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IMPROVEMENT OF QUALITY ATTRIBUTES OF BREAD BY THE APPLICATION OF PHYTASES FROM AN INDIGENOUS STRAIN OF *Aspergillus niger*

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The application of phytases in cereal products improves their nutritional profile by augmenting the availability of some vital micronutrients. In the present study phytases were produced from an indigenous strain of *Aspergillus niger* under submerged fermentation conditions. The strain was optimized at various conditions of temperature, substrate concentration and pH for hyper production of phytases. The maximum production of phytases (5.20±0.26 FTU/mL)from *A. niger* was observed at 35ºC,6% (w/v) wheat bran concentration and pH 6.0 in submerged conditions. The crude extract was partially purified through ammonium sulphate precipitation and freeze dried on lyophilizer. Various treatments comprising of T_1, T_2, T_3, T_4 and T_5 having 100, 200, 500, 1000 and 2000 FTU of partially purified and lyophilized phytases along with control T_0 were applied in bread preparation. A significant increase in volume, weight and moisture retaining ability of phytase treated bread was observed. Aroma, taste and chew-ability of the enzyme treated bread were also improved. The enzyme reduced the phytic acid content surprisingly to 45.55% which ultimately improved the free mineral status of various samples of bread treated with phytase enzyme. The results of the study imparted the potential of phytase enzyme for application in cereal, snacks and baking industry.

Keywords: Phytases, phytic acid, submerged, partially purified, bread

INTRODUCTION

Bread is consumed as a staple food not only in European countries but has also become an equally important part of breakfast in eastern countries as well. The whole wheat bread, chapatti and meals have recently been introduced as good sources of minerals. The consumption of whole wheat bread is increasing all over the world, however, the whole wheat has also sufficient amounts of phytic acid (myo-inositol hexakis phosphate) which is one of the major anti-nutritional factors found naturally in cereals, legumes and oil seed crops. It has the intrinsic property of chelating salts exclusively calcium, magnesium, potassium, iron as well as some proteins and amino acids (Hotz and Gibson, 2005; Noureddini and Dang, 2009). Human and non ruminant animals lack the enzyme required for its digestion, phytic acid hence excreted from the body unmodified while chelating many minerals making them unavailable to the human body and polluting the environment with algal blooms and eutrophication of surface water (Cromwell *et al.*, 1991). In developing countries the major daily diet of people comprises of mainly the plant sources thus the estimated daily intake of phytic acid (2 g) is much higher as compared to developed countries (200-800 mg) (Plaami, 1997), one of the reasons of under-nutrition in these countries.

Phytic acid should be addressed to avoid any losses of minerals from our daily diet. A whole wheat bread with a lower level of phytic acid and higher level of minerals would be more valuable and attractive in improving the mineral status, which will ultimately improve the nutritional status. Phytic acid content in processed foods can be reduced either mechanically or enzymatically. Enzymatic hydrolysis is considered superior over mechanical due to many added advantages like speed of reaction, protection from environmental pollution, reduced by products and enhanced targeted products. Its importance is increased many times when the food is being processed for human. A number of studies have confirmed that the enzymatic hydrolysis is more feasible for application in food and feed (Porres *et al.*, 2001; Palacios *et al.*, 2008a and b).

Phytases are the enzymes having the ability to hydrolyze phytates, which result in the formation of myo-inositol and the inorganic phosphates. They hydrolyze phytates in a stepwise manner forming myo-inositol pentakisphosphate, tetrakis-, tris-, bis- and monophosphates and as well as the liberation of inorganic phosphate. The addition of phytases in food and feeds resulted in an improved availability status

of minerals, trace elements, proteins and energy (Vats and Banerjee, 2006; Mittal *et al.,* 2013). Bread is recognized as staple food worldwide (Afinah *et al.,* 2010). Phytases are found as an excellent improver in bread manufacturing (Haros *et al.,* 2001a and b). The addition of phytases in some cereal based foods resulted in an increased availability of iron (Park *et al.,* 2011; Hurrell *et al.,* 2003). Increase in iron content in bread upto 53% by the addition of phytases has been reported by Sandberg and Svanberg (1991). On commercial scale the application of phytases has resulted in an acceleration of proofing, better bread shape, increased bread volume, soft crumb, reduced phytic acid level, improved mineral and protein status (Haros *et al.,* 2001a and b; Greiner and Konietzny, 2006; Park *et al.,* 2011).

