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Production of high activity thermostable phytase from thermotolerant *Aspergillus niger* in solid state fermentation

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The thermotolerant fungus, *Aspergillus niger* NCIM 563, was used for production of extracellular phytase on agricultural residues: wheat bran, mustard cake, cowpea meal, groundnut cake, coconut cake, cotton cake and black bean flour in solid state fermentation (SSF). Maximum enzyme activity (108 U g^{-1} dry mouldy bran, DMB) was obtained with cowpea meal. During the fermentation phytic acid was hydrolysed completely with a corresponding increase in biomass and phytase activity within 7 days. Phosphate in the form of KH_2PO_4 (10 mg per 100 g of agriculture residue) increased phytase activity. Among various surfactants added to SSF, Trition X-100 (0.5%) exhibited a 30% increase in phytase activity. The optimum pH and temperature of the crude enzyme were 5.0 and 50°C respectively. Phytase activity (86%) was retained in buffer of pH 3.5 for 24 h. The enzyme retained 75% of its activity on incubation at 55°C for 1 h. In the presence of 1 mM K^+ and Zn^{2+}, 95% and 55% of the activity were retained. Scanning electron microscopy showed a high density growth of fungal mycelia on wheat bran particles during SSF. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 237–243.

Keywords: phytase; *Aspergillus niger*; phytic acid

Introduction

Phytic acid [myo-inositol(1,2,3,4,5,6) hexakis phosphate] is the major storage form of phosphorus in seeds and pollen [26,38]. Because of its strong chelating properties it is regarded as an anti-nutritive factor. Moreover, it forms insoluble complexes with nutritionally important metals such as calcium, zinc, magnesium and iron, decreasing their bioavailability [8,10]. Phytase (myo-inositol hexaphosphate hydrolase) hydrolyzes phytic acid to myo-inositol and phophoric acid. Two types of phytases are known: 3-phytase (E.C. 3.1.3.8) and 6-phytase (E.C. 3.1.3.26), indicating the predominant attack of the susceptible phosphoester bond. Phytases are widely distributed in nature [6,45]. They have been studied most intensively in seeds of plants, such as wheat, barley, bean, corn, soybean, rice and cotton [20,32]. Phytase activity in microorganisms has been found most frequently in fungi [48], in particular Aspergilli [18,19,42–47]. It occurs also in bacteria viz *E. coli* [11], *Bacillus subtilis* [20–22,35], yeasts [4,31,40] and rumen microorganims [37]. There are recent reports on expression of the *Aspergillus niger* phytase gene (PhyA) in the yeasts *Pichia pastoris* [13] and *Saccharomyces cerevisiae* [14] and *E. coli* phytase gene in pig colon [39]. X-ray crystallographic analysis of phytase from *Bacillus amyloliquefaciens* has also been reported [12]. Among Aspergilli there are few reports of phytase production by SSF ie *A. ficcum* [7,15,27] and *A. carbonarius* [1,2].

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 phosphorus pollution in areas of intensive animal agriculture. The thermostability of this enzyme suggests potential biotechnological application in the pulp and paper industry as a novel biological agent to remove plant phytic acid. 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 [23].

Although there are reports on phytases from microorganisms there are few reports on production of high activity phytase by SSF of agricultural residues using fungi. The present communication reports production of high activity phytase by solid state fermentation using the thermotolerant fungus *Aspergillus niger* NCIM 563.

Materials and methods

Chemicals

Phytic acid sodium salt, was purchased from Sigma Chemical Company, St Louis, MO, USA. All other chemicals used were of analytical grade and obtained from leading manufacturers including BDH, Sigma and Glaxo. Various agricultural residues like wheat bran, coconut cake, groundnut cake, soya residue and soya flour were purchased from a local feedstuff outlet.

Microbial strain and its maintenance

Screening of fungal and bacterial cultures was carried out to identify a strain capable of producing thermostable phytase. Approximately 55–60 fungi and 15 thermophilic bacteria were screened for phytase production using wheat bran as a carbon source under SSF. Four fungal cultures were found to be phytase producers. Out of these, thermotolerant *Aspergillus niger* NCIM 563 was selected for further work

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because it exhibited comparatively high phytase activity. It was maintained on potato dextrose agar (PDA) slants. The fungus was maintained by periodic transfer and stored at 4°C.

Phytase production in solid state fermentation

Erlenmeyer flasks (250 ml) containing 10 g of wheat bran (or any other agricultural residue) moistened with 10 ml distilled water were autoclaved at 121°C for 40 min, cooled, inoculated with 1 ml spore suspension containing 10^6 spores ml^{-1} from a 7-day-old sporulated culture grown on a PDA slant at 30°C. The contents of each flask were mixed thoroughly with a sterile inoculating needle after inoculation for uniform distribution of fungal spores in the medium. The flasks were incubated at 30°C/37°C. A flask was harvested, its contents were extracted for enzyme activity and assayed for phytase activity every 24 h for 8 days.

