Pak. J. Agri. Sci., Vol. 54(4), 799-806; 2017
ISSN (Print) 0552-9034, ISSN (Online) 2076-0906
DOI: 10.21162/PAKJAS/17.5517
http://www.pakjas.com.pk

PRODUCTION AND CHARACTERIZATION OF PHYTASE FROM INDIGENOUS *Aspergillus niger* ISOLATES

Madeeha Tariq[1], Muhammad Nawaz[1*], Aftab Ahmad Anjum[1], Saba Sana[1], Mian Abdul Hafeez[2], Jawad Nazir[1], Wasim Shahzad[3] and Muhammad Imran Najeeb[1]

[1]Department of Microbiology, [2]Department of Parasitology, [3]Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Sciences, Lahore, Pakistan
[*]Corresponding author's e-mail: muhammad.nawaz@uvas.edu.pk

Aim of the present study was to isolate phytase producing *Aspergillus niger*, and production, purification and characterization of phytase enzyme which is responsible for conversion of organic phosphorous (phytate) into inorganic form (available). Indigenous *A. niger* isolates (n=20) were identified through macroscopic (obverse side: brown to black, granular, folded, cottony from center with smooth and white edges, reverse side: center light pale, opaque edges and ridges) and microscopic characteristics (phialospores in chains, circular vesical, metullae, phialids, septate and hyaline hyphae). Isolates were screened for their ability to produce phytase on Phytase Screening Medium. Nine isolates (45%) gave positive screening results through cobalt chloride staining (2% cobalt chloride, 6.25% ammonium molybdate and 0.42% ammonium vanadate). Diameter of colonies, zone of hydrolysis and zone of hydrolysis to colony diameter ratio were 20.97- 43.00 mm, 25.03-49.00 mm and 1.13-1.19, respectively. Phytase activity of cell free supernatants of indigenous *A. niger* isolates ranged from 68.88±2.55 to 274.99±10.14 FTU/mL. PASN01 and PASN06 exhibited highest enzyme activity (274.99±10.14 and 274.99±9.00 FTU/mL, respectively). Phytase were characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Molecular weights of phytases from *A. niger* isolates ranged from 35.21 to 107.82 kilo Daltons. It is concluded that indigenous *A. niger* PASN01 and PASN06 may have commercial application after further characterization.
Keywords: *Aspergillus niger*, Phytase, Zone of hydrolysis, Molecular weight

INTRODUCTION

Phytase is an enzyme that can initiate the release of phosphorous (P) from phytin/phytate (IUB, 1979). Phytate break down in to myo-inositol and phosphoric acid (Pandey *et al*., 2001). Phytase belong to the family of histidine acid phosphatases (HAP), a subclass of phosphatases and catalyze the hydrolysis of phytic acid (Mitchell *et al*., 1997). Phytic acid accounts 60–90% of P present in plant (Vats *et al*., 2009). Plant P is generally present as a constituent of phytin (IUB, 1979). Phytate is a poly-anionic chelating agent due its reactive groups. It reacts with proteins, amino acids, and important cations such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, and Mn^{2+} in humans and animals (Haefner *et al*., 2005). This results in malnutrition and various health problems such as poor digestion, bone weakness, iron deficiency and tooth decay (Hurrell *et al*., 2003). Ruminants contain microflora which release inorganic P from phytate. Monogastric animals (human, chickens or mice) produce little or no phytase in their intestine. Due to that, excessive phosphorus accumulations in their feces or droppings cause problems such as water pollution, algal blooms, and changes of fauna and flora in the environment (Mullaney *et al*., 1999). Phytase of fungi and bacteria have great nutritional important (Onyango *et al*., 2005). In modern era, there has been a significant interest in the use of microbial phytases to release phytate-bound P and to improve overall P availability in poultry diets. Supplementation of phytase is effective in improving the availability of P for growth, bone mineralization, metabolizable energy, nutrient digestibility and nutrient retention in broiler diets (Żyła *et al*., 2001). Phytase enzyme increase nutritive values of food and feed by hydrolyzing the phytate. Phytate hydrolysis by either plant or microbial phytases also improves human nutrition (Sandberg and Andlid, 2002). Phytase added in monogastric animal feed helps to cope with problem of non-bioavailability and environmental contamination of P (Casey and Walsh, 2004).

Different microbes have been screened for their phytase production ability on phytase screening medium (PSM) (Howson and Davis, 1983) supplemented with different substrates: Calcium phytate (Gupta *et al*., 2014) and sodium phytate (Gontia-Mishra *et al*., 2013). Molecular weights of phytases have been determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Molecular

weights of phytases generally range from 38 to 200 kilo Daltons (kDa) (Wodzinski and Ullah, 1995).

Aim of the present study was to screen phytase producing indigenous *Aspergillus niger* (*A. niger)* isolates, and partial purification and characterization of their phytase.

