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Biochemical characteristics of phytase obtained from *Aspergillus niger* Phy8 and its application in dephytinization of various flours

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Phytic acid is considered one of the main sources of organic phosphorus which is required for growth of plants and animals. However, it strongly binds to the soil solid phase and its anti-nutritional activity in feed making phytate phosphorus unavailable to the plants and animals. Phytase is able to improve the phosphorus availability from phytate and thereby phytase production is desirable. *Aspergillus niger* Phy8 has capability of phytase production. The molecular mass of the purified enzyme was about 57 KDa determined by SDS-PAGE. The purified phytase exhibited optimal pH 5.0 and optimal temperature of 50 °C. The enzyme showed acid stability, thermostability and broad substrate specificity, indicating a promising candidate for industrial application. The phytase activity exhibited significant inhibition by Cu^{2+}, Mn^{2+}, Fe^{2+} and Hg^{2+}. However, K^{+} and Mg^{2+} exhibited a significant positive enhancing effect on phytase activity. N-ethylmaleimide (NEM) inhibited the enzyme activity by approximately 25%. N-bromosuccinimide (NBS), diethylpyrocarbonate (DEPC) and phenylglyoxal (PGO) with concentration of 5 mM inhibited phytase activity. The purified enzyme was significantly released the associated phytate products (soluble proteins; phosphorus; reducing sugars), representing compatibility with feed and food applications. In addition, the enzyme phytase showed dephytinization of wheat, corn and rice flours. Therefore, phytase application can improve levels of proteins and reducing sugars, result an increment in the nutritional quality of food ingredients and availability of inorganic phosphate to plants.

Keywords: Phytic acid, *Aspergillus niger*, Phytase, Thermostability, Dephytinization, Application.

INTRODUCTION

Phytases [*myo*-inositol (1,2,3,4,5,6) hexakisphosphate phosphohydrolases] (EC 3.1.3.8) belong to the family of histidine acid phosphatases (HAPs). They catalyze the sequential dephosphorylation of phytate (*myo*-inositol hexakisphosphate) to inorganic phosphate and derivatives of *myo*-inositol (Singh et al., 2006 and Dersjant et al., 2014).

Phytate is the phosphate storage form in oil seed, legumes and cereals crops (Cao et al., 2007). Phytate display the essential percentage of organic phosphorus in soil which is strongly binding to the soil solid phase and thereby making phosphate of phytate unavailable to the plants (Gerke 2015). Plants possess different ways to enhance the phosphorus sorption from soil: the production of phosphatases and root exudates.

Therefore, the exudation of phytase activity is a potentially important step in bioavailable phosphorus acquisition from soil phytate (Vance et al. 2003 and Gaind and Nain 2015).

In contrast to other available organic phosphate molecules, phytate serves a major source of phosphorus which needed development for feed of some animals. In particular, pigs, fishes and poultry (monogastric animals) which have low or no phytase activities in their digestive tracts (Brinch-Pedersen et al., 2002; Singh and Satayanarayana, 2010). The anti-nutritional effect of phytate may be due to the combination with amino acids and proteins, thus inhibits digestive enzymes activities. Phytate also causes mineral deficiency by chelating essential minerals including calcium, magnesium, potassium, iron and zinc. This may be due to a high negative charge of phytate phosphate content over a wide pH range (Shanmugam, 2018). Obviously, the phytase is vastly utilized to manage the environmental and nutritional challenges caused by phytate.

Numerous publications have revealed that phytases are ubiquitous phosphomonoesterases found in plants and microorganisms, particularly yeasts and fungi. In addition, the most prolific extracellular producers of phytate degradation enzymes have been mostly reported by fungi, particularly from the genera *Aspergillus*, *Penicillium*, *Rhizopus* and *Mucor* (Konietzny and Greiner, 2002 and Farhat et al., 2008). *Saccharomyces cerevisiae* and *Schwanniomyces castellii* have been tested for their capability to produce phytase (Bohn et al., 2008).

In the present work, phytase from *Aspergillus niger* phy8 was isolated and purified. The biochemical characteristics of the purified phytase were studied. In addition, the dephytinizations of various foods flours by the purified enzyme were evaluated.

MATERIALS AND METHODS

Soil samples and screening for phytate-degrading fungi

Samples of soil were collected in plastic bags from different agriculture fields, compost and poultry wastes located in Egypt. To isolate the phytase producing fungi, a serial dilution of each of the collected samples were performed using sterile saline solution (10 ml, 0.9%) and shaken thoroughly for 10 min. After dilution, 100 µl supernatant of each dilution was spread on a modified PSM medium (phytase screening medium, pH 6.5) containing glucose 1.5%, KCl 0.05%, $(NH_4)_2SO_4$ 0.5%, NaCl 0.01%, $MgSO_4.7H_2O$ 0.01%, $CaCl_2.2H_2O$ 0.01%, $MnSO_4.H_2O$ 0.001%, $FeSO_4.7H_20$ 0.001% and 0.5% sodium phytate (Hosseinkhani et al., 2009).Sodium phytate was aseptically supplemented to the sterilized PSM medium using 0.45 µm Millipore sterile filters. The plates were cultivated for 5 days at 30 °C. The fungal isolates were subcultured, purified and preserved on modified PSM medium for further work.