For tray fermentations, enamel-coated metallic trays ($28 \times 24 \times 4$ cm) containing 50 g or 100 g of wheat bran and trays ($45 \times 30 \times 4$ cm) containing 200 g of wheat bran moistened with distilled water (ratio 1:1 w/v) were used. Trays were covered with aluminium foil and autoclaved at 121°C for 40 min, then inoculated with a 10% volume of a spore suspension containing 10^6 spores ml^{-1} from a 7-day-old sporulated culture grown on a PDA slant at 30°C. The contents of the trays were mixed before and after inoculation and were incubated at 30°C.

Enzyme extraction

After SSF 50 ml of 2% of an aqueous solution of $CaCl_2 \cdot 2H_2O$ was added to each of two flasks, each containing 10 g fermented wheat bran (or any other agricultural residue). Flasks were kept on a rotary shaker operated at 200 rpm for 2 h at room temperature for extraction of enzyme from fermented koji [1,7]. At the end of extraction, the suspension was squeezed through a double layer of muslin cloth and it was centrifuged at $5000 \times g$ for 20 min at 4°C. The clear supernatant was designated as the crude enzyme preparation.

Phytase assay

Phytase measurements were carried out at 50°C. The reaction mixture consisted of 3 mM sodium phytate buffered with 100 mM acetate buffer (pH 5.0). Enzymatic reactions were started by the addition of 50 μl of enzyme solution. After 30 min at 50°C, the liberated inorganic phosphate was measured by a modification of the ammonium molybdate method [17]. A freshly prepared solution of acetone: 5 N H_2SO_4: 10 mM ammonium molybdate (2:1:1 v/v/v) and 400 μl 1 M citric acid was added to the assay mixture. Absorbance was measured at 370 nm. One unit of phytase activity (U) was expressed as the amount of enzyme that liberates 1 μmol phosphorus per minute under standard assay conditions.

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.

Phytic acid reduction

Phytic acid reduction from the wheat bran during SSF was monitored by removing 2 g of solid state culture and extracting the phytic acid by using 2.4% HCl, with continuous shaking (200 rpm) for 1 h. After extraction, the suspension was centrifuged ($6000 \times g$, 15 min), the supernatant collected and phytic acid measured by the Haug and Lantzch method [16].

Factors affecting growth

(1) Biomass estimation: As it is very difficult to quantify biomass in solid state fermentations, glucosamine content was measured and taken as an equivalent value for biomass amount and growth. This parameter is quite reliable for biomass determination in solid state fermentation [5].

Glucosamine was measured according to the method of Nishio *et al* [33] in which 2 g fermented koji was removed and dried at 105°C for 24 h. The dried sample (0.5 g) was soaked in 10 ml of concentrated HCl for 24 h, centrifuged at $7000 \times g$ for 10 min and 5 ml of the supernatant was diluted with 2 ml of 8.6 N HCl solution. Then the solution, in the sealed test tube, was hydrolyzed for 2 h at 100°C to produce glucosamine. The hydrolyzed solution was then neutralized with 30% NaOH solution. Glucosamine in the supernatant of the neutralized solution was measured by the method of Blix [5].

(2) Effect of moisture level: To check the influence of moisture on phytase activity during SSF, 10 g wheat bran in 250-ml Erlenmeyer flasks was moistened with different amounts of distilled water. The ratio of wheat bran to distilled water was varied (1:0.3, 1:0.6, 1:1, 1:1.2, 1:1.5). After sterilization at 121°C for 40 min these flasks were inoculated with 1 ml spore suspension of *A. niger* and incubated at room temperature as described earlier.

(3) Effect of phosphate on enzyme production: To determine the effect of phosphate on production of phytase during SSF, 10 g wheat bran in 250-ml Erlenmeyer flasks was moistened with 10 ml distilled water containing various amounts of phosphate in the form of KH_2PO_4, 0–50 mg per 100 g of agricultural residue. After sterilization at 121°C for 40 min these flasks were inoculated with a spore suspension of *A. niger* and incubated at room temperature as described earlier.

(4) Effect of surfactants: To check the effect of surfactants on production of phytase during SSF, 10 g wheat bran in 250-ml Erlenmeyer flasks was moistened with 10 ml distilled water containing various surfactants: Tween 80, Triton X-100, sodium deoxycholate and NP-40 (0.5% w/w). After sterilization at 121°C for 40 min these flasks were inoculated with 1 ml spore suspension and incubated at room temperature as described earlier.

(5) Extraction using different salts: The effect of salts on extraction of enzyme from SSF was monitored by suspending 10 g fermented mouldy bran in 50 ml of a 2% solution of various salts like $CaCl_2 \cdot 2H_2O$, $CaCO_3$,

$CaSO_4 \cdot 2H_2O$, NaCl and KCl and it was kept on a shaker at 200 rpm for 2 h at room temperature. The enzyme was separated by passing the suspension through a double layer of muslin cloth and the filtrate was centrifuged at $5000 \times g$ for 20 min at 4°C.