Microorganisms including bacteria, yeast and mold are considered as the best phytase sources on the basis of higher yield potential and ease in cultivation (Haefner *et al.,* 2005). The phytases may be endogenous or exogenous in nature depending on the type of organism (Mitchell *et al.,* 1997). They are ubiquitous in nature and are found in plants especially in cereals e. g., wheat (Nakano *et al.,* 1999), barley (Greiner *et al.,* 2001) and in microorganisms such as *Aspergillu niger* (Papagianni *et al.,* 2001) and *Rhizomu corpusillus* (Chadha *et al.,* 2004). Phytases may be produced from microorganisms either through solid state or by submerged fermentation process. However, submerged fermentation is preferred over solid state due to easy handling, higher titers, separation of broth and ease in operation of fermenters (Shoji *et al.,* 2007).

In the study under focus, a local strain of *A. niger* was exploited to spawn indigenous source of phytases from wheat bran; a cheaper source of carbon from the wheat milling industry. The partially purified phytases were further investigated for their potential applications in bread manufacturing to reduce the phytic acid content and to improve the characteristics of bread.

MATERIAL AND METHODS

A. niger was donated by National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad. The white flour used for the production of bread was purchased from Ideal Bakers, Multan. Chemicals used were of analytical grade and purchased from Merck Chemicals, USA otherwise stated.

***Preparation of inoculum*:** Inoculum was prepared as described by Riaz *et al.,* (2012). Briefly, 50 ml Vogel's media (pH 5.0) was prepared and autoclaved at 121 ºC, 1.1 kg/cm^2 in a 250ml conical flask having 8-10 acid washed glass beads. Glucose solution (50% w/v) was autoclaved separately for 5 min and was added in Vogel's media to a final concentration of 2%. A loop full of spores was aseptically transferred from a 5-6 days old fully matured slant to the flasks containing Vogel's media and incubated for 36 h at 30 ºC and 150 rpm in an orbital shaker.

***Phytase production from A. niger*:** Phytases were produced from *A. niger* under submerged fermentation conditions on a growth medium containing (g/L): glucose, 5; peptone, 18; KCL, 0.5; $MgSO_4.7H_2O$, 1.5; KH_2PO_4, 1; $CaCl_2.2H_2O$, 2.0 at pH of 5.0-5.3 (Papagianni *et al.,* 2001). The growth media was autoclaved and the sterile wheat bran was added just before inoculation. The inoculum (10 ml) was transferred to each flask and allowed for fermentation at the orbital shaker. A flask from a continuous process of fermentation was harvested after every 4 h and the sample was drawn for enzyme assay.

***Optimization of growth conditions*:** *A. niger* was grown under submerged conditions for the bulk production of phytases by the method of Papagianni *et al.,* (2001) at various temperatures (25±1, 30±1, 35±1, 37±1, 40±1 and 45±1 ºC), concentrations of wheat bran (2%, 4%, 6%, 8% and 10%) and pH (3.0, 4.0, 5.0, 6.0 and 7.0) for the optimum production of phytases.

***Phytase activity assay*:** Phytase activity was measured after every 4 h by the method of Kim and Lei (2005). Briefly, 0.2 ml of 1% sodium phytate prepared in 0.2 M sodium acetate buffer was added in 0.2 ml sample incubated already for 5 min at 37 °C. The reaction mixture was again incubated at same temperature but for 15 min. The reaction was stopped by the addition of 0.4 ml of 15% trichloroacetic acid at ambient temperature followed by centrifugation for 10min (2000×*g*). The color reagent (2.0 ml) freshly prepared by mixing one volume each of 2.5% ammonium molybdate and 10% ascorbic acid, and three volumes of 1M sulfuric acid was added in the test tube containing 0.2 ml supernatant and 1.8 ml deionized water. The reaction mixture was incubated at 50 °C for 15 min followed by cooling at ambient temperature. The absorbance of the reaction mixture and serially diluted standard solutions (KH_2PO_4) was measured at 820 nm. One FTU is defined as the amount of the enzyme releases 1 micro mole of inorganic phosphorus per min from 0.0051 mol/L sodium phytate at pH 5 and 37 °C.