Primary screening of the fungal isolates for their capability to syntheses phytase on modified PSM agar medium supplemented with 0.5% sodium phytate as sole phosphorus source. All the plates were inoculated with spore suspension ($2x10^6$ /ml) and incubated for 5 days at 30 °C. After incubation, modified PSM agar plates were tested for clearing zone around the fungal growth. Zone of clearness around the reported fungus that can synthesis phytase was used as positive control. The result was confirmed by double staining method (Bae et al., 1999).The positive fungal isolates were preserved on PDA (potato dextrose agar) medium and stored at 4 °C. The selected isolate was sub-cultured periodically to sustain its viability during the work period.

Phytase activity assay

All positive isolates were examined for the phytase enzyme synthesis in modified PSM medium containing sodium phytate. Spore suspension of $2x10^6$ /ml was inoculated in 100 ml of modified PSM medium and then incubated on an orbital shaker (150 rpm) for 5 days at 30 °C. After incubation time, fungal mycelium was filtered using Whatman filter paper No. 1 (Whatman, Piscataway, NJ, USA) and then washed using sterile distilled water. The filtrate was centrifuged at 10,000 rpm for 30 min. The obtained supernatant was used as a source of phytase and preserved at -20 °C for further work.

The phytase activity was measured according to the modified method of previous publication (Gulatiet al.2007).The reaction mixture contained 50 µl of culture supernatant and 50 µl of phytate solution (10mM sodium phytate dissolved in sodium acetate buffer (0.2 M, pH 5.5)) was incubated at 37 °C for 1 h and terminated by adding 100 µl of trichloroacetic acid (15%), 300 µl distilled water and 0.9 µl of the color developing reagent (ammonium molybdate, 2.5%; ascorbic acid, 10%; sulfuric acid, 0.76 M; 3:1:0.5 v/v/v). The resulting mixture was determined after 20 min by a spectrophotometer at 820 nm. The phytase

activity unit was expressed as the amount of enzyme that produced one micromoles of phosphate in one minute under the assay condition. Protein content was determined according to Bradford's method (1976) for protein assay using Bovine serum albumin as a standard.

Purification of phytase enzyme

The fungal free extract was shaked constantly for 24 h at 4 °C with addition of $(NH_4)_2SO_4$ (85%). Following by centrifugation for 15 min at 10,000 rpm, the obtained precipitate was collected and dissolved in sodium acetate buffer (200 mM, pH 5.0). The resulting solution was dialyzed overnight at 4 °C in a glass beaker against the same buffer, using dialysis membrane (546-00051, Viskase Sales Corporation, Wako, Japan). The dialyzed phytase was then loaded onto a DEAE-Sepharose column which was pre-equilibrated with sodium acetate buffer (pH 5.0). Active fractions were collected, dialyzed and subjected to Sephadex G-100 column which was washed by equilibration buffer. The eluted fractions were assayed for phytase activities using UV-spectrophotometer.

Optimal pH and temperature

The optimal temperature of the enzyme was estimated by carrying out activity assays at the different temperatures from 20 to 80 °C by the standard phytase assay protocol. The enzyme thermal stability was measured after that the phytase was pre-incubated for 1 h at temperature range of 20–80 °C in pH 5.0 and the remaining activities were determined at frequent intervals.
The pH profile of phytase was determined in the pH range from 2.0 to 8.0 using 0.2 M sodium acetate (2-3), sodium citrate (4-6) and Tris-HCl (7-8) buffer. The enzyme stability at various pH values was measured after pre-incubating the enzyme at pH of 2.0–8.0 (Na acetate buffer, 0.2 M) at room temperature and the remaining activities were measured using the standard phytase assay method.

Influence of various metal ions on phytase activity

The metal ions were tested using chloride salts including $CuCl_2$, $CoCl_2$, KCl, $MgCl_2$, $CaCl_2$, $MnCl_2$, $HgCl_2$, $FeCl_2$ and NaCl with concentration of 5 mM.

Effect of chelating agent and essential group reagents on phytase activity

The impact of chelating agent (α, ά-dipyridyl) was examined at various concentrations; 0.2, 0.4, 0.6, 0.8, 1.0 mM. The nonionic surfactant (Triton-X 100) was tested at 0.2, 0.4, 0.6, 0.8, 1.0% v/v. Furthermore, the reagents of essential groups including N-ethylmaleimide (NEM), N-bromosuccinimide (NBS), diethylpyrocarbonate (DEPC) and phenyl glyoxal (PGO) at two concentrations (1 and 5 mM) on phytase activity were investigated. Remaining phytase activities were measured in accordance with the above phytase determination method.

