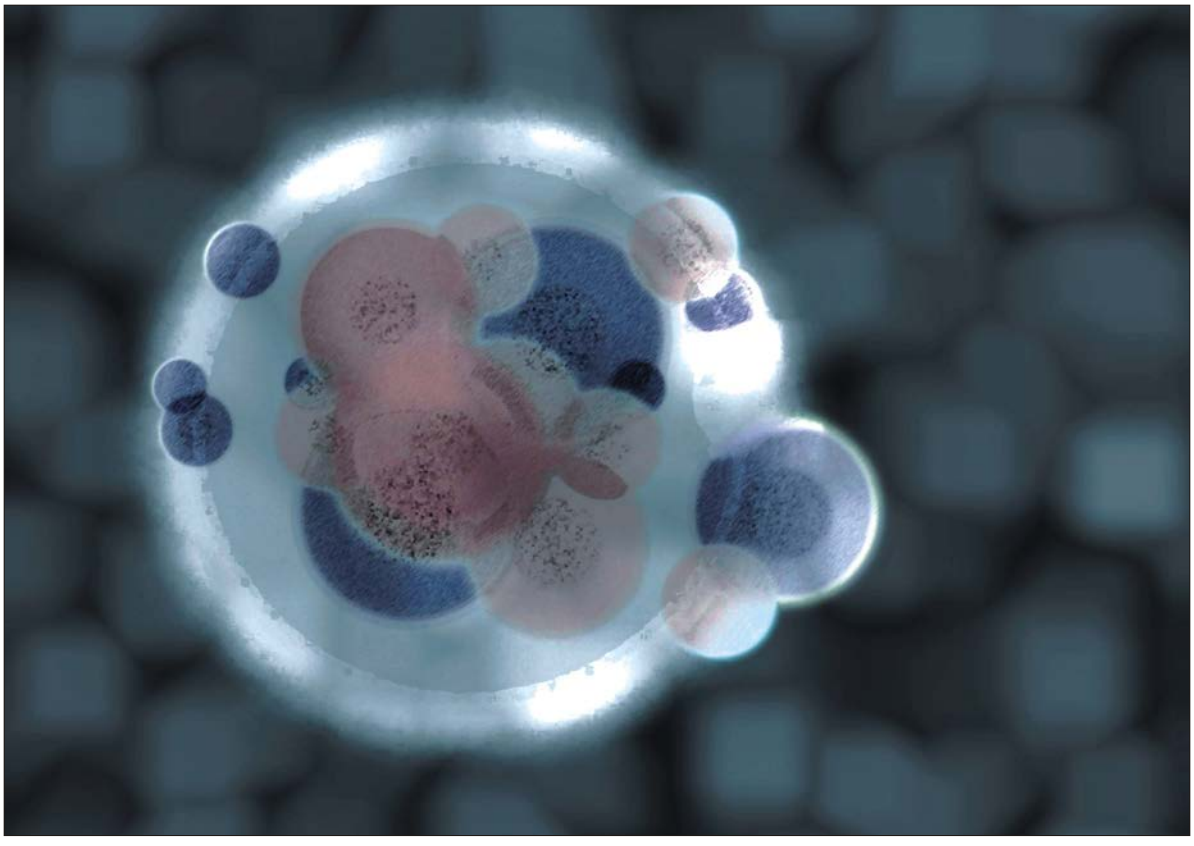

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Phytic Acid (Myo-inositol Hexakisphosphate) Phosphohydrolase from *Streptomyces hygroscopicus* NRRL B-1476

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Abstract: *Streptomyces hygroscopicus* NRRL B-1476 could produce an extracellular constitutive phytase when grown on liquid Czapek Dox's medium. Disappearance of phytase activity was observed on increasing orthophosphate concentration of the growth medium. Acetone-precipitated proteins of the growth medium showed optimum phytase activity at pH 6 and 50°C. The enzyme activity was retained when the enzyme preparation was exposed to 50°C for 30 min and was decreased and diminished on its exposure to higher degrees. Moderate activation of the enzyme action was observed on addition of Mg^{++} or Mn^{++} to the reaction mixture. Partial, considerable and complete inhibition were obtained in presence of Ag^{++}, Ca^{++} or Hg^{++} ; F^{-} and EDTA, respectively. Product inhibition was recorded on addition of orthophosphate to the reaction mixture. Orthophosphate inhibition was of the non competitive type and the K_m and the K_i values were found to be 3.03 and 1.6×10^{-3} M, respectively. *Streptomyces hygroscopicus* phytase exhibited a broad affinity for different phosphorylated compounds and the highest relative rate of hydrolysis was found with ATP.