MATERIALS AND METHODS

A. niger isolates (n=20) were procured from Department of Microbiology, University of Veterinary and Animal Sciences (UVAS) Lahore, Pakistan. These isolates were cultured on Sabouraud dextrose agar (SDA) (LAB M Limited).

***Identification of isolates*:** SDA mediam with following composition: peptone 10g, dextrose 40g and agar 15g in 1000mL distilled water was prepared according to manufacture guidelines with pH 5.6±0.2 and autoclaved at 121°C and 15lbs pressure for 15mins. *A. niger* isolates were inoculated on SDA media and incubated for 3 days at 25±3°C. Macroscopic characteristics of isolates were studied from obverse and reverse side of culture plates (Watanabe, 2010). To study the microscopic characteristics tease mount method of Leck (1999), cellophane tap method of Harris (2000) and slide culture method of Wijedasa and Liyanapathirana (2012**)** were followed. Microscopic characteristics were visualized using 400X magnification under bright field microscope.

***Screening for phytase production*:** Indigenous *A. niger* isolates were screened for phytase production following the method of Gontia-Mishra *et al*. (2013). PSM was prepared by mixing: 15g glucose, 2g NH_4NO_3, 0.5g KCl, 0.5g $MgSO_4.7H_2O$, 0.3g $MnSO_4.7H_2O$, 0.001g $FeSO_4.7H_2O$ and 20g agar in 1liter distilled water (pH 5.6). Media was autoclaved at 121°C and 15lbs pressure for 15mins. Filter sterilized sodium phytate (2%) was added in PSM as substrate. Isolates were inoculated on PSM plates and incubated at 28°C for 5-7 days.

***Confirmation of phytase production through cobalt chloride staining*:** Isolates screened on PSM media were further confirmed for phytase production through cobalt chloride staining method of Bae *et al*. (1999). Cobalt chloride solution (2%) was flooded on culture plate. After 5mins of incubation, cobalt chloride solution was decanted and a mixture of ammonium molybdate (6.25%) and ammonium vanadate (0.42%) (1:1) was flooded on culture plates and plates were re-incubated for 5minutes. Isolates were confirmed as positive for phytase if the zone of hydrolysis was retained.

***Phytase production*:** Phytase was produced following the method of Monteiro *et al*. (2015). Phytase was produced by inoculating ~10^6 spores of *A. niger* in 250mL flasks containing sterilized phytase screening broth (pH 5.6). Flasks were incubated at 25°C for 5-7 days. Phytase activity was determined through phytase activity assay.

***Phytase activity assay*:** Phytase activity assay was performed following the method of Kerovuo *et al*. (1998). Cell free supernatant (0.2mL) was mixed with (0.8mL) substrate (0.2% sodium phytate in 0.2 M acetate buffer) and incubated at 37°C for 30 minutes. Trichloroacetic acid solution (5%) was added as stop solution. One milliliter of coloring reagent (4 parts of 3% ammonium molybdate solution prepared in 5.5% sulfuric acid and 1 part of 2.7% $FeSO_4.7H_2O$) was added and absorbance was taken at 655nm. Quantity of orthophosphate was calculated from a standard curve prepared using KH_2PO_4 and enzyme units were calculated. One unit of enzyme was defined as amount of enzyme releasing one µmol of inorganic phosphorous per minute under assay conditions.

***Phytase partial purification*:** Phytases were partially purified following the method of Gunashree and Venkateswaran, (2015). Broth cultures were filtered by 0.44 µm syringe filter paper. Enzyme was precipitated by 80% saturated solution of ammonium sulfate followed by centrifugation at 13000 rpm for 30minutes at 4°C. The pellets were dissolved in 0.2M sodium-acetate buffer (pH: 5.6).

***Characterization of phytases by SDS-PAGE*:** Molecular weights of phytases produced by *A. niger* were determined by SDS-PAGE as described by Laemmli (1970). Partially purified phytases were resolved using 12.5% polyacrylamide gel. Relative flow (R_f) value of each sample was measured. Molecular weights of phytases were determined through a standard curve made between R_f values and logarithmic molecular weights of marker bands. R_f was calculated by using following formula:

R_f: distance travel by protein bands/distance travel by dye front

***Statistical analysis*:** Data of zone of hydrolysis, colony diameter and enzyme activity of different isolates were given as mean ±SD and compared by one way analysis of variance (ANOVA) followed by Duncan's multiple range test using statistical package for social sciences (SPSS version 20) at $p < 0.05$.