Substrate specificity for phytase

Phytase substrate specificity was assayed by incubating the purified phytase with different phosphate compounds (defined below) at 1.2 mM as final concentration. The evaluation of residual activities was estimated through the phytase activity assay as mentioned above.

Dephytinization of different food grains flour

Corn flour (CF), wheat flour (WF), and rice flour (RF) were purchased from a local market in Egypt. Samples (5 g) of flour were added to with 20 ml of sodium acetate buffer separately (pH 5.0, 0.2 M). The obtained sterilized flour suspension was incubated with phytase (20 U/ml) at 100 rpm and 50°C. At frequent intervals, the samples were taken and centrifuged at 10,000 rpm for 10 min and then analyzed for reducing sugar content (Miller 1959). The inorganic phosphorus content was determined according to Fiske and Subbarow (1925) and the soluble protein content was determined according to Lowry et al. (1951). All experiments were performed in three replicate and their mean values are illustrated along with the standard deviation.

RESULTS AND DISCUSSION

Screening of phytase producing strains

Qualitative screening for fungal isolates of phytase producing strains was performed on a modified PSM medium with 0.5% sodium phytate as the sole phosphorus source. Ninety three fungal isolates showed a clear halo zone as the primary indication of phytase formation. False positive results may arise as a result of acidic production by certain fungi. Therefore, the potentiality of fungal isolates for phytase production was subject to secondary screening for all positive isolates using modified PSM medium.

The results depicted in Table 1 showed that out of 93 fungal isolates only fifteen isolates from various sources exhibited a considerable phytase activity. Isolate Phy8 was selected for further work

due to its maximum phytase activity. The fungal culture was identified microscopically as *Aspergillus niger* according to Barnett and Hunter 1998.

Table 1 Phytase activities of various fungal isolates from different sources.

Fungal isolates codes	Source of isolation	Size of clear zone (mm)	Phytase activity (U mg^{-1} protein)
Phy1	Soil	7 ± 0.4	1.4 ± 0.03
Phy2	Soil	9 ± 0.3	2.5 ± 0.01
Phy3	Soil	5 ± 0.1	4.2 ± 0.01
Phy4	Soil	10± 0.3	7.2 ± 0.2
Phy5	Soil	16± 0.2	11.5 ± 0.1
Phy6	Compost	19± 0.2	15.7 ± 0.1
Phy7	Compost	7 ± 0.4	5.1 ± 0.1
Phy8	Compost	28± 0.1	22.6 ± 0.4
Phy9	Compost	8 ± 0.2	3.2 ± 0.01
Phy10	Compost	19± 0.1	14.9 ± 0.3
Phy11	Compost	12± 0.4	8.4 ± 0.1
Phy12	Poultry wastes	18± 0.1	9.2 ± 0.1
Phy13	Poultry wastes	6 ± 0.2	4.7 ± 0.1
Phy14	Poultry wastes	21± 0.1	17.4 ± 0.3
Phy15	Poultry wastes	25± 0.5	20.6 ± 0.2

*SD, standard deviation

Table 2 Purification scheme for the phytase enzyme from *A. niger* Phy8

Purification stage	Total protein (µg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	892.1	30.34	34.01	1	100
$(NH_4)_2SO_4$ (85%)	34.1	4.29	125.6	3.9	14.1
DEAE-sepharose	4.03	1.01	251.7	7.4	3.3
Sephadex G100	0.89	0.29	325.8	9.5	0.96

Phytase was most commonly found in higher plants (Greiner et al. 2000), bacteria (Hong 2011) and fungi, particularly from the *Aspergillus* species (Gontia-Mishra et al., 2013; Neira-Vielma et al., 2018).

Purification of phytase from *A. niger* Phy8

The purification of phytase produced by the *A. niger* Phy8 strain was achieved using a combination of ammonium sulfate precipitation (85%), DEAE-Sepharose chromatography and Sephadex G-100 chromatography. The phytase purified by DEAE-sepharose chromatography showed specific activity of 251.7 U mg^{-1}. Subsequently, this fraction was submitted to Sephadex G-100 showing purification fold of 9.5 and recorded with 0.96% yield (Table 2).The purification process to electrophoretic homogeneity was performed using SDS-PAGE analysis, which displayed a single protein band of 57 KDa. The purification yield of the extracellular enzyme obtained from *A. niger* was 15.5% with 7.4-fold and molecular weight of 89 KDa by SDS-PAGE (Neira-Vielma et al., 2018).The phytase from *A. niger* van Teighem showed a molecular weight of approximately 66 KDa (Vats and Banerjee, 2005; Soniet al., 2010). While the molecular weight of phytase produced by *Aspergillus niger* UFV-1 was 81 KDa using SDS-PAGE (Monteiroet al. 2015).