(6) Effect of metal ions on phytase activity: The effect of metal ions on phytase activity was carried out at 50°C for 30 min as described earlier by adding a salt of the metal ion (1 mM final concentration) to the phytase reaction mixture viz 100 mM acetate buffer, pH 5.0 containing 3 mM sodium phytate along with control, ie phytase reaction without metal ion.

Scanning electron microscopy

Samples for SEM were fixed with ultraviolet light and were mounted on brass stubs. Specimens were then coated with a thin layer of gold (100 Å) in a gold coating unit, model E 5000, Polaron Equipment Ltd (Cambridge, UK) and were viewed with an SEM Leica Stereoscan 440 (Leica, Cambridge, UK) at an accelerating voltage of 10 kV, and beam current 25 Pa.

Results

Time course of phytase production

The time course of phytase production in solid state fermentation using wheat bran as substrate is shown in Figure 1. The fungus grew rapidly on wheat bran particles intra and intercellularly as indicated by an increase in biomass in the form of glucosamine (2.5 mg g^{-1} DMB) with a corresponding increase in phytase activity (79.5 U g^{-1} DMB) till the 7th day of SSF along with a reduction of phytic acid from wheat bran. Enzyme production was associated with increased biomass and a corresponding reduction of phytic acid.

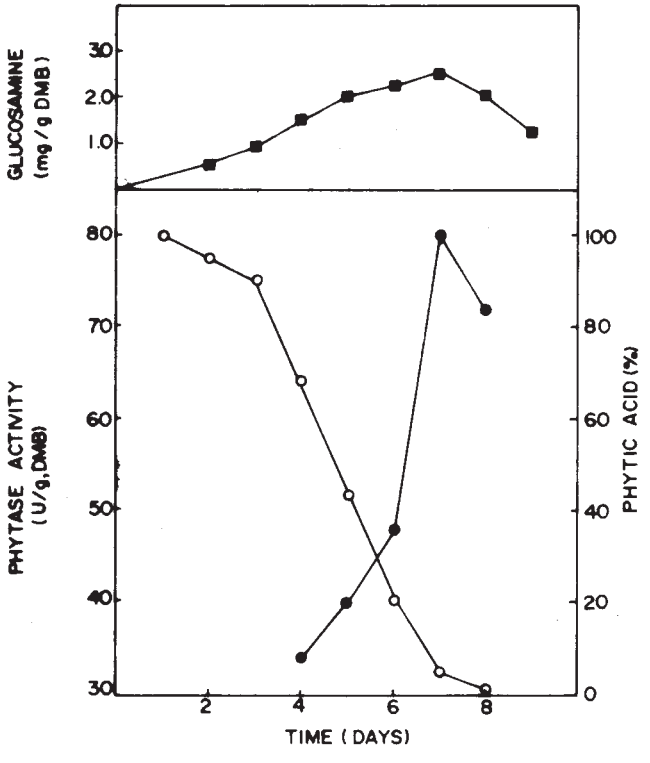

Figure 1 Phytase production by *Aspergillus niger* on wheat bran medium during SSF. ●—● Phytase activity (U g^{-1} DMB); ○—○ reduction of phytic acid content; ■—■ glucosamine (mg g^{-1} DMB).

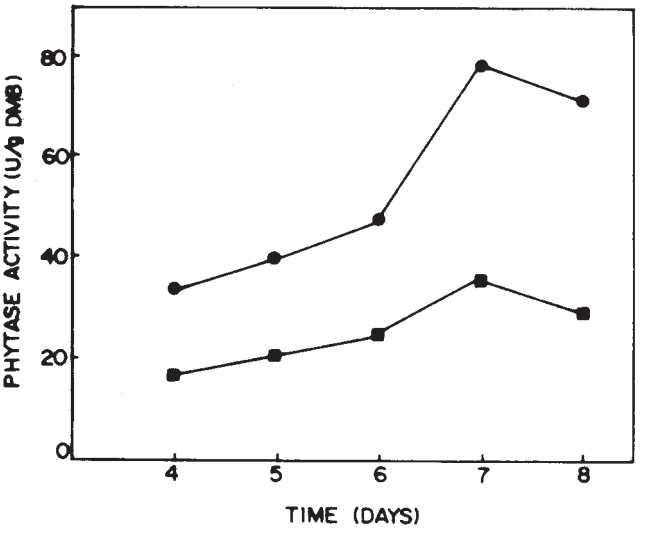

Figure 2 Effect of temperature on phytase production in SSF. ●—● Incubation at 30°C; ■—■ incubation at 37°C.

