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Production of Phytase from *Aspergillus niger* in solid state fermentation

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Extracellular phytase, a phytate hydrolyzing enzyme, was produced from *Aspergillus niger* by solid state fermentation (SSF). Different agricultural residues as substrates were investigated for phytase production by *Aspergillus niger* and maximum phytase activity was obtained with crushed rice straw (CRS). Various environmental parameters affecting phytase yield, such as, fermentation time, fermentation temperature, age of inoculum, shaking speed, hydration, amount of substrate and volume of flask were optimized for maximum production of phytase. It was found that highest yield of phytase enzyme (106.05 U/g dry substrate) was obtained with CRS as substrate (5 g/250 ml flask) having substrate-moisture ratio 1:5 at 168 hrs of fermentation and 30°C fermentation temperature under stationary condition.

Key words: Agricultural residue, *Aspergillus niger*, Phytase, Phytate-degradation, Solid state fermentation

INTRODUCTION

Phytic acid [myo-inositol (1,2,3,4,5,6) hexakis phosphate] is the major storage form of phosphorous in seeds and pollen (Maga, 1982). It is also the most frequent form of phosphorous in soil (Ehrlich *et al*, 1983). It is regarded as an antinutritive factor as it chelates vital ions like Ca^{2+}, Zn^{2+}, Mg^{2+}, Fe^{2+}, forms complexes with proteins (Graph, 1983). Thus high intakes of phytate decrease the bioavailability of metal ions and proteins (Erdman and Poneros-schneier, 1989 ; Fox and Tao, 1989). Moreover, monogastric animals are unable to use phytate phosphorous lacking endogenous phytase activity. As a result, they excrete large amounts of phytate into the environment, causing pollution (Nasi, 1990). Therefore, the enzymatic hydrolysis of phytic acid into less-phosphorylated myo-inositol derivatives in the intestine of monogastric animals is desirable (Kerovuo, 1998). Many attempts to enzymatically hydrolyze phytic acid have been made to improve the nutritional value of feed and to decrease the amount of phytate excreted by animals (Lambrechts *et al*, 1992). Phytase (E.C.3.1.3.26) hydrolyzes phytic acid to myo-inositol phosphates and inorganic phosphate. Phytases are widely distributed in nature (Dvorakova, 1998 ; Wodzinski and Ullah, 1996). Phytase activity in microorganisms has been found most frequently in fungi (Zyta, 1992), in particular Aspergilli (Hirabayashi *et al*, 1998 and Howson *et al*, 1983). Among Aspergilli, there are few reports of phytase production by SSF, such as, *A. ficcum* (Ebune *et al.*, 1995; Han *et al.*, 1987; Nair *et al.*, 1990) and *A. carbonarius* (Al-Asheh and Duvnjak,1994a,b,c). Phytases are of interest for biotechnological applications, especially for the reduction of phytate in food and feedstuff. Supplementation of animal feedstuff with phytases will increase the bioavailability of phosphate, decreasing phosphorous pollution in areas of intensive animal agriculture. The enzymatic degradation of phytic acid will not produce mutagenic and highly toxic byproducts; thus exploitation of enzymes in the industrial process would be environmentally friendly and would assist in development of novel technologies (Liu *et al.*, 1998). A large number of agricultural residues were employed as substrate for production of vari-

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ous industrial enzymes including phytases by different microorganisms in SSF (Bogar *et al.*, 2003; Mandviwala and Khire, 2000; Pandey *et al.*, 1999; Singh and Satyanarayana, 2010). But there is hardly any report on phytase production using rice straw as substrate in SSF. The present communication reports production of high activity phytase in SSF from *A. niger* using various agricultural residues as substrate.

MATERIALS AND METHODS

Chemicals: Phytic acid sodium salt was purchased from Sigma Aldrich, USA. All the other chemicals and reagents were commercially available and of analytical grade. Various agricultural residues were purchased from a local feedstuff outlet.