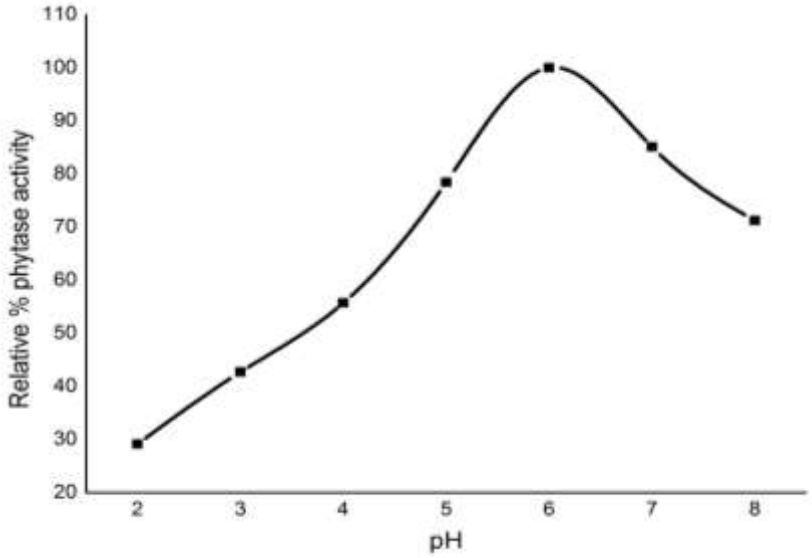
Biochemical characterization of phytase from *A. niger* Phy8

The maximum phytase activity was recorded at 50 °C (Fig.1A). In addition, the purified enzyme exhibited more than 81% and 33.7% residual activities at 60 °C and 80 °C respectively. Phytase activity declined gradually with increasing the temperature to 80 °C and this effect was attributed to the enzyme denaturation, increase in inherent energy of the system and conformational changes in protein tertiary structure (Zoldac et al., 2004). In addition, phytase stability towards temperature is demonstrated in Fig.2A. Purified enzyme showed 56.4%, 52.3% and 40.3% remaining activity at 60,

70, 80 °C, respectively.

The purified phytase activity as a function of pH exhibited its optimum at 5.0 using 0.2 M sodium citrate buffer as demonstrated in Fig. 2B.

A



B

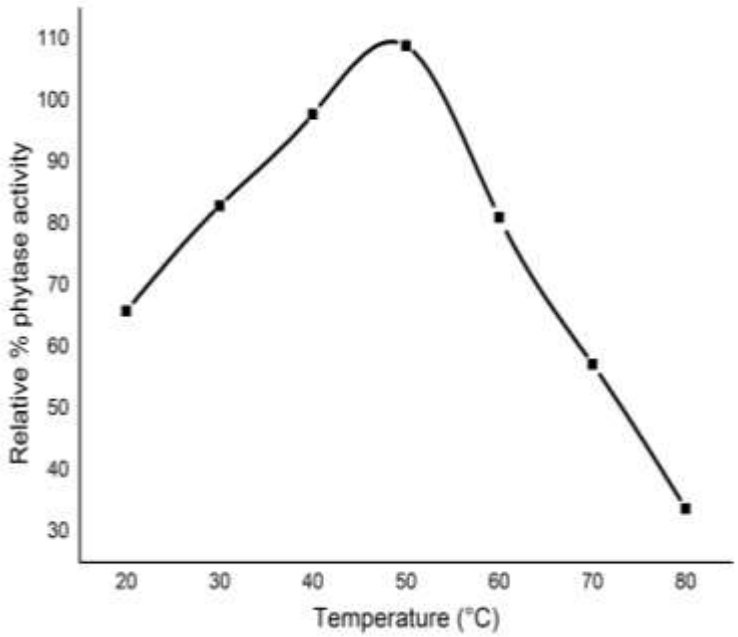


Figure. 1 Optimum temperature (A) and pH (B) of the purified phytase enzyme from *A. niger* phy8.