***Isolation of phytases*:** The crude enzyme was extracted by passing the culture through Whatman # 1 filter paper and centrifuged at 25900 x *g* at centrifuge machine of Hedolf, Germany for 15 min at 4 ºC to remove suspended particles (Riaz *et al.,* 2012).

***Partial purification of phytases*:** Phytase precipitation initiation and ending points by solid ammonium sulphate were found to be 30% and 80%, respectively. Solid ammonium sulphate was added bit by bit up to 30% in whole of the crude enzyme and kept overnight at 4 ºC. The enzyme solution was centrifuged at 29500 x *g* and the pallet was discarded. Solid ammonium sulphate was again added bit by bit to achieve final concentration of 80% saturation at 0 ºC and left over night. The solution was centrifuged at the same conditions as above. The supernatant was removed and

pallet was re-suspended in distilled water and was dialyzed overnight against many changes of distilled water at 4 ºC. The partially purified enzyme was dried on lyophilizer (VirTis 4K, USA) and was kept at -80 ºC until used (Zulfiqar *et al.,* 2013).

***Proximate analysis of flour*:** The proximate analysis (moisture, ash, protein, fat, fiber and NFE) of straight grade flour having additional 5% wheat bran used for bread making was done according to (AACC, 2000) method.

***Bread production*:** Bran (5%) was added in the white flour. Initially various doses of partially purified enzyme i.e., 0, 100, 200, 500, 1000 and 2000 FTU in treatments To (control), T_1, T_2, T_3, T_4 and T_5, respectively were used in the preparation of bread by straight dough method (AACC, 2000). The ingredients used for bread manufacturing were flour 200g, sugar 7g, yeast 4g, salt 2g, oil 10g, phytase enzyme and water. The ingredients were first mixed for 5min to form the dough. The dough was then punched for the proper mixing of ingredients for 120 to 150 min. After mixing, the dough samples were molded in pans and were kept in the proofer for 45 min at 85% relative humidity and 35 ºC. After proofing the breads were baked for 30min at 230 ºC. The bread samples in triplicate were cooled at room temperature and freeze dried for further analysis.

***Sensorial and physical characteristics of bread*:** The breads were examined for sensorial characterization by a panel of 11 trained judges according to the bread score method developed by the American Institute of Baking (Matz, 1960). The selection of judges was made by following the guidelines provided by Murray *et al.,* (2001).Moreover, some general characters like bread weight, volume and moisture were measured by weighing balance (Precisa, Switzerland), rapeseed displacement (AACC, 2000) and moisture percentage (AACC, 2000) methods, respectively.

***Estimation of phytic acid*:** Phytic acid content of samples of flour and breads after proofing and baking were determined as described by Haug and Lantzsch (1983).

***Free Mineral Content*:** The free mineral content of the bread samples was determined by the method of Park *et al.,* (2011). Briefly, 0.5g freeze dried sample of each treatment was mixed in 10 ml double distilled water and was shaken on an orbital shaker at 160 rpm for 5 min at room temperature. The samples were filtered and the filtrates were used directly for the mineral analysis by atomic absorption spectrophotometer (Thermo Scientific 3000 series).

***Statistical analysis*:** The data obtained from each parameter was subjected to statistical analysis to determine the level of significance according to the method as described by Steel *et al.* (1997).