Microbial strain and its maintenance: *Aspergillus niger*, maintained in Czapeck Dox slant by periodic transfer and stored at 4°C.

Inoculum used: Spore suspension (10^7 spores/ml) prepared from fresh 7-day old sporulated culture grown on Czapeck dox slant was used to inoculate the fermentation medium.

Phytase production in SSF

Solid State Fermentation was done by taking 5gm wheat bran (or any other agricultural residue) in 250 ml Erlenmeyer flask moistened with 2.5 ml distilled water and was sterilized by autoclaving at 121°C temperature and 15 lb pressure for 20 minutes (Ghada *et al.*, 2011) and then cooled to room temperature. It was then aseptically inoculated with spore suspension and mixed thoroughly using a sterile inoculating needle for uniform distribution of fungal spores in the medium. The flasks were then incubated at 30°C. Fermentation was done for upto 10 days. At every 24 hours interval, one flask was taken out of incubation and its contents were extracted. After SSF, 50 ml of 0.2 M sodium acetate buffer of pH-5 was added to each flask. Then flasks were kept on rotary shaker for 3 hours at 30°C and 120 r.p.m.. After 3 hours, the suspension was squeezed through a double layer of muslin cloth and then filtered through Whatman No. 1 filter paper. The filtrate was then centrifuged at 10,000 r.p.m. for 20 minutes at 4°C. The cell free clear supernatant was used as crude enzyme.

Enzyme Assay: Enzyme assay was performed according to Sigma protocol with little modifications (Das and Ghosh, 2012) taking the cell free extract. The inorganic phosphate released from the substrate by enzymatic hydrolysis was measured according to the method of King (1932). 1Unit was defined as the amount of phytase that liberates 1micromole of phosphate per minute, under the assay condition. The enzymatic activity was expressed in U/gds (i.e. gram dry substrate) according to the method described by Ramachandran *et al.*, (2004).

Optimization of environmental parameters on production of phytase by SSF :

1) ***Time course of fermentation:*** Optimization of fermentation time was done by performing fermentation upto 10 days as mentioned above. One fermented flask was harvested at every 24 hours interval its contents were extracted and checked for phytase activity.

2) ***Agricultural residues as substrate:*** To study the effect of substrate on phytase production, different agricultural residues such as pigeonpea husk, bengalgram husk, cornmeal, mustard oil cake, groundnut cake, corncob, moongbean husk, blackgram husk, red lentil husk, sesame oil cake, neem cake, wheat bran, rice bran, rice straw and wheat straw were used as substrate (5 g substrate and 2.5 ml distilled water in each of 250 ml flask) and fermentation was continued for 168 hrs. The fermented cell free extract was prepared as before and checked for phytase activity. The agricultural residue giving highest enzyme activity was selected and used as substrate in further SSF experiments.

3) ***Effect of Hydration:*** To study the effect of hydration on phytase production, fermentation was done by taking 5gm CRS in a 250ml flask moistened with different amount of distilled water. The substrate - moisture ratio was varied as 1:0.5, 1:1, 1:2, 1:3, 1:4, 1:5, 1:6 and 1:8 and fermentation was continued for 168 hrs. The effective substrate - moisture ratio giving highest enzyme activity was used in further experiments.

4) ***Effect of Amount of Substrate:*** Amount of CRS was varied as 2.5g, 5g, 7.5g and 10g per 250 ml Erlenmeyer flask and fermentation was done for 168 hours.

5) ***Fermentation Temperature:*** To study the effect of fermentation temperature on phytase production, fermentation was carried out with 5gm CRS and 25 ml distilled water taken in a 250ml flask at 25°C, 30°C and 37°C temperature for 7 days.

6) ***Effect of Volume of flask:*** To study the effect of flask volume on phytase production, fermentation was carried out by taking 5gm CRS and 25 ml distilled water in each of 50ml, 100ml, 250ml and 500ml Erlenmeyer flask and fermentation was continued for 168 hrs.