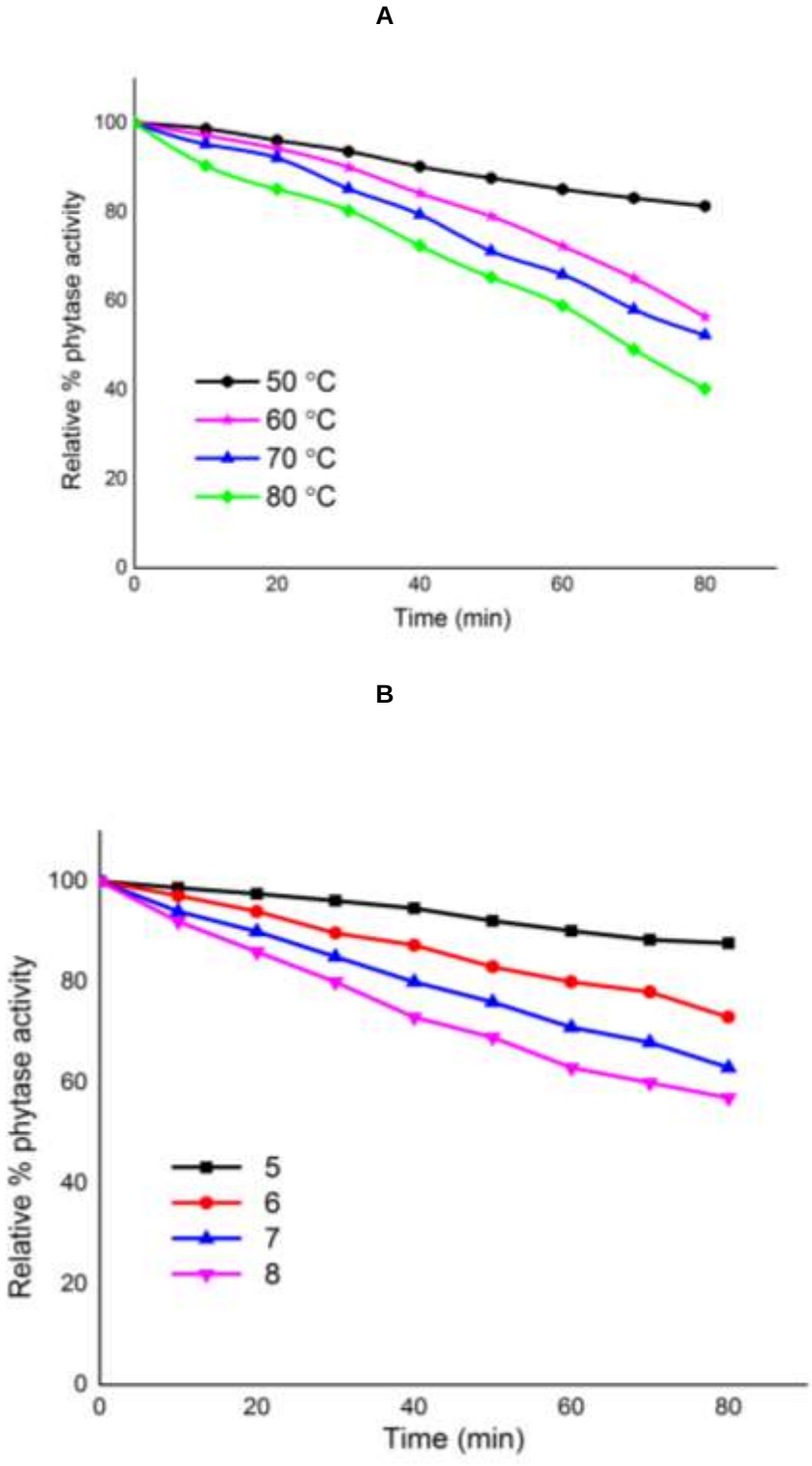


Figure 2;Thermal stability (A) and pH stability (B) of *A. niger* phy8 phytase.

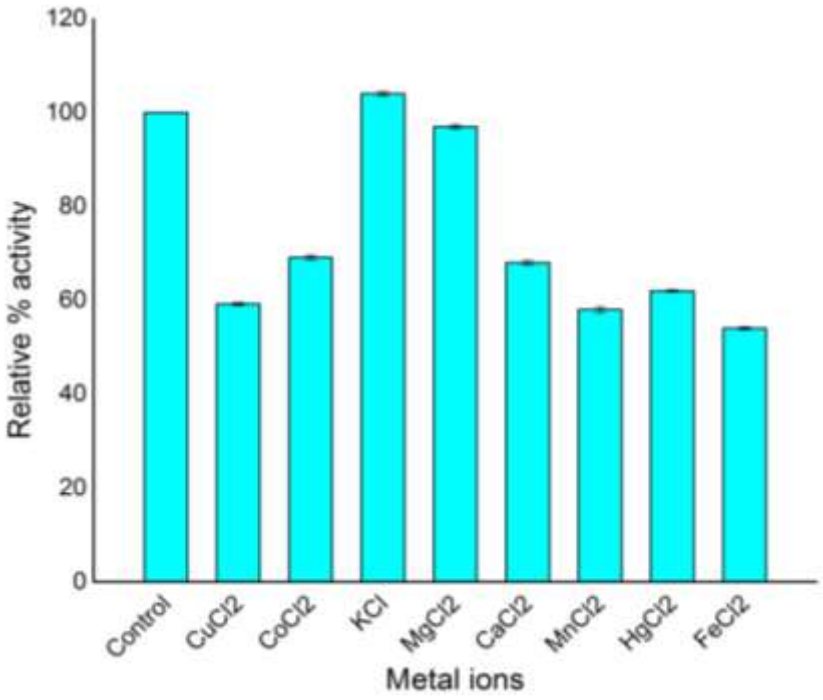


Figure. 3 Effect of different metal ions on phytase activity from *A. niger* Phy8.