RESULTS AND DISCUSSION

***Optimization of phytase production*:** Optimization of microbial sources has widely been accepted as an efficient strategy for the production of various bio-molecules. The evaluation of maximum microbial potential for the production of bio-molecules is only possible by the optimization of culture conditions. Variable substrate concentration, temperature and pH ranges were deployed for the optimization of extracellular phytase production from *A. niger*. Microbial enzymes production is critically associated with temperature and mitigates with a slight change either in upper or lower side (Bertolin *et al.,* 2003). The study in question consolidated maximum phytase production (2.2±0.15 FTU/ml) at 35°C (Fig. 1). A gradual progression was observed in phytase activity from 25 °C to 35 °C, however a vice versa results were observed beyond the optimum temperature. The low volume of enzyme titers at temperatures below optimum were perhaps due to hindrance in the flow of nutrients across the cell membrane, while at higher temperatures the enzymes and the ribosomal structural changes may affect the production and release of bio-molecules. Higher temperatures may also cause the deleterious changes in the metabolism of living organism' sand consequently low production titers of primary and secondary metabolites arise. Comparatively almost similar temperature range for *A. niger* was identified by Kim *et al.,* (1999) where optimum temperature was recorded as 37 °C however, an indigenous study from Pakistan recorded 30 °C as the most suitable temperature to enhance microbial growth and subsequent phyatse production (Tahir *et al.,* 2010).

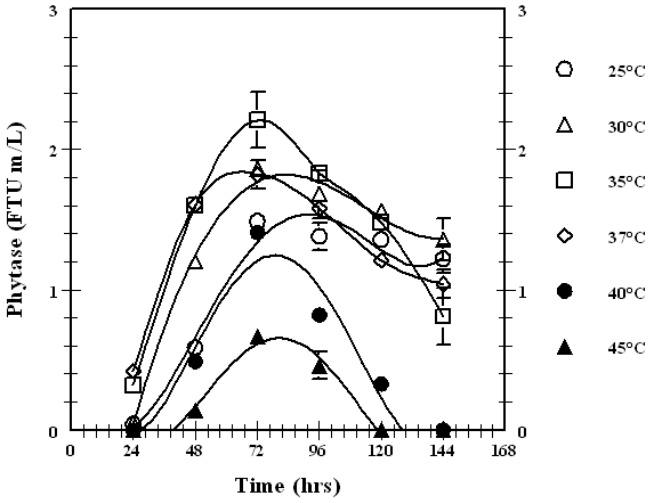

Figure 1. Effect of various temperatures on the activity of phytases from *Aspergillus niger* at pH 5 and 2% wheat bran (w/v) concentration in submerged fermentation. Data presented are average values ± SD of n = 3 experiments

The cost of enzyme production is one of the important factors that affect the ultimate usage of enzymes in the

industry. Only the enzymes bearing low production cost get their attention by industrialists. The raw material for the production of enzymes i.e. substrate is the major contributor for setting the price of enzymes. Agro-industrial wastes being cheaper substrates for the production of enzymes have been investigated in the recent years (Oliveira *et al.*, 2006). Wheat bran is one of the cheaper and potential industrial wastes for the production of various enzymes like amylases, cellulases, phytases etc. Wheat bran has been previously reported with good phytase yield i.e. 4.61 FTU/ml (Javed *et al.,* 2011). Optimizing substrate (wheat bran) concentration has some promising effects on fungal phytase production. Coherent findings were yielded from current study, where utilization of 6% wheat bran as substrate for *A. niger* contributed maximum phytase production rates (4.09±0.23 FTU/ml) as represented in Fig. 2. Wheat bran replacing *A. niger* with *Klebsiella sp*. has also been reported with very lower phytase production rate (0.51 FTU/ml) by Mittal *et al.* (2012). Perhaps the study under focus apprehends wheat bran as promising substrate for phytase production deploying *A. niger* microbial culture.