Key words: Myo-inositol phosphate, *Streptomyces hygroscopicus* phosphatase, phytase, phytate

INTRODUCTION

Phosphate is an essential nutrient for living organisms. Phytate is the major form of phosphorus storage in the cereal grains and legumes used in commercial animal feeds (Garrett *et al*., 2004). Phytases (myo-inositol hexakisphosphate phosphohydrolases) belong to a special group of phosphatases that are capable of sequentially hydrolysing phytate [myo inositol (1,2,3,4,5,6) hexakisphosphate] to a series of lower myo-inositol phosphates and phosphate (Tijskens *et al*., 2001). Phytases have been studied intensively in the last few years. Phytase was originally proposed as an animal feed additive to enhance the value of plant material by liberating orthophosphate (Mitchell *et al*., 1997). More recently, phytase addition has been considered as a means to decrease levels of phosphate pollution (Cromwell *et al*., 1995). Because phytate also can act as an antinutrient by chelating minerals such as zinc, iron, calcium and magnesium, addition of phytase can improve the nutritional value of plant-based foods. It enhances the reduction of phytate during digestion in the stomach (Sandberg *et al*., 1996) or during food processes such as soaking, grinding, malting, fermentation, heat treatment and germination (Greiner and Jany, 1996; Reddy *et al*., 1982). Furthermore, phytases are of great interest in the production of special isomers of different lower phosphate esters of *myo*inositol. Certain *myo*-inositol phosphates have been suggested to have positive effects on heart diseases (Jariwalla *et al*., 1990; Potter, 1995) and for prevention of renal stone formation (Modlin, 1980; Ohkawa *et al*., 1984). The most extensively studied positive aspect of *myo*-inositol phosphates is their potential for reducing the risk of colon cancer (Baten *et al*., 1989; Graf and Eaton, 1993; Shamsuddin *et al*., 1997; Ullah and Shamsuddin, 1990; Vucenik *et al*., 1993; Yang and Shamsuddin, 1995). Several isomers of *myo*-inositol phosphates have shown important pharmacological effects, such as prevention of diabetes complications (Carrington *et al*., 1993; Ruf *et al*., 1991) and anti-inflammatory effects (Claxon *et al*., 1990).

In spite of the fact that extensive work has been carried out on phytases of several fungal organisms and plant seeds, to our knowledge the present research represents the first report that demonstrates phytases of organisms which belong to actinomycetes.

MATERIALS AND METHODS

Chemicals: Phytic acid [myo-inositol (1,2,3,4,5,6) hexakisphosphate], adenosine monophosphate, uridine monophosphate, guanosine monophosphate, adenosine triphosphate, glucose 1-phosphate and nicotinamide adenine dinucleotide are products of Sigma Chemical Co. Pyrophosphate and phenyl disodium orthophosphate (phph) were purchased from BDH Chemicals Ltd.

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Organisms: Two *Streptomyces* strains were used in this study *Streptomyces hygroscopicus* NRRL B-1476 and *Streptomyces erythrus* NRRL 2338 were obtained from Department of Agriculture, Peoria, Illinois, USA).

Medium: The organisms were grown and kept on slants of solid modified Czapek Dox's medium containing (g L^{-1} distilled water): Glucose, 30; Na NO_3, 2.0; KH_2PO_4, 1.0; $MgSO_4.7\ H_2O$, 0.5; KCl, 0.5 and agar 20.

Preparation of crude extracts: The 4 days old mats, grown on liquid modified Czapek-Dox's medium at 30°C were harvested by filtration, washed thoroughly with distilled water, blotted dry with absorbent paper then ground with cold washed sand in a chilled mortar and extracted with cold distilled water. The slurry so obtained was centrifuged at 5500 rpm for 10 min and the supernatant was used as the crude intracellular enzyme preparation.

Preparation of extracellular enzyme: To the broth of the growth medium one and half volume of cold acetone (-5) was added and the precipitate formed was separated by centrifugation at 12,000 rpm for 10 min at -10°C, then the precipitate was dissolved in 20 mL of cold 0.02M Tris-acetate buffer pH 6 and was used as the extracellular enzyme at preparation. Dialysis was carried out against 0.02 M of the same buffer for 24 h at 7°C.