7) ***Effect of Aeration on Phytase Production:*** To study the effect of aeration, 5gm CRS and 25 ml distilled water were taken in each flask and flasks were incubated at 30°C at different shaking speed upto 150 r.p.m.. One flask was kept under stationary condition at 30°C. Fermentation was done for 168 hours.

8) ***Effect of Age of Inoculum on Phytase Production:*** To study the effect of age of inoculum on phytase production, 5gm CRS and 25 ml distilled water were taken in each of 250ml flask and fermentation was done with spore suspension (as 0 day), one day, two days and three days old germinated spore suspensions (final spore count 10^7 cells/flask).

Optimum values of each of these environmental parameters were determined with respect to highest enzyme activity in respective cases.

Each experiment was carried out in triplicate and the values reported are the mean of three such experiments in which a maximum of 3-5% variability was observed.

RESULTS AND DISCUSSION

Phytase production by filamentous fungi including different species of *Aspergillus* in SSF were reported by several workers (Al Asheh and Duvunjak, 1994; Ebune *et al.*, 1995; Han *et al.*, 1987; Hirabayashi *et al.*, 1998; Howson and Davis, 1983; Mandviwala and Khire, 2000; Nair and Duvunjak, 1990; Zyta, 1992). But there is hardly any report on phytase production with CRS. In the present study, fifteen different agricultural residues were employed as substrates and maximum phytase production (106.05 U/g dry substrate) was obtained with crushed rice straw (Table 2). A highly active

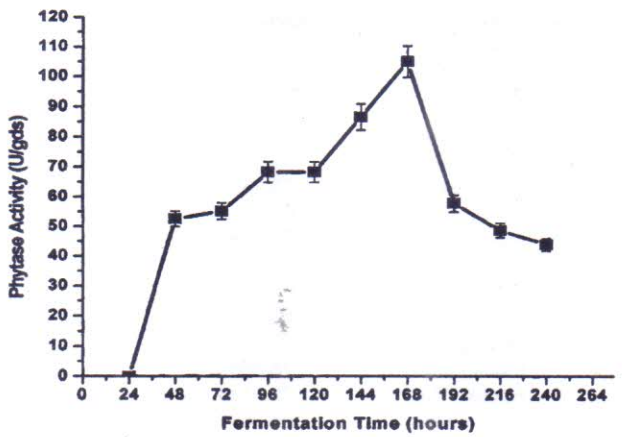

Fig . 1: Phytase production at different time of fermentation

Table 1: Phytase production at different time of fermentation

Fermentation Time (hrs)	Phytase Activity (U/gds)
24	-
48	52.5±1.3
72	55.125±0.125
96	68.25±0.75
120	68.25±0.25
144	86.62±1.62
168	105±0.2
192	57.75±0.25
216	48.64±0.64
240	43.92±0.64

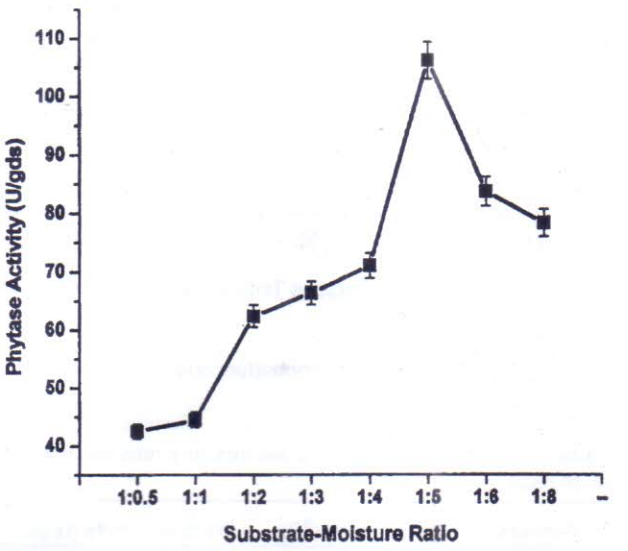

Fig .2: Effect of different substrate-moisture ratio on production of phytase

phytase was produced by this strain of *A. niger* using CRS as substrate compared to that of by other strains, such as, 108 U/g dry mouldy bran (Mandviwala and Khire, 2000); 66.4 U/g dry solid using wheat bran (Gunashree and Venkateswaran, 2008) and 0.6 U/ml using rice bran as substrate (Cha *et al*, 2010).