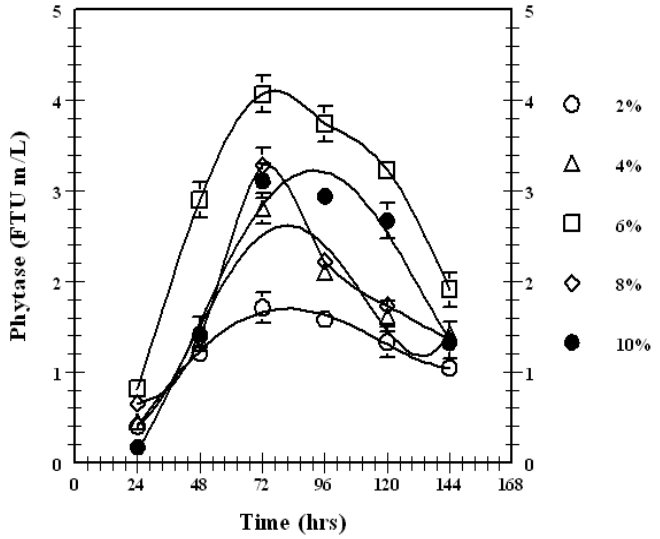

Figure 2. Effect of various concentrations of wheat bran (w/v) on the activity of phytase from *Aspergillus niger* at pH 5 and 35 ˚C in submerged fermentation. Data presented are average values ± SD of n = 3 experiments

Optimum pH is very important for the growth of microbes and their metabolic activities. A sharp decline in activities beyond optimum pH observed on both sides was due to the fact that metabolic activities are very sensitive towards pH change (Bokhari *et al.,* 2008). Parallel to substrate and temperature optimization, slight acidic pH i.e. 6.0 was found to contribute tremendously in highest peak formation yielding 5.20±0.26 FTU/ml phytase from *A. niger* (Fig. 3). Comparatively similar approach was identified in series of experimentation made in *A. niger* growth for phytase production at 5.0, 6.0 and 7.0 pH (Tahir *et al.,* 2010; Bhavsar *et al.,* 2010).

A peak graph built between incubation time and phytase activity for all optimization parameters concealed a gradual increase in phytase production up to 72h, however, further incubation of microbial cultures on substrates revealed a significant and rapid decline in phytase production. The decline in production of phytase after 72 h of incubation was due to initiation of secondary metabolites production, consequently depression in the production of primary products and reduction in the activity of phytase. Possibilities like elimination of nutrient from the substrate, reduction in microbial activity or unfavorable growth conditions for *A. niger* might be some leading factors in rapid reduction of phytase production.

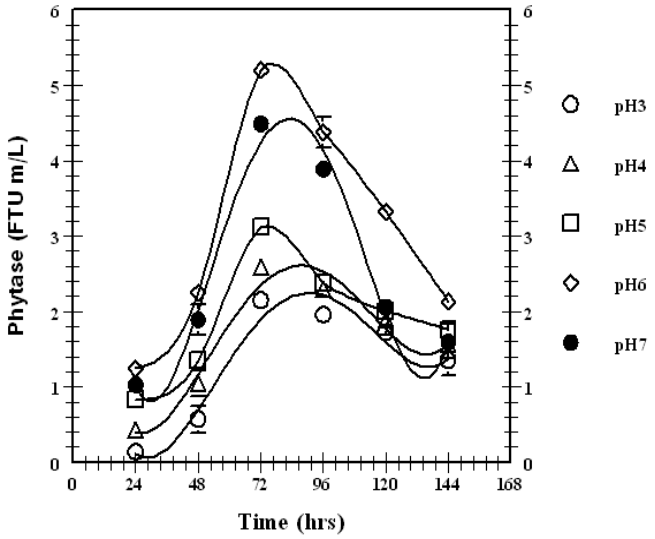

Figure 3. Effect of various pHs on the activity of phytase from *Aspergillus niger* at 35 ˚C and 6% (w/v) wheat bran concentration in submerged fermentation. Data presented are average values ± SD of n = 3 experiments

***Flour characterization*:** White wheat flour being used for bread preparation was supplemented with 5% wheat bran. Proximate composition of wheat bran supplemented flour (Table 1) revealed normal concentration of crude protein (10.1%) however conspicuously quite lower recovery levels of crude fiber (1.23%) and ash (0.587%) were observed probably due to lesser bran concentration as compared to whole wheat flour. Wheat bran has also been characterized as a source of some anti-nutritional complex chemical compounds including phytic acid that chelates mineral elements noticeably iron, manganese and zinc thereby

reducing their availability to the body on dietary intake (Garcia-Estepa *et al.,* 1999). Quite higher level of phytic acid (44.9 mg/g) has been previously reported from whole wheat flour by Frontela *et al.* (2011). Lower bran concentration in flour being used for product development was found to contribute in reduced flour phytic acid concentration i.e. 16.2 mg/g.

