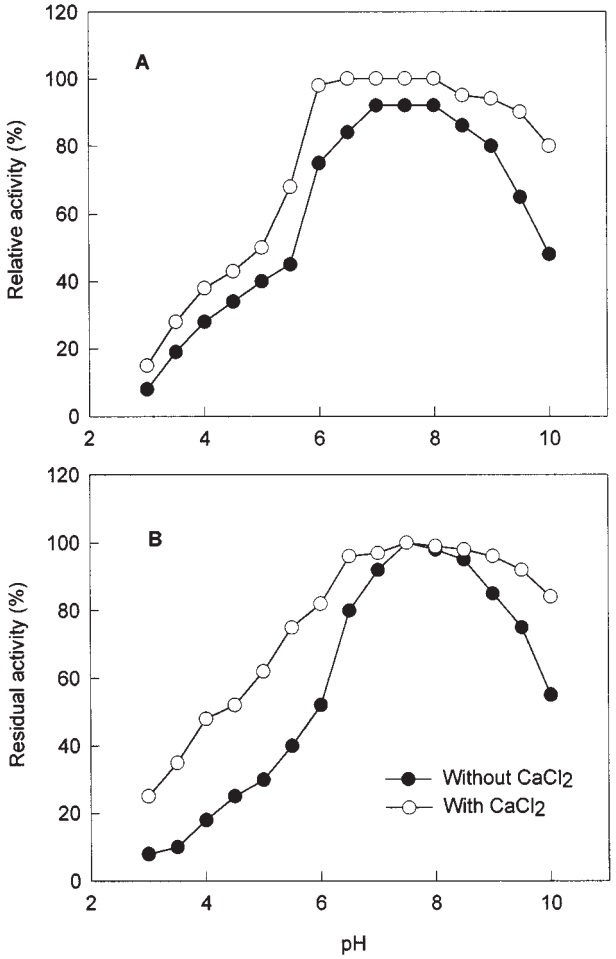

Fig. 3. Effect of pH on the phytase activity and stability. The enzyme activity (A) was assayed at various pH buffers without and with 10 mM $CaCl_2$. For determining pH stability (B), the enzyme was preincubated at various pH buffers without and with 10 mM $CaCl_2$ for 30 min and the remaining activity was measured using the standard reaction mixture. The buffers used are as follows: pH 3.0–5.5, *trans* aconitate-NaOH; pH 6.0–7.0, Tris–malate; pH 7.5–8.5, Tris–HCl; pH 9.0–10.0, glycine–HCl.

4. DISCUSSION

The phytase of *Bacillus* sp. KHU-10 is not synthesized in the exponential phase of growth. This stationary phase induction suggests that the phytase is not

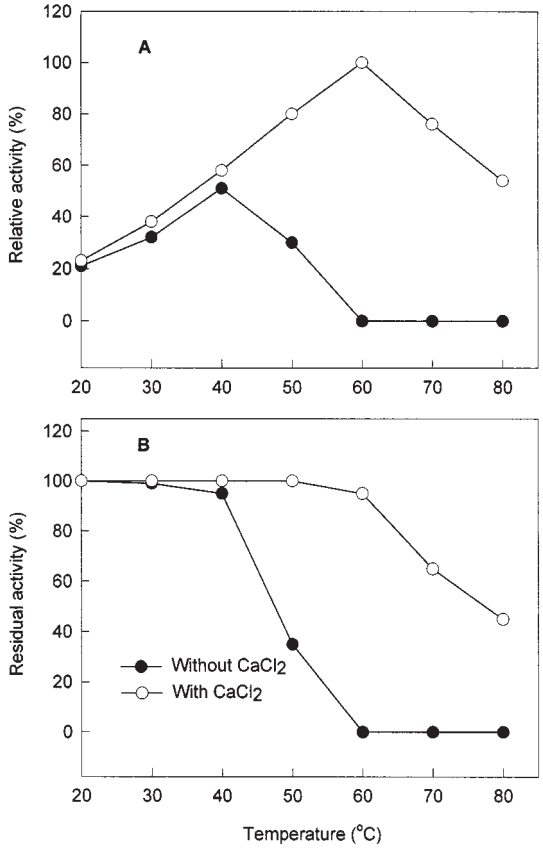

Fig. 4. Effect of temperature on the phytase activity and stability. The enzyme activity (A) was assayed at various temperatures without and with 10 mM $CaCl_2$. For determining thermal stability (B), the enzyme was preincubated at various temperatures without and with 10 mM $CaCl_2$ for 10 min. The remaining activity was measured using the standard reaction mixture.