The effect of temperature of enzyme production is shown in Figure 2. The enzyme is secreted at both 30°C and 37°C. The fungus produced only 35 U g^{-1} DMB phytase activity at 37°C compared to 79.5 U g^{-1} DMB at 30°C on the 7th day.

Effect of agricultural residues on phytase production

All the substrates except rice husks showed phytase activity (Table 1). Wheat bran gave 79.5 U g^{-1} DMB phytase activity while cowpea meal supported the highest enzyme production (108 U g^{-1} DMB). Since wheat bran was very economical compared to cowpea meal, all fermentation parameters for SSF were standardized using wheat bran.

Tray fermentation

The yield of enzyme activity (75–77 U g^{-1} DMB) was comparable to that in Erlenmeyer flasks (79.5 U g^{-1} DMB) (Table 2).

Table 1 Effect of various substrates on phytase production

Substrate	Activity (U g^{-1} DMB)
Wheat bran	79.5
Mustard cake	40.6
Cowpea meal	108.0
Groundnut cake	50.9
Coconut cake	37.3
Black bean flour	84.7
Cotton cake	47.5
Rice husk	0

Table 2 Cultivation of *Aspergillus niger* NCIM 563 in trays

Equipment	Capacity	Substrate quantity (g)	Activity (U g^{-1} DMB)
Flask	250 ml	10	79.5
Tray	28 × 24 × 4 cm	50	77.0
Tray	28 × 24 × 4 cm	100	77.0
Tray	40 × 30 × 4 cm	200	75.0

Extraction using different salt solutions

Salt solutions like 2% $CaCl_2 \cdot 2H_2O$, $CaCO_3$, $CaSO_4 \cdot 2H_2O$, NaCl and KCl can be used for extraction of the enzyme from fermented wheat bran from SSF. Compared to 2% $CaCl_2 \cdot 2H_2O$ however, other salts recovered only 82–85% enzyme activity.

Effect of moisture on phytase production

The effect of initial moisture content of the substrate on enzyme yield is shown in Figure 3. Maximum phytase activity was obtained when wheat bran was moistened with distilled water in a 1:1 ratio (79.5 U g^{-1} DMB). Above this ratio enzyme production was decreased as the porosity of the medium was decreased.

Effect of phosphate in the growth medium

Enzyme production increased as the phosphate concentration in SSF increased till 10 mg phosphate per 100 g wheat bran (125.5 U g^{-1} DMB). Above this concentration phytase activity reduced sharply. At 50 mg phosphate per 100 g wheat bran in SSF, only 70 U g^{-1} DMB phytase activity was produced (Figure 3).

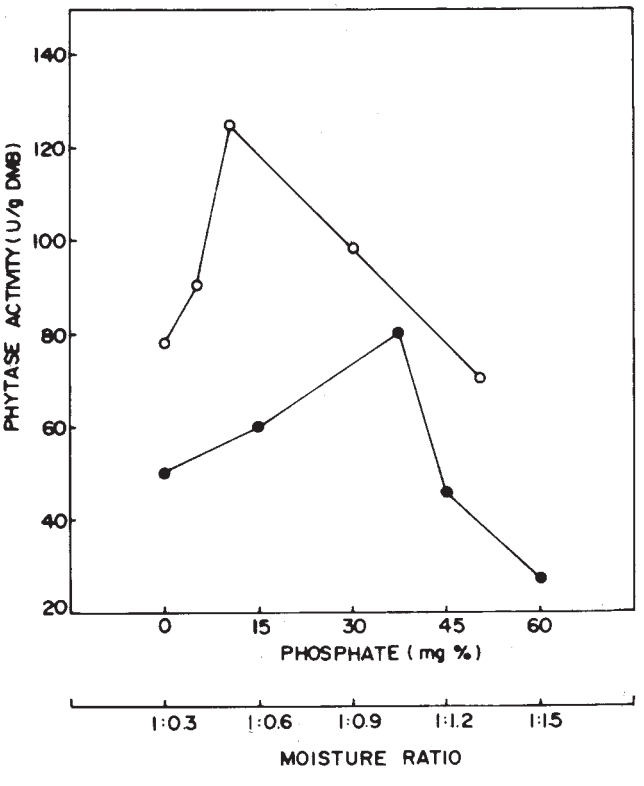

Figure 3 Effect of moisture level and concentration of phosphate on phytase production in SSF. ●—● Moisture; ○—○ phosphate.

Table 3 Effect of surfactants on phytase production during SSF by *Aspergillus niger* NCIM 563

Surfactant (0.5%)	Phytase activity (U g^{-1} DMB)
None	79.5
NP 40	83.0
Tween 80	91.0
Sodium deoxycholate	92.0
Triton X-100	108.0

Effect of surfactants

The effects of surfactants on phytase production during SSF of wheat bran are shown in Table 3. The system with surfactants produced more enzyme than the control, which was not supplemented with any surfactant. Triton X-100 supported a 30% increase in phytase production (108 U g^{-1} DMB) whereas Tween 80 and sodium deoxycholate increased phytase production by 14% (90 U g^{-1} DMB).