Table 2: Effect of various substrates on production of phytase

Substrates	Phytase Activity (U/gds)
Rice bran	17.23±0.33
Pegionpea bran	27.84±1.25
Bengalgram bran	44.41±0.51
Cornmeal	78.21±1.09
Wheat straw	84.18±2.77
Mustard oilcake	98.76±0.24
Rice straw	106.05±2
Groundnut cake	100.75±0.75
Corncob	86.17±1.17
Moongbean bran	83.52±1.48
Blackgram bran	78.88±0.12
Redlentil bran	72.91±1.09
Sesame oilcake	66.95±0.95
Wheat bran	55±0.8
Neem cake	54.35±0.65

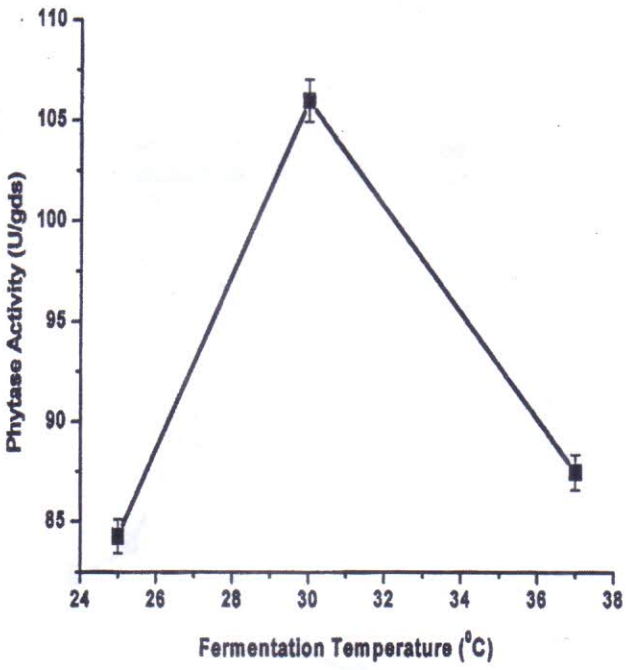

Fig. 3 : Effect of fermentation temperature on production of phytase

Table 3: Effect of different substrate-moisture ratio on production of phytase

Fermentation Temperature (°C)	Phytase Activity (U/gds)
25	84.27±0.27
30	106±0.8
37	87.45±0.71

Phytase production gradually increased with time and became maximum at 168 hrs (Table 1 and Fig. 1) and fermentation beyond 168 hrs showed decreased yield of phytase. In SSF, moisture level is crucial for product biosynthesis by filamentous fungi (Ebune *et al.*, 1995; Mandviwala and Khire,