Assay of phytase activity: The assay reaction mixture of the extracellular enzyme contained (in 1 mL vol.): 0.4 mL of enzyme preparation, 10 μmoles substrate and 80 μmoles Tris-acetate buffer at pH 6. The time of the reaction was 30 min and the temperature was 50°C. Enzyme activity was estimated colorimetrically by monitoring the release of inorganic phosphate from phytic acid according to the method of Ames (1966).

Protein determination: Protein of the extracts and of the enzyme preparation was estimated by the method of Sutherland *et al.* (1949).

Specific activity was expressed as μmoles of inorganic phosphate released from 10 μmoles substrate per mg protein per 30 min at 50°C. Appropriate control reaction mixtures where the enzyme source or the substrate was omitted were used as blanks through out the work. Each experiment cited in this work was repeated at least three times and all the results were reproducible.

RESULTS AND DISCUSSION

Detection of phytase activity in the extracts and in the growth medium of *Streptomyces hygroscopicus* and *Streptomyces erythrus*: Table 1 shows detection of intra- and extracellular phytase activities in cell extracts and in the growth medium of two different *Streptomyces* species when grown on the Dox's medium. The results indicate that the highest activity was obtained with extracellular enzyme of *S. hygroscopicus* NRRL B-1476 at pH 4. Thus, the acid extracellular phytase of this organism was chosen for the present study.

Constitutivity of *S. hygroscopicus* phytase: From results of Table 1 it appears that the phytase of this organism is a constitutive enzyme as it was formed by the cells in absence of phytic acid or other organophosphorus compounds.

Disappearance of activity on using higher concentrations of orthophosphate in the growth medium: Data of Table 2 show that when orthophosphate concentration of the growth medium was decreased, phytase activity was increased, while when the concentration was increased phytase activity completely disappeared. These data may suggest repression of the enzyme synthesis by high concentrations of orthophosphate in the growth medium.

Determination of the optimum pH for the phytase activity: When the effect of the pH on the phytase-catalyzed reaction was tested, it was found that the optimum pH for enzyme activity was around pH 6 (Fig. 1). This indicates that the enzyme belongs to the acid phytases and not to the alkaline ones. A rapid decline of the enzyme activity was observed on both sides of the pH optimum.

Table 1: Detection of phytase activity in the extracts and in the growth medium of *Streptomyces hygroscopicus* and *Streptomyces erythrus*

	Phytase units			
	Intracellular		Extracellular	
Organism	pH 4	pH 8	pH 4	pH 8
Streptomyces hygroscopicus	2.5	1.25	4.2	0.0
Streptomyces erythrus	2.13	0.94	2.8	0.0

Reaction mixture contained (in 1 mL vol): Substrate, 10.0 μmol; protein, 0.7 mg of extracellular enzyme preparation and 1.5 mg for intracellular enzyme; Tris-acetate buffer at pH 6, 80 μmol; temp, 50°C; incubation period, 30 min

Table 2: Effect of different concentrations of inorganic phosphate in the growth medium on phytase formation

Inorganic phosphate conc. (%)	Specific activity (units/mg protein)
0.05	7.5
0.1	6.4
0.2	0.0
0.5	0.0
1.0	0.0

Specific activity is expressed as μmol liberated from 10 μmol phytic acid mg^{-1} protein 30^{-1} min in presence of 80 μmol Tris-acetate buffer, at pH 6

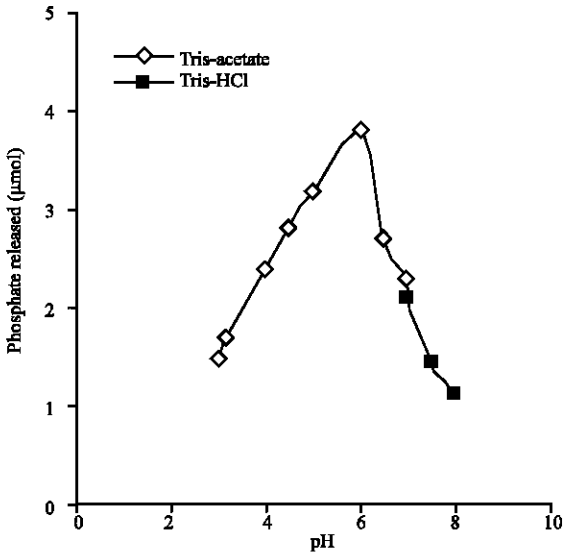

Fig. 1: pH dependence of the phytase activity. Reaction mixture contained: substrate, 10 µmol; buffer, 80 µmol Tris-acetate pH 3-7; Tris- HCl pH 7-8; protein, 0.3 mg; temperature, 50°C; total vol.,1 mL; time, 30 min