Table 1. Composition of 5% (w/w) bran reconstituted wheat flour

Parameters	Composition of wheat flour (5% Bran)
Phytic Acid (mg/g)	16.20±0.130
Moisture (%)	11.93±0.110
Crude protein (%)	10.10±0.270
Crude fat (%)	0.98±0.046
Crude fiber (%)	1.23±0.035
Ash (%)	0.59±0.080
N.F.E. (%)	75.17±0.220

Data presented are average values ± SD of n = 3 experiments.

Bread quality parameters: Technological changes in bread recipes are only accepted if such changes do not significantly affect major qualitative characteristics of the finished product. Seemingly, such changes are often recorded with negative sensorial appeals that ultimately reject new inclusions on consumer acceptability grounds. Apparently as conceived from the sensorial and general characters study of bread prepared from phytase treated wheat flour, slight or no effect on most of the bread characters were identified (Table 2 & 3).

Significant but positive effect of phytase treatment was observed on the volume, taste, aroma and chewability of bread. In the sensorial study the treatment, T_4 showed the best results for volume, taste and chewability of bread, while T_3 showed best results for aroma. The results of some general characters of bread under study showed that the bread volume, weight and moisture percentage were improved by the addition of phytase enzymes. T_5 (2000FTU/kg) showed the best results for bread weight (392.54±11.49) and moisture% (41.56±0.09) while T_4 imparted maximum bread volume increase (546.39±3.34). Maximum quality attributes were satisfied at 1000 FTU/kg phytases supplementation (T_4), revealing overall acceptability of bread qualitative aspects.

Haros*et al.* (2001a) observed improved shape and crumb and a slightly increased bread specific volume by the addition of a commercial phytase from *A. niger*. In another study Zyla *et al.* (2005) also observed increased bread volume and chewiness and an improved bread crumb by the addition of phytases. The role of phytase enzyme as bread improver is thought to be associated with improved α-amylase activity. The addition of phytases breaks the phytate and calcium complex resulting in an increased calcium concentration. These calcium ions accelerate the α-amylase activity consequently the rate of breakdown of starch into simpler sugars is increased which ultimately improves the bread qualitative parameters including bread taste and aroma. Moreover, simpler sugars are available excessively to the yeast activity resulting higher release of CO_2 and H_2O. Hence, greater will be the volume of bread with increased retaining ability of moisture content (Haros*et al.*, 2001b; Park*. et al.*, 2011).

Table 2. Some sensory characteristics of *Aspergillus niger* phytase treated baked bread