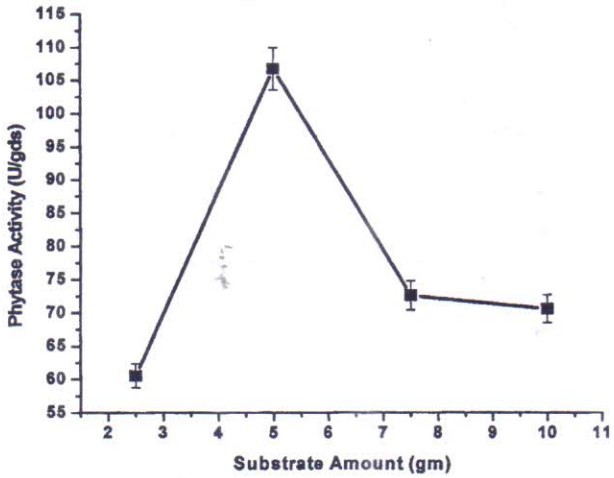

Fig. 4 : Effect of different amount of substrate on production of phytase

Table 4: Effect of fermentation temperature on production of phytase

Substrate Amount (gm)	Phytase Activity (U/gds)
2.5	60.5±0.3
5	106.72±0.72
7.5	72.5±1.5
10	70.5±0.5

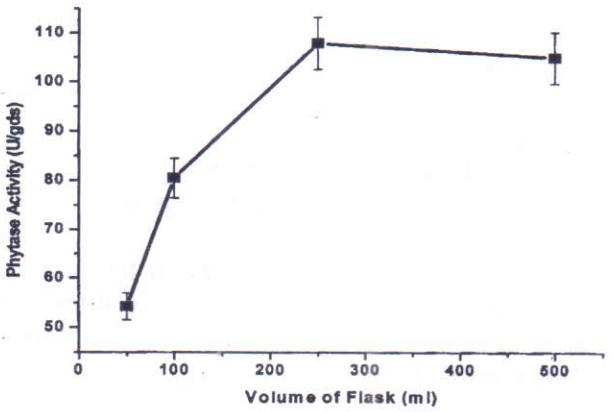

Fig. 5 : Effect of volume of flask on production of phytase

Table 5 : Effect of different amount of substrate on production of phytase

Volume of Fermentation Flask (mL)	Phytase Activity (U/gds)
50	54.2±0.8
100	80.5±0.5
250	108.04±1.25
500	105.2±1.56

2000).We also worked out such crucial ratio of substrate to distilled water as 1:5 for maximum phytase production (Table 3 and Fig. 2). The optimum tem-

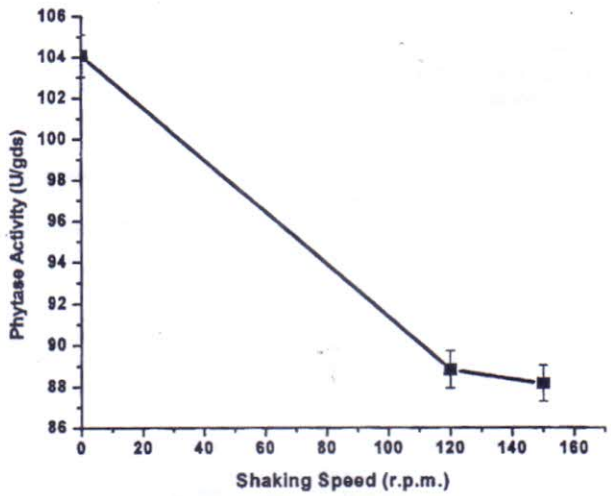

Fig. 6 : Effect of shaking speed on production of phytase

Table 6 : Effect of volume of flask on production of phytase

Shaking Speed (r.p.m.)	Phytase Activity (U/gds)
0	104.06±0.98
120	88.82±1.42
150	88.16±2

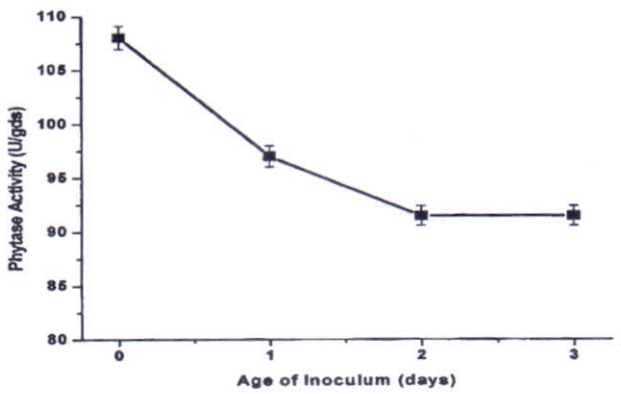

Fig. 7 : Effect of age of inoculation on production of phytase

Table 7 : Effect of shaking speed on production of phytase

Age of Inoculum (days)	Phytase Activity (U/gds)
0	108.04±2.04
1	97±2.06
2	91.47±0.98
3	91.47±0.02