RESULTS

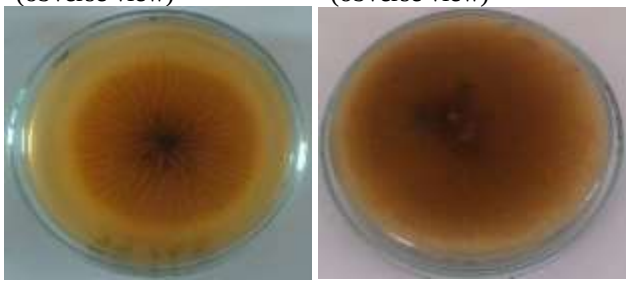
***Identification of Fungi*:** Macroscopic characters including colony color and texture specific for *A. niger* are given in Table 1. Representative pure culture plates photographed from obverse and reverse side are presented as figure (1a, b, c and d). Microscopic characters were observed by Cellophane tape method, tease mount method and slide culture. Unicellular circular black colored spores arranged in chains were observed by cellophane tape method (Fig. 2a). Phialospores arranged in the form of long chains covering vesicle along with hyaline phialophore were visualized (Fig. 2b). Microscopic characters recorded during growth of fungal isolates on slide culture were hyaline septate hyphae, vesicle, metullae and conidiophores (Fig. 2c and d).

Table 1. Macroscopic and microscopic characters of *Aspergillus niger* isolates.

Sr. No.	Isolate No.	Macroscopic Characters	Microscopic Characters
1	PASN 01		
2	PASN 02	**Obverse side**	**Hyphae**
3	PASN 03		
4	PASN 04	Initially white turning to brown to black in colour with white periphery. Texture cottony to granular, folds present	Septate, hyaline hyphae
5	PASN 05		
6	PASN 06		**Structures**
7	PASN 07		
8	PASN 08		Presence of vesicle on conidiophore, phialids and metullae.
9	PASN 09		
10	PASN 10	**Reverse side**	
11	PASN 11		
12	PASN 12	Pale to colorless. Ridges present	**Phialospores**
13	PASN 13		
14	PASN 14		Circular conidia in chains all around the vesicle on phialids. Conidia pigmented (black in color)
15	PASN 15		
16	PASN 16		
17	PASN 17		
18	PASN 18		
19	PASN 19		
20	PASN 20		

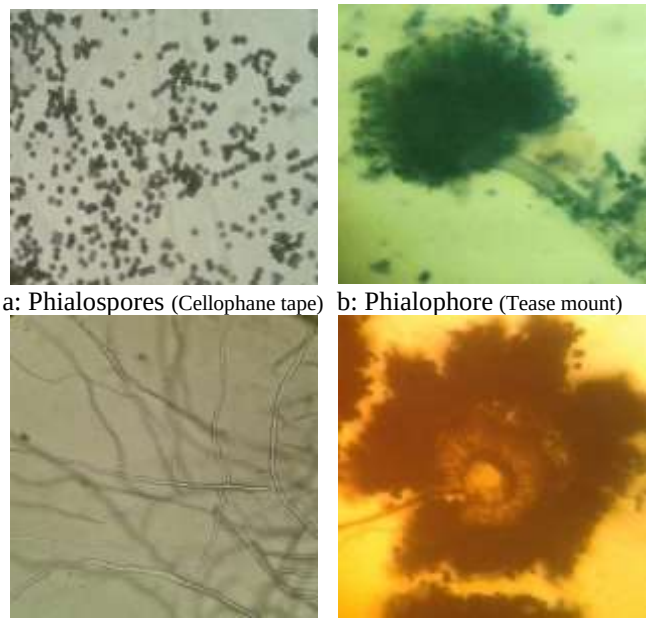
PASN: Phytase producing *Aspergillus niger*

a: Immature colony (obverse view) b: Mature colony (obverse view)



c: Immature colony (reverse view) d: Mature colony (reverse view)

Figure 1. Colony characters of *Aspergillus niger* isolates.



a: Phialospores (Cellophane tape) b: Phialophore (Tease mount)

c: Hyphae (Slide culture) d: Mature phialophore (Slide culture)

Figure 2. Microscopic characters of *Aspergillus niger* isolates at 400X magnification.

***Screening for phytases production*:** Morphologically identified *A. niger* isolates were screened for phytase production using Phytase screening agar supplemented with sodium phytate. Zone of hydrolysis around *A. niger* colonies were observed. Representative zone of hydrolysis of phytate are shown in figure 3a, 3b, 3c and 3d. Out of twenty, nine isolates (45%) (PASN 01, PASN 03, PASN 04, PASN 06, PASN 08, PASN 09, PASN 10, PASN 11 and PASN 12) were positive for phytases production. Diameter of colony, zone of hydrolysis on PSM media (Mean ± SD) and zone of hydrolysis to colony diameter ratio of each isolate are given in table 2. PASN04 showed highest (43.00±0.1mm) ($p<0.05$) while PASN12 showed significantly ($p<0.05$) lowest (20.97±0.06mm) colony diameter. Colony diameters of PASN01, PASN03, PASN06, PASN08, PASN09, PASN10 and PASN11 were 41.03±0.54, 30.09±0.77, 24.04±0.06, 31.03±0.05, 36.06±0.12, 31.03±0.15 and 31.00±0.20mm, respectively. Statistically significant differences were observed among colony diameters of *A. niger* isolates ($p<0.05$). Zone of hydrolysis observed around colony of PASN04 isolate was largest (49.00±0.10 mm), while PASN12 produced smallest zone of hydrolysis (25.03±0.06mm). Zone of hydrolysis of PASN01, PASN03, PASN06, PASN08, PASN09, PASN10 and PASN11 were 47.12±0.52, 36.01±0.02, 28.00±0.01, 36.00±0.20, 41.00±0.00, 35.01±0.01 and 37.03±0.15 mm, respectively. Zone of hydrolysis to colony diameter ratios was highest (1.19) for PASN12 and lowest (1.13) for PASN10.