Enzymatic properties

Phytase assay was performed from pH 2.0 to 9.0 using a variety of buffers at 100 mM in the reaction mixture at 50°C for 30 min, using 3 mM sodium phytate as a substrate. Glycine-HCl (pH 2.0–5.0), acetate buffer (pH 4.0–6.0) and Tris-HCl (pH 7.0–9.0) were used. The enzyme has an optimum pH of 5.0 and was virtually inactive above pH 7.5. However, the enzyme was quite stable towards the lower pH range (Figure 4). The effect of pH on enzyme stability was tested in the range of 2.0–9.0 at 4°C. After 24 h the enzyme was fairly stable from pH 2.5–5.5, while 86% of the phytase activity was retained for 24 h at pH 3.5 (Figure 4).

The enzyme's optimum temperature was 50°C as shown in Figure 5. The enzyme was incubated at various temperatures for 15, 30, 45 and 60 min. At 55°C the enzyme retained 75% of its original activity after 60 min and at 60°C it lost 84% of its activity after 15 min (Figure 5).

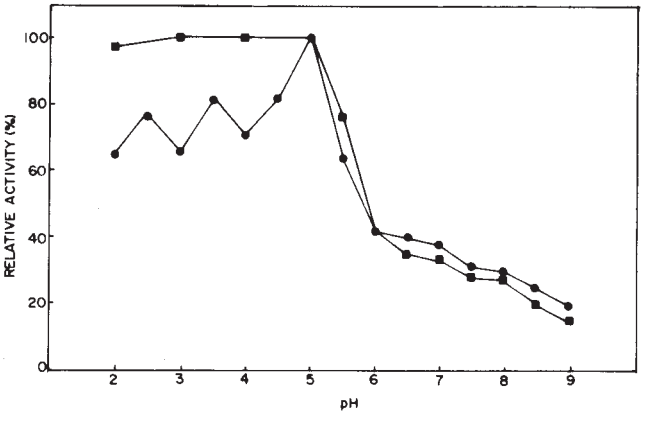

Figure 4 Effect of pH on phytase activity (●—●) and stability (■—■).

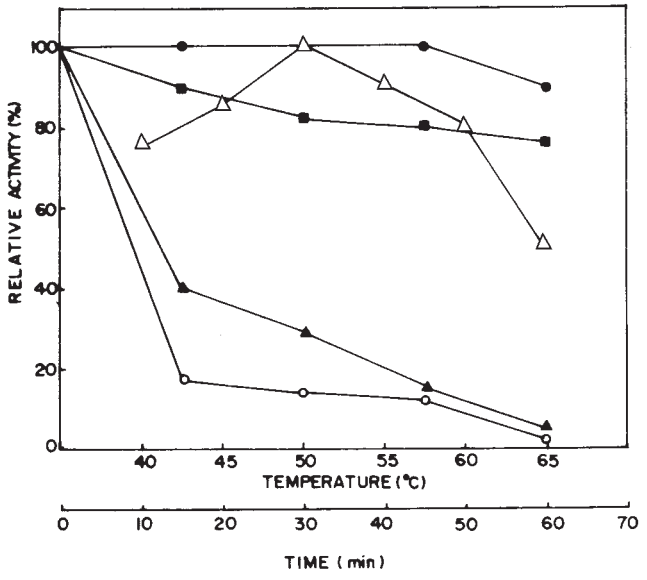

Figure 5 Effect of temperature on enzyme activity (△—△) and stability at 50°C (●—●), 55°C (■—■), 58°C (▲—▲) and 60°C (○—○).

Effect of metal ions on phytase activity

The effect of metal ions was studied by adding a metal ion at 1 mM final concentration in a reaction mixture using 100 mM acetate buffer, pH 5.0 at 50°C for 30 min using 3 mM sodium phytate as a substrate. The enzyme retained 55% of its activity in the presence of 1 mM Zn^{2+} while all other metal ions (Ca^{2+}, Mn^{2+}, Mg^{2+}, Ni^{2+} and Fe^{2+}) did not show any activation or inhibition.

Scanning electron microscopy

A. niger grew rapidly on wheat bran and formed a dense mat of vegetative mycelium which sporulated (Figure 6). The biomass formed was directly related to enzyme activity secreted into the medium.