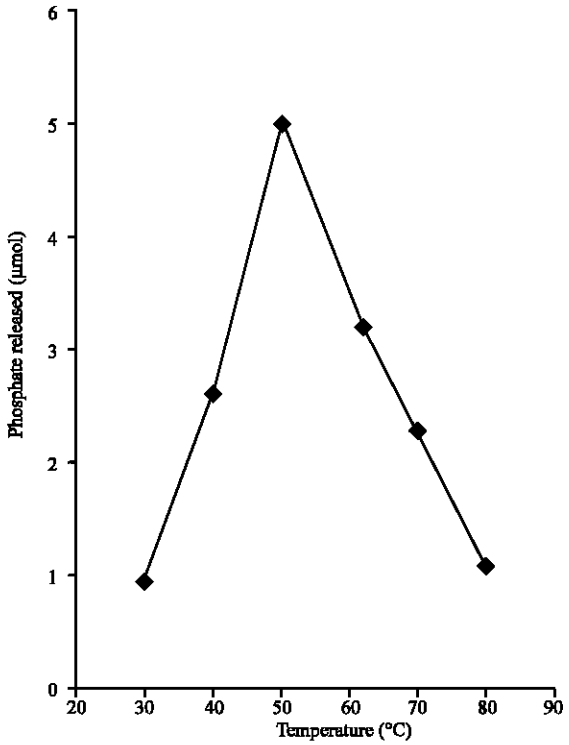

Fig. 2: Temperature-activity relationship of phytase. Reaction mixture contained: substrate, 10 µmol; buffer, 80 µmol Tris-acetate pH 6; protein, 0.3 mg; temperature, as indicated; total vol., 1 mL; time, 30 min

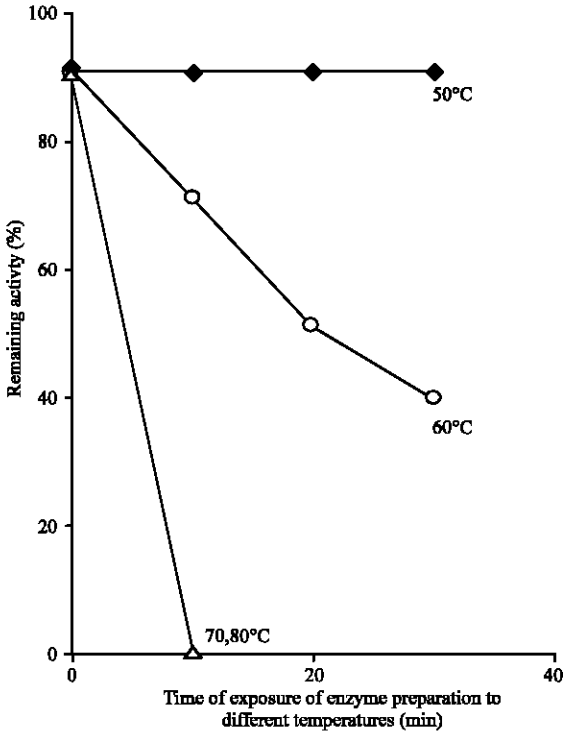

Fig. 3: Heat inactivation kinetic of the phytase. Reaction mixture contained: Substrate, 10 µmol; buffer, 80 µmol Tris-acetate pH 6; protein, 0.3 mg; temperature, 50°C; total vol., 1 mL; time, 30 min

Temperature-activity relation ship and heat inactivation kinetics of the phytase: Figure 2 demonstrates the pattern of dependence of the phytase activity on the temperature of the reaction. 50°C seems to be the optimum temperature. In this connection when heat inactivation kinetics was studied, it was found that incubation of the enzyme preparation at 50°C for 30 min in absence of the substrate before assay of the enzyme activity, no loss in this activity was observed (Fig. 3). However, when the incubation temperature was above 50°C, partial and complete loss of activity was recorded at 60 and 70°C, respectively. This indicates that the enzyme is not thermostable.