Table 2. Screening of *Aspergillus niger* isolates for phytases production potential

Sr. No	Isolates	Diameter of colony	Diameter of zone of hydrolysis	Zone of hydrolysis to Colony diameter ratio
1	PASN 01	41.03±0.54[a]	47.12±0.52[a]	1.15
2	PASN 03	30.09±0.77[b]	36.01±0.02[b]	1.19
3	PASN 04	43.00±0.10[a]	49.00±0.11[a]	1.14
4	PASN 06	24.04±0.06[c]	28.00±0.10[c]	1.16
5	PASN 08	31.03±0.05[b]	36.00±0.20[b]	1.16
6	PASN 09	36.07±0.12[d]	41.00±0.00[d]	1.14
7	PASN 10	31.03±0.15[b]	35.01±0.01[c]	1.13
8	PASN 11	31.00±0.20[b]	37.03±0.15[b]	1.19
9	PASN 12	20.97±0.06[e]	25.03±0.06[e]	1.19

[a,b,c,d]Means carrying different superscript within same column differ significantly

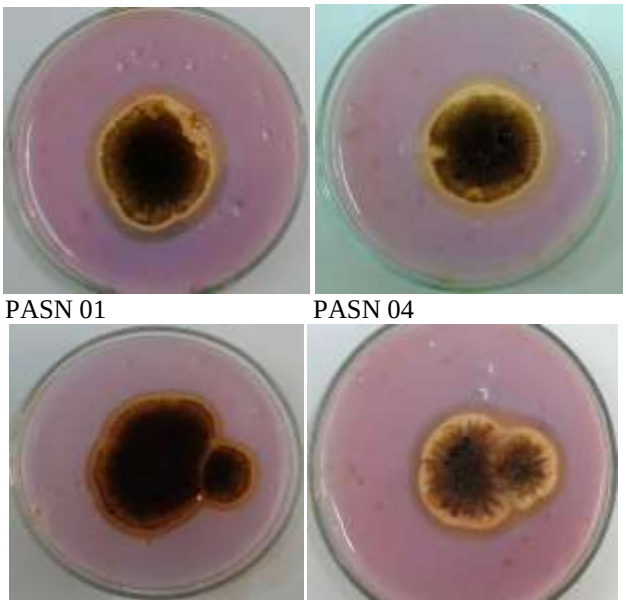


Figure 3. Screening of *Aspergillus niger* isolates for phytases production potential.

***Phytase activity*:** After 5-7 days incubation phytase activity was determined. PASN01 and PASN06 showed highest activity units 274.99±10.14 and 274.99±9.0 FTU/mL, respectively. PASN08 showed lowest enzyme activity 68.88±2.55 FTU/mL. Activities of PASN03, PASN04, PASN06, PASN12, PASN09, PASN10 and PASN11 phytase were 109.99±7.27, 258.88±9.77, 274.99±.00, 77.77±7.88, 187.77±2.55, 88.88±6.94 and 139.99±10.14 FTU/mL respectively. There were non-significant (p>0.05) differences observed between enzyme activity units of PASN01 and PASN06. There were also non-significant differences observed among enzyme activity units of PASN08, PASN10 and PASN12 (Table 3).

Table 3. Quantitative analysis of Phytase production potential of *Aspergillus niger* isolates.

Sr. No	Isolates	Mean FTU/ml
1	PASN 01	274.99±10.14[a]
2	PASN 03	109.99±7.27[b]
3	PASN 04	258.88±9.77[c]
4	PASN 06	274.99±9.00[a]
5	PASN 08	68.88±2.55[d]
6	PASN 09	187.77±2.55[e]
7	PASN 10	88.88±6.94[f]
8	PASN 11	139.99±10.1[g]
9	PASN 12	77.77±7.88[d]

[a,b,c,d.e.,f.g]Means carrying different superscript within columns differ significantly and with same non-significantly

***Characterization of phytases by SDS-PAGE*:** Phytases produced by different isolates in broth cultures were concentrated using saturated ammonium sulfate and their molecular weights were determined by SDS-PAGE using 12.5 percent polyacrylamide resolving gel (Fig. 4). Molecular weights of phytases produced by different isolates ranged between 35.21 to 107.82 kDa (Table 4). Molecular weight of phytase produced by isolate PASN06 was highest (107.82 kDa) and of PASN01 lowest (35.21 kDa). Molecular weights of enzymes produced by PASN03, PASN04, PASN08, PASN09, PASN10, PASN11 and PASN12 were 70.87, 100.54, 105.34, 98.22, 87.41, 72.54 and 57.46 kDa, respectively.