Tr.	Volume	Color of crust	Symmetry of form	Evenness of Bake	Character of Crust	Bake and Shred	Grain	Color of Crumb	Aroma	Taste	Texture	Chweability
T_0	7.13±0.21^{f}	6.51±0.16^{b}	2.30±0.05^{a}	2.32±0.06^{d}	2.32±0.08^{d}	2.08±0.04^{e}	7.06±0.27^{b}	7.12±0.15^{f}	6.99±0.36^{d}	9.98±0.38^{e}	9.99±031^{a}	6.49±0.21^{f}
T_1	7.30±0.14^{e}	6.50±0.19^{b}	2.30±0.06^{a}	2.35±0.07^{c}	2.33±0.07^{cd}	2.09±0.07^{e}	7.44±0.13^{a}	7.33±0.24^{e}	7.21±0.29^{c}	10.50±019^{d}	9.94±041^{b}	7.06±0.24^{e}
T_2	7.42±0.34^{d}	6.55±0.21^{b}	2.25±0.07^{ab}	2.39±0.12^{a}	2.28±0.05^{e}	2.16±0.07^{d}	7.48±0.14^{a}	7.52±0.13^{d}	7.53±0.21^{ab}	10.61±0.32^{c}	9.83±032^{c}	7.08±0.45^{d}
T_3	8.00±0.26^{b}	6.90±0.25^{a}	2.22±0.08^{ab}	2.31±0.04^{d}	2.34±0.07^{c}	2.23±0.11^{c}	7.50±0.26^{a}	7.71±0.46^{c}	7.54±0.18^{a}	10.63±0.46^{c}	9.38±033^{d}	7.56±0.46^{c}
T_4	8.23±0.29^{a}	6.89±0.16^{a}	2.18±0.06^{b}	2.35±0.06^{c}	2.41±0.05^{a}	2.42±0.10^{a}	7.50±0.16^{a}	8.11±0.22^{a}	7.50±0.18^{ab}	11.13±0.19^{a}	9.22±020^{f}	7.97±0.66^{a}
T_5	7.92±0.58^{c}	6.91±0.17^{a}	2.22±0.07^{ab}	2.37±0.05^{b}	2.37±0.03^{b}	2.36±0.17^{b}	7.47±0.26^{a}	8.02±0.49^{b}	7.46±0.37^{b}	10.84±0.43^{b}	9.31±052^{e}	7.68±0.70^{b}

Values are expressed as means standard ± deviation (n = 11). Means followed by the same letter within a column are not significantly different($P < 0.05$).

Table 3. Physical characteristics of *Aspergillus niger* phytases treated baked bread

Treatments	Bread Vol. (cc)	Bread Wt. (g)	Specific Vol. (cc/g)	Bread density (g/cc)	Moisture%
T_0	486.47±2.71^{f}	380.11±1.18^{f}	1.28±0.02^{d}	0.78±0.01^{a}	37.44±0.17^{e}
T_1	492.46±4.83^{e}	385.14±1.29^{e}	1.28±0.03^{d}	0.78±0.02^{a}	39.96±0.18^{d}
T_2	500.66±2.73^{d}	390.32±0.09^{d}	1.28±0.03^{d}	0.78±0.03^{a}	40.52±0.30^{c}
T_3	523.22±3.72^{c}	390.68±1.01^{c}	1.35±0.02^{c}	0.75±0.02^{ab}	41.19±0.21^{b}
T_4	546.39±3.34^{a}	392.24±0.08^{b}	1.39±0.02^{b}	0.72±0.02^{ab}	41.23±0.23^{b}
T_5	539.09±1.87^{b}	392.54±0.09^{a}	1.42±0.01^{a}	0.70±0.02^{b}	41.56±0.09^{a}

Values are expressed as means standard ± deviation (n = 3). Means followed by the same letter within a column are not significantly different ($P < 0.05$).

Table 4. Effect of phytases on free minerals in various treatments of bread

Sample	Iron (mg/g)	Calcium (mg/g)	Zinc (mg/g)	Magnesium (mg/g)	Phosphorus (mg/g)
Contro1	0.0011±0.0001^{f}	0.063±0.002^{f}	0.00121±0.00013^{f}	0.13±0.01^{f}	0.53±0.01^{f}
100 FTU/kg	0.0019±0.0001^{e}	0.071±0.001^{e}	0.00126±0.00011^{e}	0.19±0.01^{e}	0.57±0.01^{e}
200 FTU/kg	0.0024±0.0002^{d}	0.074±0.003^{d}	0.00131±0.00016^{d}	0.24±0.01^{d}	0.65±0.01^{d}
500 FTU/kg	0.0031±0.0002^{c}	0.083±0.002^{c}	0.00136±0.00020^{c}	0.28±0.01^{c}	0.72±0.01^{c}
1000 FTU/kg	0.0039±0.0001^{b}	0.088±0.002^{b}	0.00152±0.00022^{b}	0.35±0.01^{b}	0.77±0.01^{b}
2000 FTU/kg	0.0049±0.0001^{a}	0.095±0.001^{a}	0.00181±0.00019^{a}	0.41±0.01^{a}	0.83±0.01^{a}

Values are expressed as means standard ± deviation (n = 3). Means within a column followed by different letter are significantly different ($P < 0.05$).