Partial dephosphorylation of phytate by the phytase preparation: When 10 µmol of phytate were incubated with *S. hygroscopicus* phytase preparation under the optimum pH and temperature, only about 20% of phosphate content of this amount of phytic acid (about 60 µmol orthophosphate) was released after 3 h incubation (Fig. 4). This might be due to product inhibition by phosphate. Also slower hydrolysis rates of the lower phosphate esters of myo-inositol might be the reason. Both factors might probably be responsible for the observed results.

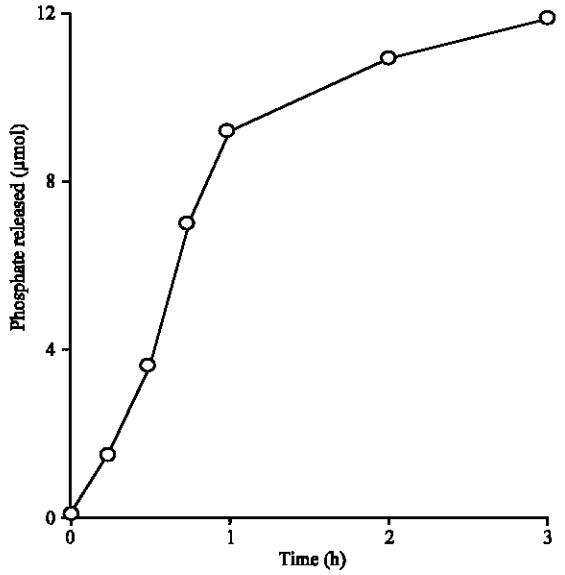

Fig. 4: Extent of Pi liberation from phytic acid. Reaction mixture contained: Substrate, 10 µmol; buffer, 80 µmol Tris-acetate pH 6; protein, 0.3 mg; temperature, 50°C; total vol., 1 mL; time, as indicated

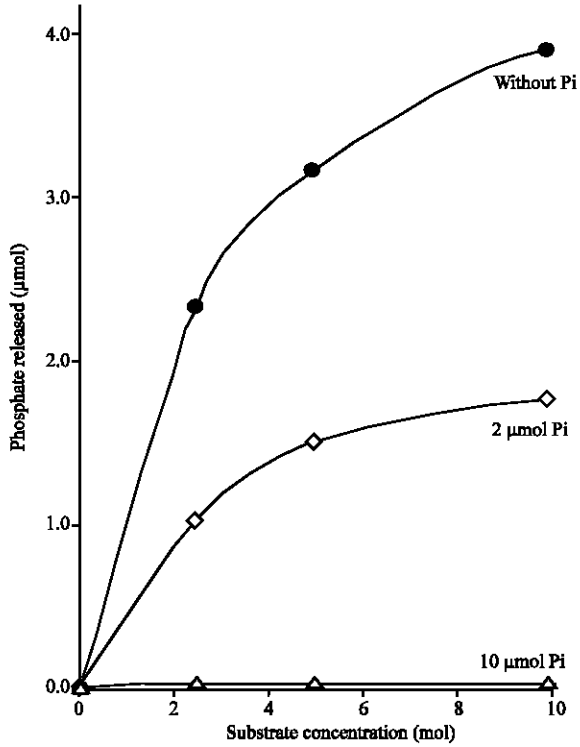

Fig. 5: Non-competitive inhibition of the phytase activity. Reaction mixture contained: substrate, 10 µmol; buffer, 80 µmol Tris-acetate pH 6; protein, 0.3 mg; temperature, 50°C; total vol., 1 mL; time, 30 min

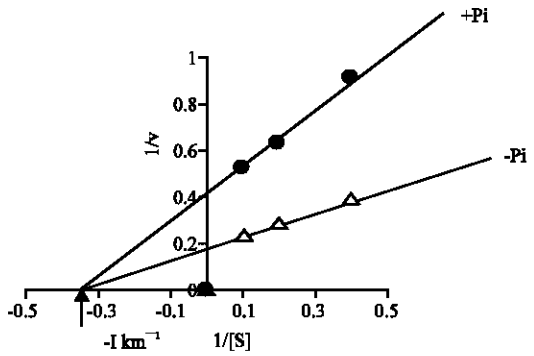

Fig. 6: Lineweaver Burk plot in presence and absence of phosphate

Inhibition of phytase activity by the products of the reaction: Incompletion of the dephosphorylation reaction observed in Fig. 4 led to the suggestion that this could be probably due to product inhibition. Substantiation of this assumption can be observed from results of Table 3 which show that, addition of phosphate or myo-inositol to the reaction mixture containing phytic acid as substrate, at the beginning of the reaction, revealed about 100 and 77% inhibition, respectively.