Table 4. Relative flow values, logarithmic molecular weights and molecular weights of phytases produced by *Aspergillus niger* isolates.

Isolates	Lane No.	Peptide No.	R_f value	Logarithmic molecular wt.	Molecular weight (kDa)
PASN01	3	1	0.58	1.55	35.21
PASN03	4	1	0.41	1.85	70.87
PASN04	5	1	0.32	2.00	100.54
PASN06	6	1	0.30	2.03	107.82
PASN08	7	1	0.31	2.02	105.34
PASN09	8	1	0.32	1.99	98.22
PASN10	9	1	0.35	1.94	87.41
PASN11	10	1	0.40	1.86	72.54
PASN12	11	1	0.46	1.76	57.46

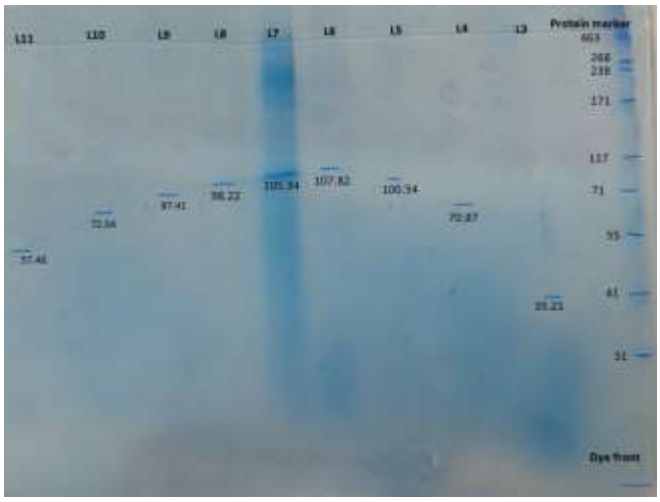


Figure 4. Bands (manually marked) of phytase as resolved by Sodium dodecyl sulphate Polyacrylamide gel electrophoresis

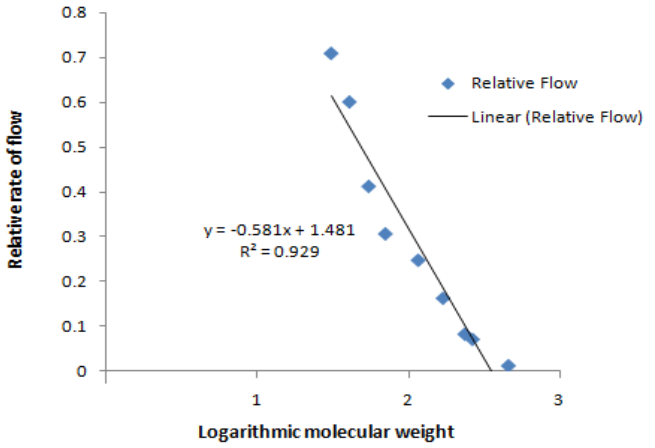


Figure 5. Standard curve between relative flow and logarithmic molecular weights of protein markers.

DISCUSSION

***Identification of fungi*:** Different culture media have been used to culture and identify *A. niger*. Habib *et al*. (2015) used Rose Bengal chloramphenicol agar as selective media to study the characteristics *of* aspergilli from obverse and reverse side of culture plate after 72 hrs incubation at room temperature. Macroscopic characteristics included dark brown to black colony, entire margins, umbonate elevation. In another previous report, *Aspergillus* species was studied by culturing on potato dextrose agar (PDA) and czapek dox agar medium (CZA) (Gautam and Bhadauria, 2012).

According to Diba *et al*. (2007) microscopic characteristics including stipe with slightly brown, smooth surface, biseriate-large vesicle covered with metullae, glubose and rough conidia were presented by *A. niger* isolates on different culture media i.e czapek dox agar (CZ), czapek yeast agar (CYA), malt extract agar (MEA) and czapek yeast 20% sucrose agar media. Microscopic results of Present study were comparable with Diba *et al*. (2007). Gautam and Bhadauria, (2012) studied microscopic characteristics (septate and branched hyphae, glubose vesicle, blackish brown conidia and biseriate phialids covering entire vesicle) were in agreement with microscopic characters of present study.