perature for SSF was 30°C (Table 4 and Fig. 3) and at temperatures lower or higher than 30°C, the enzyme yield was less. Critical relation between amount of substrate and volume of flask was found to be 5 g substrate (CRS) in 250 ml flask for maximum production of phytase (Table 5 & 6 and Fig. 4&5). The yield of phytase at stationary condition was better than that of shaking conditions (Table 7 and Fig. 6). Inoculum as spore suspension showed better yield of phytase than germinated spores of different ages as shown in Fig.7.

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REFERENCES

Al-Asheh, S and Duvnjak, Z. 1994a. Characteristics of phytase produced by *Aspergillus carbonarius* NRC 401121 incanola meal. *Acta. Biotechnol.* **3**: 223-233.

Al-Asheh, S and Duvnjak, Z. 1994b. Effect of glucose concentration on the biomass and phytase productions and the reduction of phytic acid content in canola meal by *Aspergillus carbonarius* during a solid state fermentation process. *Biotechnol. Prog.* **10**: 353-359.

Al-Asheh, S and Duvnjak, Z. 1994c. The effect of surfactants on the phytase production and the reduction of the phytic acid in canola meal by *Aspergillus carbonarius* during a solid state fermentation process. *Biotechnol Lett.* **2**: 183–188.

Al-Asheh, S and Duvnjak, Z. 1995. Phytase production and decrease of phytic acid content in canola meal by *Aspergillus carbonarius* in solid-state fermentation. *World J Microbiol Biotechnol.* **11**: 228–231.

Bogar, B.; Szakaes, G.; Linden, J.C.; Pandey, A. and Tengerdy, R.P.. 2003. Optimization of phytase production by solid state fermentation. *J. Ind. Microbiol Biotechnol.* **30**: 183-189.

Cha, J.Y.; Dower, N.; Luechai, S. & Dharmsthiti, S.C.. 2010. Multiple non-polysaccharide-degrading enzyme production from solid state fermentation of *Aspergillus niger* AK 10. *As. J. Food Agri-Ind.* **3**(10): 108-119.

Das, S.; and Ghosh, U..2012. Production of Phytase by Submerged Fermentation. *J. Mycopathol. Res.* **50**(1): 93-96.

Dvorakova, J.. 1998. Phytase: sources, preparation and exploitation. *Folia Microbiol.* **43**: 323–338.

Ebune, A.; Al-Asheh, S. and Duvnjak, Z.. 1995. Effects of phosphate, surfactants and glucose on phytase production and hydrolysis of phytic acid in canola meal by *Aspergillus ficuum* during solid state fermentation. *Biores Technol.* **54**: 241–247.

Ehrlich, K.C.;Montalbano, B.G.;Mullaney, E.J.;Dischinger, H.C.;Ullah, A.H..1983.Identification and cloning of a second phytase gene (*phyB*) from *Aspergillus niger* (ficuum).*Biochem.Biophys.Res .Commun.***195:**53-57.

Enzymatic Assay of Phytase. www.sigmaaldrich.com/life sciences.

Erdman, J.W. and Poneros-Schneier, A.. 1989. Phytic acid interactions with divalent cations in food and in the gastrointestinial tract. *Adv Exp Med Biol.* **249**: 167–171.

Fox, M.R.S. and Tao, S.H.. 1989. Antinutritive effects of phytate and other phosphorylated derivatives. *Nutr Toxicol.* **3**: 59–96.