Discussion

Phytases from bacteria, yeasts, fungi and plant sources have been produced by solid state fermentation as well as liquid fermentation. Nair and Duvnjak [28] reported use of *Rhizopus oligosporus* NRRL 2990, *Aspergillus niger* NRC 5765, *A. carbonarius* NRC 401121, *A. ficuum* and *Saccharomyces cerevisiae* in an SSF process for reduction of the phytic acid content in canola meal. However, among the reports on phytase by fungi using the SSF technique, the present strain of *A. niger*, NCIM 563, produced a high activity thermostable phytase (108.5 U g^{-1} DMB) with cowpea meal as substrate compared to *A. ficuum* (4 U g^{-1} solid state culture) [7] and *A. carbonarius* (2.5 U g^{-1} solid state culture) [1] when canola meal was used as a substrate for SSF. A low level of phytase appeared in the early stages of incubation and enzyme levels reached a maximum by the 7th day of fermentation. A prolonged incubation time beyond this period did not help to further increase the yield (Figure 1). The duration of incubation is generally dependent on the growth rate and enzyme production pattern of the strain [34]. Phytic acid was completely hydrolyzed after 7 days of fermentation (Figure 1).

Figure 6 Scanning electron micrograph of wheat bran degradation by *Aspergillus niger* during SSF after 3 days of fermentation. Marker, magnigication (× 250).

The culture grew and produced phytase (35 U g^{-1} DMB) at 37°C, demonstrating the thermotolerant nature of the fungus (Figure 2).

There have been reports on the use of 2% $CaCl_2 \cdot 2H_2O$ as an extractant [1,7] of phytase activity from solid substrates used for the fermentation. But we have observed that various other salt solutions like $CaCO_3$, $CaSO_4 \cdot 2H_2O$, NaCl and KCl at 2% can also be used to extract phytase with a recovery of 80–85%. However, 2% $CaCl_2 \cdot 2H_2O$ proved to be the best salt solution amongst the ones tested.

The result of tray fermentation was encouraging for large-scale production of phytase. Similar trials for large-scale production of α-amylase from a thermophilic *Bacillus coagulans* in enamel trays have been reported [3]. Phytase production in enamel trays was comparable with that in culture flasks, indicating that scaling up does not result in a reduction of phytase titers. Such tray fermentors have been described as simple yet efficient in operation even though the need for a large area is one of their drawbacks [24,25].

The moisture content in a solid state fermentation is a crucial factor that determines the success of the process [9]. The importance of moisture level in SSF media and its influence on microbial growth and product biosynthesis may be attributed to the impact of moisture on the physical properties of the solid substrate [9,30,36]. A higher than optimum moisture level causes decreased porosity, alteration in wheat bran particle structure, lower oxygen transfer and enhanced formation of aerial mycelia [9,36]. Similarly, a moisture level lower than optimum leads to a higher water tension, lower degree of swelling and reduced solubility of the nutrients of the solid substrate [9]. We have seen that maximum phytase activity was produced when the ratio of wheat bran to distilled water was 1:1. Similar results were reported during SSF of canola meal by *A. ficuum* [7].

The optimal amount of phosphorus in the growth medium should be determined [41]. We have observed that 10 mg % phosphate during SSF of wheat bran yielded the highest enzyme activity (125.5 U g^{-1} DMB) (Figure 3). Similar effects of phosphate on phytase production by *A. ficuum* in liquid medium were also reported [41]. In addition, Han *et al* [15] obtained a similar trend for phytase production by *A. ficuum* on semisolid substrate using

soybean meal. They found that 10 mg phosphate per 100 g substrate in the growth medium resulted in high phytase activity (82.5 U g^{-1} substrate) compared to a control without added phosphate (8.0 U g^{-1} substrate) while higher phosphate levels inhibited phytase production.

The use of a surfactant during SSF gave higher phytase activity. Maximum phytase activity (108 U g^{-1} DMB) was obtained when Triton X-100 at 0.5% (w/w) was used. Ebune *et al* [7] also showed increased phytase production by addition of sodium oleate and Tween-80 during SSF of canola meal by *A. ficuum* while Triton X-100 had a negative effect. In the case of phytase production by *A. carbonarius* [1] using canola meal as substrate for SSF, sodium oleate and Tween-80 (each at 0.5%) showed a higher rate of biomass and phytase production than the control which did not contain surfactants. Increased phytase production may be explained by an effect of the surfactant on cell permeability. However, it is also possibile that the surfactant in lower concentration stimulated growth, as in the case of phytase production by *A. carbonarius* [1].

Enzymatic properties

Most isolated phytases are active within the pH range 4.5–6.0 and the stability of enzyme activity is decreased dramatically by pH values lower than 3 or higher than 7.5 [23]. However, the phytases from *Enterobacter*, mungbean and *Lilium longiflorum* (pollen) have pH optima around 7.5. The enzymes isolated from animal tissues also have alkaline optimum pH values. This wide range of pH optima could be reflected in the molecular structure or the stereo specificity of the enzyme from different sources. Phytases show high activity in the temperature range of 50–70°C but optimum temperatures for the enzyme are mainly between 45° and 60°C [23]. The optimum temperature of the spelt phytase is 45°C which is the lowest optimal temperature recorded.