Determination of the type of inhibition exerted by inorganic phosphate: The plot of enzyme activity versus phytic acid concentration (mM) in absence and presence of orthophosphate clearly show the significant inhibition in phytase activity (Fig. 5 and 6). At 2 µmol mL^{-1} Pi concentration, approximately 45% inhibition of phytase activity was observed and as the concentration was increased to 10 µmol mL^{-1}, there was complete inhibition in the enzyme activity. From Fig. 5 and 6 it was found that the enzyme was inhibited in a non competitive manner by Pi. The apparent K_m value for phytic acid and the apparent K_i value for orthophosphate were calculated from the Line weaver Burk plot and found to be 3.03 and 1.6×10^{-3} M, respectively.

Effect of some compounds on phytase activity: As it appears from Table 3, Mg^{2+} and Mn^{2+} moderately activated the enzyme as about 30% increase in the enzyme activity was observed. Ag^{2+}, Ca^{2+} and Hg^{2+} moderately inhibited the activity. Fluoride has considerable inhibitory effect as it caused about 60% inhibition. Presence of EDTA led to almost complete inhibition of the enzyme activity, a result which may indicate that the *S. hygroscopicus* enzyme is a metalloenzyme.

Substrate specificity of phytic acid dephosphorylating enzyme: When *S. hygroscopicus* phytase was tested with

Table 3: Effect of addition some compounds on phytase activity of *S. hygroscopicus* NRRL B-1476

Compound (10^{-2}M)	Relative activity (%)
None	100.0
$AgCl_2$	67.8
$MnCl_2$	135.6
$CaCl_2$	65.5
$HgCl_2$	70.0
$MgSO_4$	127.8
Na_2HPO_4	0.0
Na F	41.1
$FeCl_3$	91.1
Myo-inositol	23.0
Mercaptoethanol	90.2
EDTA	5.6

Reaction mixture contained (in 1 mL vol): substrate, 10.0 μmol; protein, 0.30 mg; Tris-acetate buffer at pH 6, 80 μmol; temp, 50°C; incubation period, 30 min. conc of the added compounds, 10 μmol

Table 4: Substrate specificity of phytic acid dephosphorylating enzyme

Phosphorylated substances	Inorganic phosphate	Relative activity (%)
Sodium phytate	3.55	100.0
Pyrophosphate	6.46	182.0
Phenyl phosphate	3.35	94.0
Uridine monophosphate	3.38	95.0
Adenosine monophosphate	3.35	94.0
Guanosine monophosphate	3.32	93.0
Glucose 1-phosphate	3.36	94.6
Nicotinamide adenine dinucleotide	3.36	94.6
Adenosine triphosphate	22.09	622.0

Reaction mixture contained (in 1 mL vol): Substrate, 10.0 μmol; protein, 0.30 mg; Tris-acetate buffer at pH 6, 80 μmol; temp, 50°C; incubation period, 30 min

different phosphorylated compounds including phosphate esters and pyrophosphates, it was observed that the enzyme catalyzed phosphate liberation from all these substrates but with different rates (Table 4). Hence, the enzyme appears to have a broad substrate specificity and the highest relative rate of hydrolysis was with ATP.

As far as the authors are aware, the present study is the first that demonstrates phytases in organisms which belong to the actinomycetes.

From the results obtained, it can be suggested that *Streptomyces hygroscopicus* contains an extracellular and intracellular dephosphorylating enzyme that can dephosphorylate phytic acid to lower phosphorylated myo-inositol and Pi. This enzyme has constitutive nature as, it has been found in mats grown on synthetic medium containing glucose plus mineral salts only.

The acid phytase detected is one of two main types of phytases that have been previously identified: acid phytases with optimum pH around 5.5 and alkaline phytases with optimum pH around 8.0. The optimum pH of extracellular *Streptomyces hygroscopicus* enzyme indicates that it belongs to the acidic phytases, as are most of the so far-characterized cereal phytases: Oat: pH 5.0, (Greiner and Alminger, 1999), rye: pH 6.0, (Greiner *et at.*, 1998) spelt: pH 6.0 (Konietzny *et al.*, 1995) wheat: pH 5.5 (Nagai and Funahashi, 1962) and it is also similar to the optimum pH of fungal phytases that has been reported from *A. niger* (Vats and Banerjee, 2006), *A. oryzae* (Chantasartrasamee *et al.*, 2005) and six fungi reported by Tarafdar *et al.* (2002) that their optimum pHs were 2.5, 5.0 and 4.5, respectively. Also the yeast phytase reported by Kaur and Satyanarayana (2005) has its optimum pH at 4. The optimum pH of the *S. hygroscopicus* phytase was also similar to optimum pH of enzymes from seven different origins (five from grain species and two from bacterial species) reported by Tijskens *et al.* (2001). Only Lim and Tate (1973) detected a phytate degrading enzyme from wheat bran having its optimum pH at 7.2.