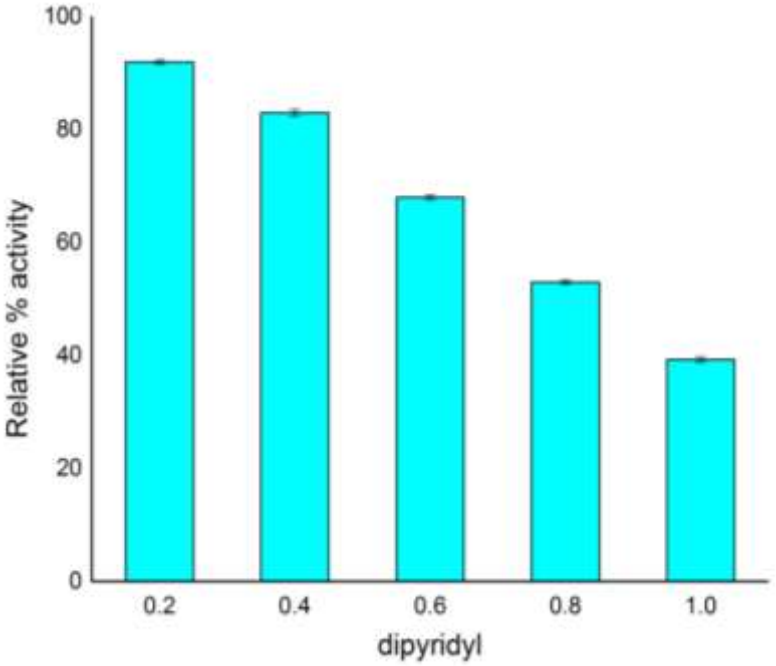


Figure. 4 Susceptibility of phytase from *A. niger* Phy8 towards α, ά-dipyridyl.

The purified phytase had more than 80% residual activity in the pH range from 5.0 to 7.0. These observations are in agreement with the findings of phytase obtained from *A. niger* which has most phytase activity at pH 5.3 and 56 °C

(Neira-Vielma et al., 2018). Phytase from *E. coli* (ATCC 25922) and *A. niger* (ATCC 16888) have maximal activity at temperature of 45 °C and at pH range from 3.5 to 5.0 (Mogal et al., 2017).

The inhibitory response of phytase activity towards various pH values, suggests a declension in enzyme production and variation in enzyme ionization state. Furthermore, different microbial metabolic activities are sensitive to lower or higher pH than the optimum value because of modification of enzyme surface charge and dissociation of coenzyme or subunits (Suresh and Radha, 2015).

The effect of various metal ions on phytase activity is shown in Fig.3. It was observed that Cu^{2+}, Mn^{2+}, $Fe^{2+}$and Hg^{2+} has markedly inhibition on phytase activity. On the other hand, the purified phytase exhibited a significant positive stimulatory effect by K^{+} and Mg^{2+}. Previous studies have reported the inhibition of phytase by Fe^{2+}, Co^{2+},Cu^{2+}, and Mg^{2+} on *Bacillus subtilis* CF92 phytase activity (Hong, 2011; Zhang et al., 2013). In addition, Monteiro et al. (2015) formed slight inhibition of phytase by K^{+}, Mg^{2+}, Ca^{2+} and Cd^{2+}.

The obtained results are similar to that of purified phytase derived from *A. niger* which inhibited by Ca^{2+} and K^{+}. While, Mg^{2+}, Mn^{2+}, Cu^{2+}, Cd^{2+} and Ba^{2+} showed enhancing effect on phytase activity (Neira-Vielma et al., 2018).The inhibition of phytase by metal ions can speculate on two possible reasons. First, the formation of soluble complexes with sodium phytate causes a drastic decrease in the active concentration of phytate in assay procedure. Second, metal ions have an inhibitory effect on enzyme productivity (Wyss et al., 1999; El-Shora et al., 2004).

The results shown in Fig. 4 exhibited that α, ά-dipyridyl, chelating agent, drastically inhibited enzymatic activity and occurred depending on the concentration tested. The obtained results revealed that phytase is metalloenzyme which in consistent with the result obtained by El-Shora et al., (2016) who mentioned that α-α-dipyridyl showed phytase activity inhibition in a dose-dependent manner. Shin et al., (2001) reported that phytases are enzymes in which metal ion cofactor is required for the catalysis process. Phytase was susceptible towards nonionic surfactant (Triton-X 100), even at low concentrations (Fig. 5), suggesting unmasking of the hidden catalytic sites and induce conformational changes (Mandviwala and Khire, 2000).