***Screening of Aspergillus niger isolates for phytase production*:** *A. niger* isolates were screened for phytase production on PSM agar. In present study 0.2 % sodium phytate was used as substrate for screening in PSM agar. Gupta *et al*. (2014) screened isolated fungal isolates on PSM agar with Ca-phytate 0.5% as substrate. In another study PSM agar with 0.5% sodium phytate as substrate was used to screen different fungal isolates (Gontia-Mishra *et al*., 2013). Gangoliya *et al*. (2015) used 0.1% Ca phytate as substrate to screen the *A. fumigatus* on PSM agar of pH 5.5.

A. niger isolates were confirmed as positive for phytase production through cobalt chloride staining method in a previuous study (Bae *et al*., 1999). Cobalt chloride 0.2%, ammonium molybdate 6.25% and ammonium vanadate 0.42% were used. Gontia-Mishra *et al*. (2013) also screened *Aspergillus* spp. for phytase production through cobalt chlorie staining. Cobalt chloride staining is used to rule out false positive results (Coban and Demirci, 2014).

Out of 20, only 9 (45%) *A. niger* isolates were screened as positive on PSM media. In a previous study, eight *A. fumigatus* isolates out of 50 were selected as phytase producers (Gangoliya *et al*., 2015). Betancur *et al*. (2012) also selected 26 for phytase producing fungal isolates.

Zone of hydrolysis (12 ± 0.3 to 32 ± 0.2 mm) of different fungal isolates reported by Gontia-Mishra *et al*. (2013) were comparable with zone of hydrolysis (25.03±0.1 to 49.00±0.11mm) observed in present study. Zones of hydrolysis were in contrast with reported zone of hydrolysis (8 ± 0.2 to 19 ± 0.1 mm) of Gupta *et al*. (2014). Using method of cobalt chloride staining proposed by Bae *et al*. (1999), all nine *A. niger* isolates were confirmed as positively for phytase production.

***Phytase activity*:** Enzymes are generally produced solid state or submerged fermentation with different advantages of each. Moisture level is one of the critical factors for optimum enzyme production (Shivanna and Venkateswaran, 2014). Phytase enzyme activity of cell free supernatant of aspergilli has been reported to be dependent on temperature, moisture level, production media, inducing substrate and inoculum size (Shivanna and Venkateswaran 2014) Increased or decreased moisture level impact the enzyme production by affecting the growth of fungal isolates (Gautam *et al*., 2002; Al-Asheh and Duvnjak, 1995). In present study, phytase activities of cell

free supernatant of *A. niger* isolates grown in PSM broth ranged from 68.88±2.55 to 274.99±10.14 FTU/mL which were comparable with previous reports. Monteiro *et al*. (2015) analyzed phytase production by *A. niger* UFV-1 and reported that it had 138.6 U/mL phytase enzyme activity. In another previous study by Rani and Ghosh (2011), phytase enzyme has been reported form *Aspergillus oryzae*. Lata *et al*. (2015) reported that Glucose 0.5% in medium supported higher phytase production. Phytase enzyme produced at a wide range of pH has also been reported previously (Casey and Walsh, 2003; Oh *et al*., 2004).

***Molecular weight of phytase*:** Phytases were characterized and their molecular weight was estimated by SDS PAGE. Previous reports indicate that molecular weight of phytases range from 38 to 200 kDa (Wodzinski and Ullah, 1995). In present study, molecular weight (35.21kDa) of phytase from PASN01 was in contrast with earlier findings on molecular weights of phytase from *A. niger* isolates. Molecular weight of PASN03 (70.87 kDa), PASN11 (72.54 kDa), and PASN12 (57.46 kDa) phytases were compareable with weights of phytases from *A. ficuum* NTG-23 (65.5 kDa) (Zhang *et al*., 2010) and *A. niger* CFR 335 (66kDa) (Gunashree and Venkateswaran, 2015). Molecular weight (87.41kDa) of phytase of PASN10 was comparable with molecular weight of *A. niger* NICM (87kDa) (Bhavsar *et al*., 2011) and *A. niger* UFV-1 (81kDa) (Monteiro *et al*., 2015). Molecular weights PASN04, PASN06 and PASN08 were 100.54, 107.82 and 105.34 kDa, respectively which were similar to the molecular weights of phytase from *A. niger* FS3 (108kDa) (Spier *et al*., 2011) and *R. oligospora* (120kDa) (Casey and Walsh, 2004). In a previous report, molecular weight of purified phytase of *Streptococcus thermophillus* was ~90kDa which is comparable to molecular weight of PASN09 (98kDa), reported in this study (Singh and Satyanarayana, 2009)

Conclusion: It is concluded that indigenous isolates of aspergilli contain phytase enzyme activity which may be optimized for their enhanced production. *A. niger* PASN01 and *A. niger* PASN06 have excellent phytase activity and may be used for industrial production of phytase under optimum conditions. It is also insinuated that phytases reported in this study may have commercial application after further characterization.