required during balanced growth, and that it may be synthesized in response to a limitation in some nutrient. The synthesis has been shown to be stimulated by a limitation in phosphate (Greiner *et al.*, 1997) or anaerobiosis (Greiner *et al.*, 1993). The reduction of the growth rate observed when phosphate became rate limiting was not followed by a significant synthesis of phytase (Greiner *et al.*, 1997). Although phophate salts increased cell mass in our results, phytase activity decreased in the cell broth (data not shown). The addition of Ca^{2+} ion greatly stimulated the enzyme production with a concentration of 0.1% in the medium (Choi *et al.*, 1999). Phytase and wheat bran containing phytate were reported to enhance the phytase production in cell broth for *Bacillus subtilis* (Powar and Jagannathan, 1982), but there are no effects on phytase production for *Bacillus* sp. KHU-10. In *Escherichia coli* (Greiner *et al.*, 1997), phytate has no influence on the synthesis of phytate-degrading enzyme, but in *Klebsiella terrigena*, the phytase was produced only in the presence of phytate (Greiner *et al.*, 1997).

Table III. Effect of Metal Ions on the Phytase Activity from *Bacillus* sp. KHU-10

Metal	Relative activity (%)	
	Without $CaCl_2$	1 mM $CaCl_2$
Control	100	100
EDTA	6	120
$BaCl_2$	35	146
$CaCl_2$	102	100
$CdCl_2$	0	0
$CoCl_2$	60	102
$CrCl_3$	45	68
$CuCl_2$	59	84
$HgCl_2$	43	73
KCl	95	102
$LiCl_2$	96	102
$MgCl_2$	85	106
$MnCl_2$	17	82
NaCl	98	101

The purified phytase from *Bacillus* sp. KHU-10 shares some enzymatic properties in common with other phytases, but the enzyme shows some differences to phytases from the other *Bacillus* species (Shimizu, 1992; Kerovuo *et al.*, 1998). It showed a maximum activity at 60°C between pH 6.0 and 8.0, whereas phytases from other *Bacillus* sp. showed the maximum activity under acidic conditions (pH 4.5–6.0) and the activity was sharply decreased above pH 6.0. In addition, phytases from other *Bacillus* sp. showed an optimum temperature above 55°C (Pandey *et al.*, 2001).

The apparent subunit molecular mass of the purified phytase was approximately 44 kDa by SDS–polyacryamide gel electrophoresis (Fig. 2). The molecular mass of the native enzyme was determined as 46 kDa on a calibrated Sephadex G-100 gel chromatography. The extracellular phytase from *A. niger* had a molecular weight approximately 100 kDa (Dvorakova *et al.*, 1997). An extracellular phytase from *B. subtilis* (natto) N-77 had a smaller molecular weight (36 kDa) than our results. The purified phytase from *Bacillus* sp. showed an isoelectric point at 6.8, whereas the enzyme from other *Bacillus* sp. showed an isoelectric point at 6.25 (Shimizu, 1992).

The K_m values of other phytases are reported to range from 0.08 to 10 mM (Irving, 1980; Nayini and Markakis, 1986), but the K_m values of phytases for sodium phytate from *Bacillus subtilis* (natto) (Shimizu, 1992) and *Bacillus* sp. DS11 (Kim *et al.*, 1998) were reported to be 500 and 55 μM, respectively. In *Bacillus* sp. KHU-

10, the K_m value for sodium phytate was 50 μM. *Bacillus subtilis* phytase (Powar and Jagannathan, 1982) was specific for only sodium phytate and *Bacillus* sp. DS11 phytase (Kim *et al.*, 1998) and *Bacillus subtilis* (natto) N-77 phytase (Shimizu, 1992) were reported to have a high specificity for sodium phytate. *Aspergillus ficuum* phytase (Han and Gallagher, 1987) has broad specific activity for phosphorylated compounds. KHU-10 phytase was specific for only sodium phytate (Table II).

The turnover number of sodium phytate was determined to be 26.6 sec^{-1} for KHU-10 phytase. The only data available on molecular activities of other phytases are 6209 sec^{-1} for the *E. coli* phytase (Greiner *et al.*, 1993), 368 sec^{-1} for the spelt phytase (Konietzny *et al.*, 1995), 220 sec^{-1} for *Aspergillus ficuum* NRRL 3135 phytase (Ullah, 1988), and less than 10 sec^{-1} for soybean phytase (Gibson and Ullah, 1988). These results imply that *Bacillus* sp. KHU-10 phytase is specific for inositol polyphosphate. This property makes KHU-10 phytase distinct from *Bacillus subtilis* (natto) N-77 phytase (Shimizu, 1992).