Scanning electron microscopy

The degree of substrate transformation in SSF depends upon the capability of fungal mycelia to penetrate deep into the intracellular and intercellular spaces [29]. In the present study we have observed that *A. niger* attacks wheat bran particles rapidly and forms a full mat growth with spores on wheat bran particles which was necessary for high phytase activity as biomass was related to phytase activity.

Further work on purification and characterization of phytase enzyme is in progress.

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References

1 Al-Asheh S and Z Duvnjak. 1994. 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.
2 Al-Asheh S and Z Duvnjak. 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.
3 Babu KR and T Satyanarayana. 1995. α-Amylase production by thermophilic *Bacillus coagulans* in solid state fermentation. Proc Biochem 30: 305–309.
4 Bindu S, D Somashekar and R Joseph. 1998. A comparative study on permeabilization treatments for in situ determination of phytase of *Rhodotorula gracillis*. Appl Microbiol Lett 27: 336–340.
5 Blix G. 1948. The determination of hexosamine according to Elson and Morgan. Acta Chem Scand 2: 467–473.
6 Dvorakova J. 1998. Phytase: sources, preparation and exploitation. Folia Microbiol 43: 323–338.
7 Ebune A, S Al-Asheh and Z Duvnjak. 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.
8 Erdman JW and A Poneros-Schneier. 1989. Phytic acid interactions with divalent cations in food and in the gastrointestinial tract. Adv Exp Med Biol 249: 167–171.
9 Feniksova RV, AS Tikhomrova and BE Rakhleeva. 1960. Conditions for forming amylase and proteinase in surface culture of *Bacillus subtilis*. Mikrobiologica 29: 745–748.
10 Fox MRS and SH Tao. 1989. Antinutritive effects of phytate and other phosphorylated derivatives. Nutr Toxicol 3: 59–96.
11 Greiner R, U Konietzny and K-D Jany. 1993. Purification and characterization of two phytases from *Escherichia coli*. Arch Biochem Biophys 303: 107–113.
12 Ha NC, YO Kim and BH Oh. 1999. Preliminary X-ray crystallographic analysis of a novel phytase from a *Bacillus amyloliquifaciens* strain. Acta Crystallographia Section D-Biological Crystallography 55: 691–693.
13 Han YM and XG Lei. 1999. Role of glycosylation in the functional expression of an *Aspergillus niger* phytase (PhyA) in *Pichia pastoris*. Arch Biochem Biophys 364: 83–90.
14 Han YM, DB Wilson and XG Lei. 1999. Expression of an *Aspergillus niger* phytase gene (PhyA) in *Saccharomyces cerevisiae*. Appl Environ Microbiol 65: 1915–1918.
15 Han YM, DJ Gallagher and AG Wilfred. 1987. Phytase production by *Aspergillus ficuum* on semisolid substrate. J Ind Microbiol 2: 195–200.
16 Haug W and H Lantzsch. 1983. Sensitive method for the rapid determination of phytate in cereal and cereal products. J Sci Food Agric 34: 1423–1426.
17 Heinohen JK and RJ Lahti. 1981. A new and convenient colorimetric determination of inorganic orthophosphate and its application to the assay to inorganic pyrophosphatase. Anal Biochem 113: 313–317.
18 Hirabayashi M, T Matsui and H Yano. 1998. Fermentation of soyabean flour with *Aspergillus usamii* improves availabilities of zinc and iron in rats. J Nutritional Science and Vitaminology 44: 877–886.
19 Howson SJ and RP Davis. 1983. Production of phytase hydrolyzing enzyme by some fungi. Enz Microbiol Technol 5: 377–382.
20 Irving GCJ. 1980. Inositol Phosphates: Their Chemistry, Biochemistry and Physiology (Cosgrove DJ, ed), pp 85–98, Elsevier, Amsterdam.
21 Kerovuo J, M Lauraeus, P Nurminen, N Kalkkinen and J Apajalahti. 1998. Isolation, characterization, molecular genecloning and sequencing of a novel phytase from *Bacillus subtilis*. Appl Environ Microbiol 64: 2079–2085.
22 Kim YO, HK Kim, KS Bae, JH Yu and TK Oh. 1998. Purification and properties of a thermostable phytase from *Bacillus* sp DS-11. Biotechnol Appl Microbiol 22: 2–7.
23 Liu BL, A Rafiq, YM Tzeng and A Rob. 1998. The induction and characterization of phytase and beyond. Enz Microbiol Chem 22: 415–424.
24 Lonsane BK, NP Ghildyal, S Budaitman and SV Ramakrishna. 1985. Engineering aspects of solid state fermentation. Enzyme Microb Technol 7: 258–265.
25 Lonsane BK, G Saucedo-Castenada, M Raimbault, S Roussos, G Viniegra-Gonzalez, NP Ghildyal, M Ramakrishna and MM Krishnaiah. 1992. Scale-up strategies for solid state fermentation systems. Proc Biochem 27: 259–273.
26 Maga JA. 1982. Phytate: its chemistry, occurrence, food interactions, nutritional significance, and methods of analysis. J Agric Food Chem 30: 1–9.
27 Nair VC and Z Duvnjak. 1990. Reduction of phytic acid content in