Recording product inhibition for the *Streptomyces hygroscopicus* phytase on addition of inorganic phosphate or myo-inositol to the reaction mixture is similar to what has been found with the phytase of *A. niger* (Vats and Banerjee, 2006) that was inhibited noncompetitively by phosphate and showed competitive inhibition with myo-inositol.

The *Streptomyces hygroscopicus* phytase exhibited a broad affinity for various phosphorylated compounds. In this respect, it resembles the phytases of oat, (Greiner and Alminger, 1999), of rye (Greiner *et at.*, 1998), of spelt: (Konietzny *et al.*, 1995) and of wheat (Nagai and Funahashi, 1962). The highest relative rate of hydrolysis was found with ATP in the present research.

The optimum temperature for the *Streptomyces hygroscopicus* phytase was found to be 50°C which is in accordance with most of the investigated phytases which exhibited an optimum temperature between 45 and 60°C. The rye and spelt enzymes show optimal phytate hydrolysis at 45°C. An optimum temperature of 55°C was reported for the maize seedling phytases (Hubel and Beck 1996). In general, the phytases from plants are less stable at higher temperatures than the corresponding enzymes from micro-organisms (Greiner and Alminger, 1999).

REFERENCES

Ames, B.N., 1966. Assay of Inorganic Phosphate, Total Phosphate and Phosphatases Methods in Enzymology. Colowick, S.P. and N.O. Kaplan (Eds.), Academic Press, New York and London, 8: 115-116.

Baten, A., A. Ullah, V.J. Tomazic and A.M. Shamsuddin, 1989. Inositolphosphate-induced enhancement of natural killer cell activity correlates with tumor suppression. Carcinogenesis, 10: 1595-1598.

Carrington, A.L., N.A. Calcutt, C.B. Ettlinger, T. Gustafsson and D.R. Tomlinson, 1993. Effects of treatment with myo-inositol or its 1,2,6-trisphosphate (PP56) on nerve conduction in streptozotocin-diabetis. Eur. J. Pharmacol., 237: 257-263.

Chantasartrasamee, K., D.A. Israngkul, S. Intarareugsorn and S. Dharmsthiti, 2005. Phytase activity from *Aspergillus oryzae* AK9 cultivated on solid state soybean meal medium. Process Biochemistry. 40: 2285-2289.

Claxon, A., C. Morris, D. Blake, M. Siren, B. Halliwell, T. Gustafsson, B. Löfkvist and I. Bergelin, 1990. The anti-inflammatory effects of D-myoinositol-1,2,6-trisphosphate (PP56) on animal models of inflammation. Agents Actions, 29: 68-70.

Cromwell, G.L., R.D. Coffey, G.R. Parker, H.J. Monegue and J.H. Rondolph, 1995. Efficacy of a recombinant-derived phytase in improving the bioavailability of phosphorus in corn-soybean meal diets for pigs. J. Anim. Sci., 73: 2000-2008.

Garrett, J.B., K.A. Kretz, O.D. Eileen, J. Kerovuo, K. William, N.R. Barton, G.P. Hazlewood and J. Short, 2004. Enhancing the thermal tolerance and gastric performance of a microbial phytase for use as a phosphate-mobilizing monogastric-feed supplement. Applied Environ. Microbiol., 70: 3041-3046.

Graf, E. and J.W. Eaton, 1993. Suppression of colonic cancer by dietary phytic acid. Nutr. Cancer, 19: 11-19.

Greiner, R., K.D. Jany, 1996. Ist Phytat ein unerwu¨nschter Inhaltsst off in Getreideprodukten. Getreide Mehl Brot., 50: 368-372.

Greiner, R., U. Konietzny, K.D. Jany, 1998. Purification and characterization of a phytase from rye. J. Food Biochem., 22: 143-161.

Greiner, R., M. Larsson Alminger, 1999. Purification and characterization of a phytate-degrading enzyme from germinating oat (*Avena sativa*). J. Sci. Food Agric., 79: 1453-1460.