Ghada, E.A. Awad; Elnashar, Magdy M.M. and Danial, Enas N.. 2011. Optimization of phytase production by *Penicillium funiculosum* NRC467 under solid state fermentation by using full factorial design. *World Applied Sciences Journal.* **15** (11): 1635-1644.

Graf, E..1983.Calcium binding to phytic acid.*J.Agric.Food Chem.* **31:**851-855.

Gunashree, B.S & Venkateswaran, G.. 2008. Effect of different cultural conditions for phytase production by *Aspergillus niger* CFR 335 in submerged and solid state fermentations. *J Ind Microbiol Biotechnol.* **35**: 1587-1596.

Han, Y.M,; Gallagher, D.J. and Wilfred, A.G.. 1987. Phytase production by *Aspergillus ficuum* on semisolid substrate. *J Ind Microbiol.* **2**: 195–200.

Hirabayashi, M.;Matsui, T. and Yano, H.. 1998. Fermentation of soyabean flour with *Aspergillus usamii* improves availabilities of zinc and iron in rats. *J Nutritional Science and Vitaminology.* **44:** 877-886.

Howson, S.J. and Davis, R.P.. 1983. Production of phytase hydrolyzing enzyme by some fungi. *Enz Microbiol Technol.* **5:** 377-382.

Kerovuo, J.. 1998. Isolation, characterization, molecular gene cloning, and sequencing of a novel phytase from *Bacillus subtilis Applied and environmental microbiology.* **64:** 2079-2085.

King, E.J.. 1932. The colorimetric determination of phosphorous. *Biochem. J.* **26:** 292-297.

Lambrechts, C.; Boze, H.;Moulin, G.;Galzv, P.. 1992. Utilization of phytate by some yeast. *Biotechnol. Lett.* **14:** 61-66.

Liu, B.L.; Rafiq, A.; Tzeng, Y.M. and Rob, A.. 1998. The induction and characterization of phytase and beyond. *Enz Microbiol Chem.* **22:** 415-424.

Maga, J.A.. 1982. Phytate : its chemistry, occurence, food interactions, nutritional significance, and methods of analysis. *J Agric Food Chem.* **30:** 1-9.

Mandviwala, T.N. and Khire, J.M..2000. Production of high activity thermostable phytase from thermotolerant *Aspergillus niger* in solid state fermentation *J. Industrial Microbiol & Biotechnol.* **24:** 237-243.

Nair, V.C. and Duvnjak, Z.. 1990. Reduction of phytic acid content in canola meal by *Aspergillus ficuum* in solid state fermentation. *Appl Microbiol Biotechnol.* **34:** 183-188.

Nasi. M.. 1990. Microbial phytase supplementation for improving availability of plants phosphorous in the diets of growing pigs. *J. Agric. Sci.* **62:** 435-442.

Pandey, A.; Selvakumar, P.; Soccol, R. and Nigam, P.. 1999. Solid state fermentation for the production of Industrial enzymes. *Curr. Sci.* **77:** 149-162.

Ramchandran, S.; Patel, A.K.; Nampoothiri, K.M.; Chandran, S.; Szakacs, G.; Soccol, C.R. and Pandey, A.. 2004. Alpha amylase from a fungal culture grown on oil cakes and its properties. *Brazillian Archives of Biology and Technology.* **47**:309-317.

Singh, B. and Satyanarayana, T.. 2010. Applications of phytase of thermophilic mould, *Sporotrichum thermophile :* A review. *J. Scientific and Industrial Research.* **69:** 411-414.

Wodzinski, R.J. and Ullah, A.H.J.. 1996. Phytase. In : Advances in Applied Microbiology (Neidleman SL and Al Laksin, eds), Vol 42, pp 263 - 302, Academic Press, San diego, California, USA.

Zyta, K.. 1992. Mould Phytases and their application in food industry. *world J Microbiol Biotechnol.* **8:** 467-472.