REFERENCES

Al-Asheh, S. and Z. Duvnjak. 1995. The effect of phosphate concentration on phytase production and the reduction of phytic acid content in canola meal by *Aspergillus carbonarius* during a solid-state fermentation process. Appl. Microbiol. Biotechnol. 43:25-30.

Bae, H.D., L.J. Yanke, K.J. Cheng and L.B. Selinger. 1999. A novel staining method for detecting phytase activity. J. Microbiol. Metheds. 39:17-22

Betancur, M.O., L.F.P. Cervantes, M.M. Montoya, M. S. Yepes and P.A.G. Sánchez. 2012. Isolation and characterization of potential phytase-producing fungi from environmental samples of Antioquia (Colombia). Rev. Fac. Nal. Agr. Medellín. 65:6291-6303.

Bhavsar, K., V.R. Kumar and J.M. Khire. 2011. High level phytase production by *Aspergillus niger* NCIM 563 in solid state culture: Response surface optimization, up-scaling, and its partial characterization. J. Ind. Micbiol. Biotechnol. 38:1407-1417.

Casey, A. and G. Walsh. 2003. Purification and characterization of extracellular phytase from *Aspergillus niger* ATCC 9142. Bioresour. Technol. 86:183-188.

Casey, A. and G. Walsh. 2004. Identification and characterization of a phytase of potential commercial interest. J. Biotechnol. 110:313-322.

Coban, H.B. and A. Demirci. 2014. Screening of phytase producers and optimization of culture conditions for submerged fermentation. Bioprocess Biosyst. Eng. 37:609-616.

Diba, K., P. Kordbacheh, S.H. Mirhendi, S. Rezaie and M. Mahmoudi. 2007. Identification of *Aspergillus* species using morphological characteristics. Pak. J. Med. Sci. 23:867-872.

Gangoliya, S.S., R.K. Gupta and N.K. Singh. 2015. Phytase production through response surface methodology and molecular characterization of *Aspergillus fumigatus* NF191. Ind. J. Exp. Biol. 53:350-355.

Gautam, A.K. and R. Bhadauria. 2012. Characterization of *Aspergillus* species associated with commercially stored triphala powder. Afr. J. Biotechnol. 11:16814-16823.

Gautam, P., A. Sabu, A. Pandey, G. Szakacs, and C.R. Soccol. 2002. Microbial production of extra-cellular phytase using polystyrene as inert solid support. Bioresour. Technol. 83:229-233.

Gontia-Mishra, I., D. Deshmukh, N. Tripathi, K. Bardiya-Bhurat, K. Tantwai and S. Tiwari. 2013. Isolation, morphological and molecular characterization of phytate-hydrolysing fungi by 18S rDNA sequence analysis. Braz. J. Microbiol. 44:317-323.

Greiner, R., L.G. Silva and S. Couri. 2009. Purification and characterization of an extracellular phytase from *Aspergillus niger* 11T53A9. Braz. J. Microbiol. 40:795-807.

Gunashree, B.S. and G. Venkateswaran. 2015. Extracellular phytase from *Aspergillus niger* CFR 335: purification and characterization. J. Food. Sci. Technol. 52:4558-4564.

Gupta, R.K., S.S. Gangoliya and N.K. Singh. 2014. Isolation of thermotolerant phytase producing fungi and optimization of phytase production by *Aspergillus niger*

NRF9 in solid state fermentation using response surface methodology. Biotechnol. Bioproc. Eng. 19:996-1004.

Habib, M., P. Abdul, C.N. Kwanashie, J. Kabir and A. Negedu. 2015. Isolation and identification of *Aspergillus* species from poultry feeds in Kaduna State, Nigeria. Micbiol. Res. Int. 3:27-32.

Haefner, S., A. Knietsch, E. Scholten, J. Braun, M. Lohscheidt and O. Zelder. 2005. Biotechnological production and applications of phytases. Appl. Microbiol. Biotechnol. 68:588-597.

Harris, J.L. 2000. Safe, low-distortion tape touch method for fungal slide mounts. J. Clin. Microbiol. 38:4683-4684.

Howson, S.J. and R.P. Davis. 1983. Production of phytate-hydrolyzing enzyme by some fungi. Enzyme Microb. Technol. 5:377-382.

Hurrell, R.F., M.B. Reddy, M-A. Juillerat and J.D. Cook. 2003. Degradation of phytic acid in cereal porridges improves iron absorption by human subjects. Am. J. Clin. Nutr. 77:1213-1219.

International Union of Biochemists (IUB). 1979. Enzyme nomenclature. Page 247 in Recommendations of the Nomenclature Committee of the International Union of Biochemistry. Academic Press, New York.