The effect of NEM, NBS, DEPC and PGO on the purified phytase was presented in Table 3. Phytase activity was moderately reduced approximately 25% activity by 5 mM NEM, suggesting that the SH group may be essential in the enzyme-catalytic activity. The results revealed that 5 mM NBS gradually inhibited phytase activity (13% decrease), indicating that tryptophanyl group seems essential for enzyme catalysis. DEPC suppressed phytase activity at a concentration of 5 mM by 24%. This outcome suggested that the histidyl group is necessary for enzyme catalysis. Phytase activity is more susceptible to 5 mM PGO and decreased by about 30 %. It is suggested that arginyl residue may essential for the enzyme-catalytic activity. Phytases have a several amino acid residues which are involved in cleavage and affinity sites of enzyme (Shin et al., 2001).

Table 3; Effects of different reagents of essential groups on phytase activity from *A. niger* Phy8.

Reagents	Relative activity (%)	
	1 mM	5 mM
Control	100	100
NEM	98.7 ± 1.9	75.1 ± 1.3
NBS	92.1 ± 1.6	86.7 ± 1.7
DEPC	89.4 ± 1.4	76.1 ± 1.5
PGO	90.3 ± 1.5	68.4 ± 1.0

Table 4; Susceptibility of phytase towards various phosphate compounds.

Substrate	Relative activity (%)
Sodium phytate*	100
β-Glycerophosphate	41.2 ± 0.7
AMP	81.3 ± 1.5
ATP	85.4 ± 1.3
Phenyl phosphate	62.7 ± 0.6
1-Naphthyl phosphate	57.8 ± 0.8
3-phosphoglycerate	55.4 ± 0.9
p-Nitrophenylphosphate	48.7 ± 0.7
Glycerol-2-phosphate	72.1 ± 1.1
Glucose-6-phosphate	77.5 ± 1.5

*The phytase activity obtained when sodium phytate (5 mM) used as substrate was defined as 100%.

The results shown in Table 4 demonstrated the phytase substrate specificity and the capability of the phytase to dephosphorylate different phosphate compounds. It was shown that the enzyme displayed broad substrate specificity, and, in particular, the highest phytase activity was reached using sodium phytate. The purified enzyme dephosphorylated adenosine monophosphate (AMP) and adenosine

triphosphate (ATP) with relative activity above 80%. Similar observations were previously mentioned in few studies (Wyss et al., 1999; Zhang, et al., 2013; Neira-Vielma et al., 2018).

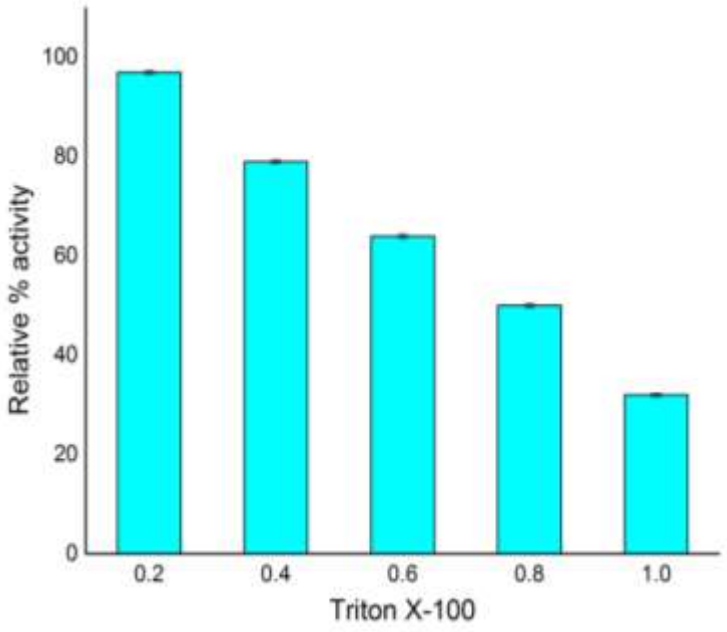
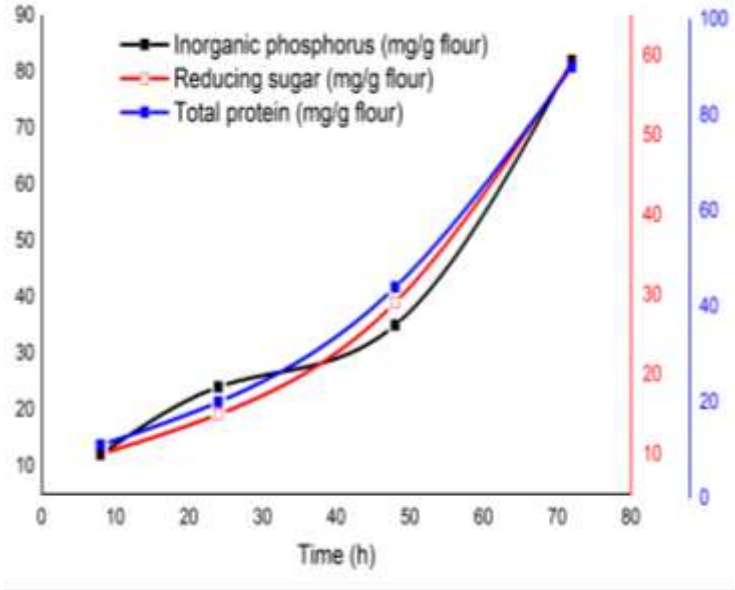


Figure 5;Susceptibility of phytase from *A. niger* Phy8 towards Triton-X 100.