Hubel, F. and E. Beck, 1996. Maize root phytase. Plant Physiol., 112: 1429-1436.

Jariwalla, R.J., R. Sabin, S. Lawson and Z.S. Herman, 1990. Lowering of serum cholesterol and triglycerides and modulation of divalent cations by dietary phytate. J. Applied Nutr., 42: 18-28.

Kaur, P. and T. Satyanarayana, 2005. Production of cell-bound phytase by *Pichia anomala* in an economical cane molasses medium: Optimization using statistical tools. Process Biochem., 40: 3095-3102.

Konietzny, U., R. Greiner and K.D. Jany, 1995. Purification and characterization of a phytase from spelt. J. Food Biochem., 18: 165-183.

Lim, P.E. and M.E. Tate, 1973,. The phytases. II. Properties of phytase fractions F1 and F2 from wheat bran and the myo-inositol phosphates produced by fraction F2. Biochim. Biophys. Acta, 302: 326-328.

Mitchell, D.B., K. Vogel, B.J. Weimann, L. Pasamontes and A.P.G.M. van Loon, 1997. The phytase subfamily of histidine acid phosphatases: Isolation of genes for two novel phytases from the fungi *Aspergillus terreus* and *Myceliophthora thermophila*. Microbiology, 143: 245-252.

Modlin, M., 1980. Urinary phosphorylated inositols and renal stone. Lancet, 11: 1113-1114.

Nagai,Y. and S. Funahashi, 1962. Phytase from wheat bran. Agric. Biol. Chem., 26: 794-803.

Ohkawa, T., S. Ebisuno, M. Kitagawa, S. Morimoto, Y. Miyazaki and S. Yasukawa, 1984. Rice bran treatment for patients with hypercalciuric stones. Experimental and clinical studies. J. Urol., 132: 1140-1145.

Potter, S.M., 1995. Overview of proposed mechanisms for the hypocholesterolemic effect of soy. J. Nutr., 125: 606s-611s.

Reddy, N.R., S.K. Sathe and D.K. Salunkhe, 1982. Phytates in legumes and cereals. Adv. Food Res., 28: 1-92.

Ruf, J.C., M. Ciavatti, T. Gustafsson and S. Renaud, 1991. Effects of PP-56 and vitamin E on platelet hyperaggregability, fatty acid abnormalities and clinical manifestations in streptozotocin-induced diabetic rats. Diabetes, 40: 233-239.

Sandberg, A.S., L. Rossander and M. Tu rk, 1996. Dietary *Aspergillus niger* phytase increases iron absorption in humans. J. Nutr., 126: 476-480.

Shamsuddin, A.M., I. Vucenik and K.E. Cole, 1997. IP-6: A novel anti-cancer agent. Life Sci., 61: 343-354.

Sutherland, E.W., C.R. Cori, R. Haynes and N.S. Olson, 1949. Purification of the hyperglycemic glycogenolytic factor from insulin and gastric mucosa. J. Biol. Chem., 180: 825-837.

Tarafdar, J.C., R.S. Yadav and R. Niwas, 2002. Relative efficiency of fungal intra-and extracellular phosphatases and phytase. J. Plant Nutr. Soil Sci., 165: 17-19.

Tijskens, L.M.M., R. Greiner, E.S.A. Biekman and U. Konietzny, 2001. Modeling the Effect of Temperature and pH on activity of enzymes: The Case of Phytases. Biotechnol. Bioeng., 72: 323-330.

Ullah, A. and A.M. Shamsuddin, 1990. Dose-dependent inhibition of large intestinal cancer by inositol hexaphosphate in F344 rats. Carcinogenesis, 11: 2219-2222.

Vats, P. and U.C. Banerjee, 2006. Catalytic characterization of phytase (myo-inositolhexakisphosphate phosphohydrolase) from *Aspergillus niger* van Teighem: Glycosylation pattern, kinetics and molecular properties. Enzyme Microbial. Technol., 39: 596-600.

Vucenik, I., K. Sakamoto, M. Bansal and A.M. Shamsuddin, 1993. Mammary carcinogenesis inhibition by inositol compounds. Cancer Lett., 75: 95-102.

Yang, G. and A.M. Shamsuddin, 1995. IP-6-induced growth inhibition and differentiation of HT-29 human colon cancer cells: Involvement of intracellular inositol phosphates. Anticancer Res., 15: 2479-2488.