The isolated enzyme required calcium for its activity and stability. In the addition of 10 mM $CaCl_2$, Ca^{2+} ion has effects not only on the enzyme stability, but the enzyme activity against temperature and pH (Figs. 2 and 3). Kerovuo *et al.* (1998) also studied the metal ion requirement of a *Bacillus subtilis* phytase. Removal of metal ions from the enzyme by EDTA resulted in complete inactivation. The loss of enzymatic activity was most likely due to 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. It was concluded that *B. subtilis* phytase required calcium for active conformation. Many other phytases, however, are not metalloenzymes. The most potent inhibitors of other phytases are Cu^{2+}, Zn^{2+}, fluoride, molybdate, vanadate, and phosphate.

The properties of the phytase from *Bacillus* sp. KHU-10 are favorable for the use of this enzyme in increasing the availability of phytate phosphate and minerals in food and feedstuff to nonruminants. Further work is in progress to elucidate the structure of the phytase and to overexpress the enzymes.

REFERENCES

Ahmad, T., Rasool, S., Sarwar, M., Haq, A. U., and Hasan, Z. U. (2000). *Anim. Feed Sci. Tech.* **83**, 103–114.

Choi, Y. M., Noh, D. o., Cho, S. H., Lee, H. K., Suh, H. J., and Chung, S. H. (1999). *J. Microbiol. Biotech.* **9**, 223–226.

Dvorakova, J., Volfova, O., and Kopecky, J. (1997). *Folia Microbiol.* **42**, 349–352.

Gibson, D. M. and Ullah, A. H. J. (1988). *Arch. Biochem. Biophys.* **260**, 503–513.

Greiner, R., Konietzny, U., and Jany, K. D. (1993). *Arch. Biochem. Biophys.* **303**, 107–113.

Greiner, R., Haller, E., Konietzny, U., and Jany, K. D. (1997). *Arch. Biochem. Biophy.* **341**, 201–206.

Han, Y. W. and Gallagher, D. J. (1987). *J. Ind. Microbiol.* **2**, 295–301.

Irving, G. C. J. (1980). In *Inositol Phosphates: Their Chemistry, Biochemistry, and Physiology* (Cosgrove, D. J., ed.), Elsevier Press, Amsterdam, pp. 85–98.

Kerovuo, J., Lauraeus, M., Nurminen, P., Kalkkinen, N., and Apajalahti, J. (1998). *Appl. Environ. Microbiol.* **64**, 2079–2085.

Kim, D. S., Godber, J. S., and Kim, H. R. (1999). *Biotechnol. Lett.* **21**, 1077–1081.

Kim, Y. O., Kim, H. K., Bae, K. S., Yu, J. H., and T. K. (1998). *Enzyme Microbiol. Technol.* **22**, 2–7.

Konietzny, U., Greiner, R., and Jany, K. (1995). *J. Food Biochem.* **18**, 165–183.

Martinez, C., Ros, G., Periago, M. J., Lopez, G., Ortuno, J., and Rincon, G. (1996). *Food Sci. Technol. Int.* **2**, 201–209.

Mayer, A. F., Hellmuth, K., Schlieker, H., Lopez-Ulibarri, R., Oertel, S., Dahlems, U., Strasser, A. W. M., and van Loon, A. P. G. M. (1999). *Biotechnol. Bioeng.* **63**, 373–381.

Nayini, N. R. and Markakis, P. (1986). In *Phytic acid; Chemistry and Applications* (Graf, E., ed.), Pilatus Press, Mineapolis, MN, pp. 101–118.

Pandey, A., Szakacs, G., Soccol, C. R., Rodriguez-Leon, J. A., and Soccol, V. T. (2001). *Bioresource Technol.* **77**, 203–214.

Powar, V. K. and Jagannathan, V. (1982). *J. Bacteriol.* **151**, 1102–1108.

Shimizu, M. (1992). *Biosci. Biotechnol. Biochem.* **56**, 1266–1269.

Sreeramulu, G., Srinivasa, D. S., Nand, K., and Joseph, R. (1996). *Lett. Appl. Microbiol.* **23**, 385–388.

Sztajer, H., Lnsdorf, H., Erdmann, H., Menge, U., and Schmid, R. (1992). *Biochim. Biophys. Acta* **1124**, 253–261.

Ullah, A. H. J. (1988). *Prep. Biochem.* **18**, 459–471.