Phytic acid content: Prevalence of natural phytases degrades a considerable amount of phytate during proofing of cereal products. Addition of phytases in wheat flour in a range of 100-2000 FTU/kg yielded a significant reduction in phytate contents of the bread. Quantification after proofing indicated highest fungal phytase activity yielding 44.41% reduction in phytate content. Reduction in phytate was least in control samples (19.34%), while it was observed maximum (45.56%) in baked bread manufactured from flour treated with 2000 FTU/kg phytases (Fig. 4).

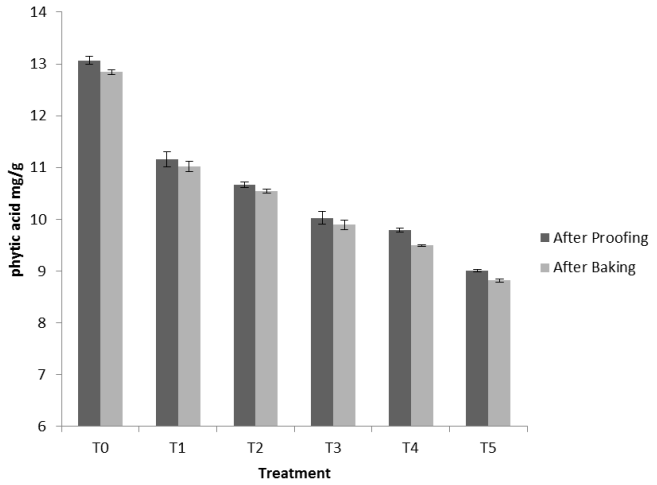

Figure 4. Effect of phytase activity on the reduction in phytic acid content after proofing and baking of 5% bran reconstituted wheat bread. Data presented are average values ± SD of n = 3 experiments

A similar approach has been identified by Park *et al.* (2011) in a recent study on reduction of phytic acid after one hour proofing of dough treated with commercial acidic phytase revealing almost comparative rate of reduction in phytates i.e. 43.4%. A slight higher extent of phytate reduction in current study as compared to work of Park *et al.* (2011) might be associated with type of flour as whole wheat flour carrying higher bran proportion was used in referred study. A similar approach has been identified by Penella and Collar (2008) revealing higher rate of phytate reduction in flour carrying lesser bran fraction. A very slight further reduction in phytic acid content in baked bread might be associated with activity of phytases after proofing prior to their complete inactivation at baking temperature (Haros *et al.*, 2001a). The reduction in phytic acid content by phytases is also reported by Mittal *et al.* (2013).

Free Minerals Content: The results of present study clearly showed that the degradation of phytic acid by various treatments of phytase enzyme resulted in an improved free minerals status of bread (Table 4). The application of various treatments of phytase enzyme showed a direct relation with the mineral content of bread samples. The maximum mineral content was observed in T_5 having 2000FTU/kg phytase enzyme. The maximum increase was observed in iron and magnesium content of bread samples. The increasing trend of mineral content in Ca, Zn and P was almost similar. The results of present study are concomitant to that of Park *et al.* (2011). The maximum increase in the present study was observed in the Fe content revealing the potential of the phytase enzymes for industrial application including baby formulas and diets prepared especially for iron deficient patients.

Conclusion: The findings of the present study indicated the potentials of phytases produced from the indigenous *A. niger* for its applications in food and feed industry. The ideal conditions recorded for maximum phytase yield were pH 6.0, wheat bran 6% (w/v) and a temperature of 35 ºC. The enzyme reduced the phytic acid content up to 44.41% consequently increase in minerals up to a considerable level and improvement in the quality attributes of bread had been observed. The enzyme has potentials for its applications in bread, baking, baby formulas and ready to serve breakfasts. Moreover, bioavailability of iron revealed phytase application in pharmaceutical industry.

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