canola meal by *Aspergillus ficuum* in solid state fermentation. Appl Microbiol Biotechnol 34: 183–188.
28 Nair VC and Z Duvnjak. 1991. Phytic acid content reduction in canola meal by various micro-organisms in a solid state fermentation process. Acta Biotechnologia 11: 211–218.
29 Nako M, M Harada, Y Kodama, T Nakayama, Y Shibano and T Amachi. 1994. Purification and characterization of a thermostable β-galactosidase with high transglycosylation activity from *Saccharopolyspora rectivingula*. Appl Microbiol Biotechnol 40: 657–663.
30 Narahara H, Y Koyama, T Yoshida, S Pichangkura, R Ueda and H Taguchi. 1982. Growth and enzyme production in a solid state fermentation culture of *Aspergillus oryzae*. J Ferment Technol 60: 311–319.
31 Nayini NR and P Markakis. 1984. The phytase of yeast. Lebensm Wiss U Technol 17: 24–46.
32 Nayini NR and P Markakis. 1986. Phytic Acid: Chemistry and Applications (Graf E, ed), pp 101–118, Pilatus Press, Minneapolis, MN.
33 Nishio N, K Tai and S Nagai. 1979. Hydrolase production by *Aspergillus niger* in solid state cultivation. Eur J Appl Biotechnol 8: 263–270.
34 Park YK and BC Rivera. 1982. Alcohol production from various enzyme converted starches with or without cooking. Biotechnol Bioeng 24: 495–500.
35 Powar VK and V Jagannathan. 1982. Purification and properties of phytase-specific phosphatase from *Bacillus subtilis*. J Bacteriol 151: 1102–1108.
36 Raimbault M and D Alazard. 1980. Culture method to study fungal in solid state fermentation. Eur J Appl Microbiol Biotechnol 9: 199–209.
37 Raun A, E Cheng and WJ Burroughs. 1956. Agric Food Chem 4: 869–871.
38 Reddy NR, SK Sathe and DK Salunkhe. 1982. Phytases in legumes and cereals. Adv Food Res 28: 1–92.
39 Rodriguez E, YM Han and XG Lei. 1999. Cloning, sequencing and expression of *Escherichia coli* phosphatase/phytase gene (AppA2) isolated from pig colon. Biochem Biophys Res Commun 257: 117–123.
40 Sano K, H Fukuhara and Y Nakamura. 1999. Phytase of the yeast *Arxula adeninivorans*. Biotechnol Lett 21: 33–38.
41 Shieh TR, J Wodzinski and JH Ware. 1969. Regulation of the formation of acid phosphatases by inorganic phosphate in *Aspergillus ficuum*. J Bacteriol 100: 1161–1165.
42 Ullah AHJ. 1988. Production, rapid purification and catalytic characterization of extracellular phytase from *Aspergillus ficuum*. Prep Biochem 18: 443–458.
43 Yamamoto S, Y Minoda and K Yamada. 1972. Chemical and physiochemical properties of phytase from *Aspergillus terreus*. Agric Biol Chem 36: 2097–2103.
44 Volfova O, J Dvorakova, A Hanzlikova and A Jandera. 1994. Phytase from *Aspergillus niger*. Folia Microbiol 39: 479–482.
45 Wodzinski RJ and AHJ Ullah. 1996. Phytase. In: Advances in Applied Microbiology (Neidleman SL and AI Laksin, eds), Vol 42, pp 263–302, Academic Press, San Diego, California, USA.
46 Wyss M, L Pasamontes, A Friedlein, R Remy, M Tessier, A Kronenberger, A Middendorf, M Lehmann, L Schnoeblen, U Rothlisberger, E Kusznir, G Wahl, F Muller, H Lahm, K Vogel and APGM van Loon. 1999. Biophysical characterization of fungal phytases (myo-inositol hexakisphosphate phosphohydrolases): molecular size, glycosylation pattern and engineering of proteolytic resistance. Appl Environ Microbiol 65: 359–366.
47 Wyss M, R Burgger, A Kronenberger, R Remy, R Fimbel, G Oesterhelt, M Lehmann and APGM van Loon. 1999. Biochemical characterization of fungal phytases (myo-inositol hexakisphosphate phosphohydrolases): catalytic properties. Appl Environ Microbiol 65: 367–373.
48 Zyta K. 1992. Mould phytases and their application in the food industry. World J Microbiol Biotechnol 8: 467–472.