A



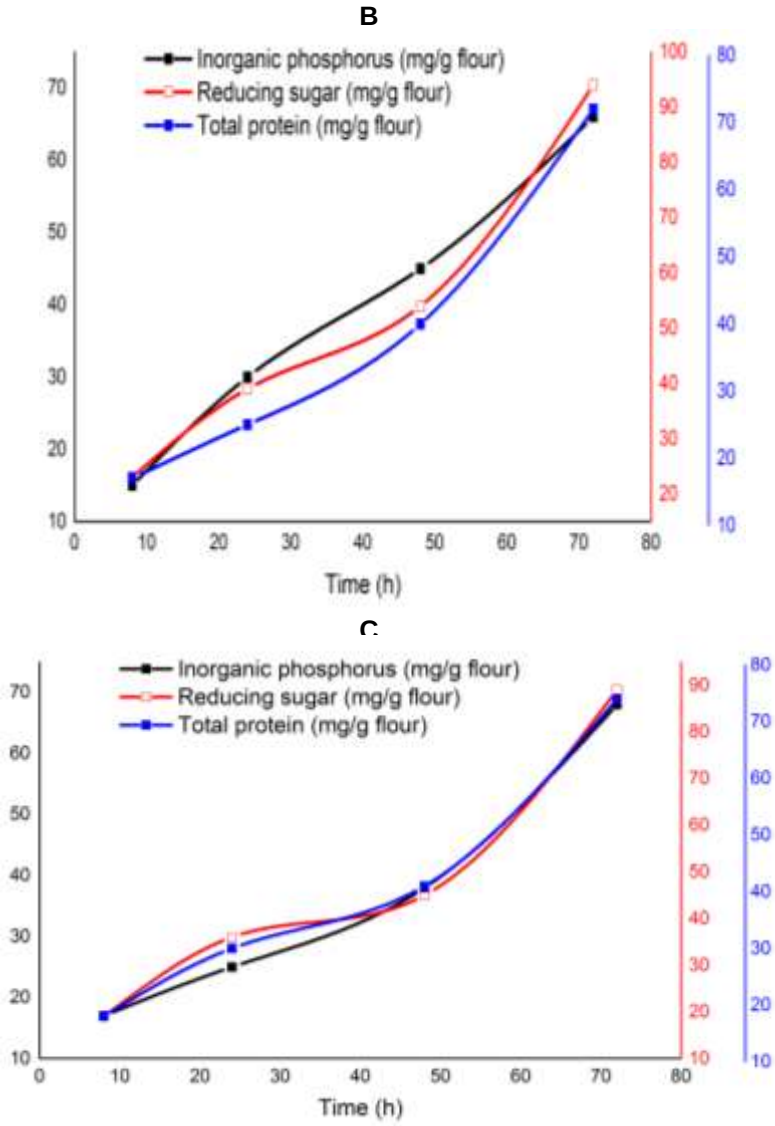


Figure 6; Dephytinization of wheat flour (a), corn flour (b) and rice flour (c) using *A. niger* Phy8 phytase at 55 °C and pH 6.0.

Dephytinization of various flours by phytase from *A. niger* Phy8

Inorganic phosphate, soluble protein and reducing sugars were liberated from rice flour (RF), wheat flour (WF) and corn flour (CF) (Fig. 6). The highest liberation of inorganic phosphorus was obtained using wheat flour, followed by rice flour and corn flour. This may attributed to different quantity of released inorganic phosphorus by phytase and nature of the phosphorylated substrate (Ali et al., 2007). Phytase from *A. niger* Phy8 was considerably efficient using WF (90%) with a higher rate of soluble protein released, followed by RF (74%) and CF (72%). In addition, the released of reducing sugars showed better performance in CF, followed by RF and WF. The highest release of phytate associated products (soluble proteins and reducing sugars), indicating insoluble complex formation between phytic acid, chelating and anti-nutritional agent, and minerals, proteins, as well as carbohydrates (Singh and Satyanarayana, 2014).

CONCLUSION

The characterization of phytase from *A. niger* Phy8 was investigated and revealed that enzyme displayed a maximum activity at 50 °C, a broad pH range and substrate specificity towards various phosphate compounds. Interestingly, the extracellular phytase obtained from *A. niger* Phy8 was effective for dephytinization of wheat, rice and corn flours, releasing inorganic phosphorus, reducing sugars and soluble proteins. Therefore, phytase from *A. niger* Phy8 seems can function as a promising enzyme for industrial applications.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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