Kerovuo, J., M. Lauraeus, P. Nurminen, N. Kalkkinen and J. Apajalahti. 1998. Isolation, Characterization, Molecular Gene Cloning, and Sequencing of a Novel Phytase from *Bacillus subtilis*. Appl. Environ. Microbiol. 64:2079-2085.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227:680-685.

Lata, S., S. Rastogi, A. Kapoor and M. Imran. 2015. Influence of culture conditions on production of phytase by *Zygosaccharomyces bailii* var *bailii*. J. Environ. Biol. 36: 947-954

Leck, A. 1999. Preparation of lactophenol cotton blue slide mounts. Community Eye Health. 12: 24.

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

Monteiro, P.S., V.M. Guimarães, R.R. Melo and S.T. Rezende. 2015. Isolation of a thermostable acid phytase from *Aspergillus niger* UFV-1 with strong proteolysis resistance. Braz. J. Microbiol. 46:251-260.

Mullaney, E.J., C.B. Daly and A.H. Ullah. 1999. Advances in phytase research. Adv. Appl. Microbiol. 47:157-199.

Oh, B.C., W.C. Choi, S. Park, Y.O. Kim, and T.K. Oh. 2004. Biochemical properties and substrate specificities of alkaline and histidine acid phytases. Appl. Microbiol. Biotechnol. 63:362-372.

Onyango, E., M.R. Bedford and O. Adeola. 2005. Efficacy of an evolved *Escherichia coli* phytase in diets of broiler chicks. Poult. Sci. 84:248-255.

Pandey, A., G. Szakacs, C.R. Soccol, J.A. Rodriguez-Leon and V.T. Soccol. 2001. Production, purification and properties of microbial phytases. Bioresour. Technol. 77:203-214.

Rani, R. and S. Ghosh. 2011. Production of phytase under solid-state fermentation using *Rhizopus oryzae*: Novel strain improvement approach and studies on purification and characterization. Bioresour. Technol. 102:10641-10649.

Sandberg, A.S. and T. Andlid. 2002. Phytogenic and microbial phytases in human nutrition. Int. J. Food. Sci. Technol. 37:823-833.

Shivanna, G.B. and G. Venkateswaran. 2014. Phytase production by *Aspergillus niger* CFR 335 and *Aspergillus ficuum* SGA 01 through submerged and solid-state fermentation. Sci. World J. 2014:1-6

Singh, B. and T. Satyanarayana. 2009. Characterization of a HAP–phytase from a thermophilic mould *Sporotrichum thermophile*. Bioresour. Technol. 100:2046-2051.

Spier, M.R., R.C. Fendrich, P.C. Almeida, M. Noseda, R. Greiner, U. Konietzny, A.L. Woiciechowski, V.T. Soccol and C.R. Soccol. 2011. Phytase produced on citric by products: Purification and characterization. World. J. Microbiol. Biotechnol. 27:267-274.

Ullah, A.H.J. and B.J. Cummins. 1987. Purification, N-Terminal Amino Acid Sequence and Characterization of pH 2.5 Optimum Acid Phosphatase (EC 3.1.3.2) from *Aspergillus ficuum*. Prep. Biochem. 17:397-422.

Vats, P. and U.C. Banerjee. 2005. Biochemical characterization of extracellular phytase (myo-inositol hexakisphosphate phosphohydrolase) from a hyper-producing strain of *Aspergillus niger* van Teighem. J. Ind. Microbiol. Biotechnol. 32:141-147.

Vats, P., B. Bhushan and U.C. Banerjee. 2009. Studies on the dephosphorylation of phytic acid in livestock feed using phytase from *Aspergillus niger* van Teighem. Bioresour. Technol. 100:287-291.

Watanabe, T. 2010. Pictorial atlas of soil and seed fungi: Morphologies of cultured fungi and key to species, 3rd Ed. Taylor and Francis Gp. CRC press., New York.

Wijedasa, M.H. and L.V.C. Liyanapathirana. 2012. Evaluation of an alternative slide culture technique for the morphological identification of fungal species. Sri J. Infect. Dis. 2:47-52

Wodzinski, R.J. and A.H. Ullah. 1995. Phytase. Adv. Appl. Microbiol. 42:263-302.

Zhang, G.Q., X.F. Dong, Z.H. Wang, Q. Zhang, H.X. Wang and J.M. Tong. 2010. Purification, characterization, and cloning of a novel phytase with low pH optimum and

strong proteolysis resistance from *Aspergillus ficuum* NTG-23. Bioresour. Technol. 101:4125-4131.

Żyła, K., J. Koreleski, S. Świątkiewicz, D.R. Ledoux and J. Piironen. 2001. Influence of supplemental enzymes on the performance and phosphorus excretion of broilers fed wheat-based diets to 6 weeks of age. Anim. Feed. Sci. Technol. 89:113-118.