DIVERSITY OF PHYTASE PRODUCING NON-TOXIGENIC FUNGI ISOLATED FROM SOIL

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ABSTRACT

In present study diversity of phytase producing fungi isolated from soil of livestock farms in 10 percent villages of Lahore district of Punjab was explored. A total of 260 fungal isolates were obtained by soil dilution technique from soil samples (n=29) and qualitatively screened for phytase production on plates of phytase screening medium. Twenty eight (n=28) isolates were positive for phytase production. Out of 28 phytase producing fungi,13 isolates were toxigenic as detected by thin layer chromatography. Out of 15 non-toxigenic fungi, selected isolates (n=12) were identified as *Aspergillus niger* (n=7) and *Aspergillus flavus* (n=5) by morphological method using macroscopic and microscopic characters. The native non-toxigenic phytase producing fungi can be used for cost effective enzyme production on industrial level.

Key words: Soil, livestock farms, phytase, thin layer chromatography, morphological method and cost effective

INTRODUCTION

Phytate (myo-inositol hexakisphosphate) is a major storage form of organic phosphorus in plant seeds (Mitchell *et al.,*1997). Monogastric animals lack enzyme to hydrolyse the phytate into inorganic phosphorus. This has a number of undesirable consequences. Phytic acid chelates multivalent cations and some proteins, thereby rendering these biologically unavailable to the animal (Harland and Morris,1995). As a result, the phytate acts as anti nutritional agent and undigested phosphorus passes out as excreta into the environment where it causes phosphorus pollution in areas of intensive livestock production. When phosphorus goes into aquatic environment, it disturbs water quality which is known as eutrophication. Thus, inadequately used phosphorus results in environmental complications and economical loss (Cromwell and Coffey, 1991).

Phytases (myo-inositol-hexakisphosphate3-phosphohydrolases) are acid phosphatase enzymes, which efficiently cleave phosphate moieties from phytic acid (myo-inositol-hexakisphosphate), thereby generating myo-inositol and inorganic phosphate (Mitchell *et al.,*1997). Phytases have important applications in human and animal nutrition because they hydrolyze the phytate present in legumes, cereal grains and oil seeds. This results in increased availability of minerals, trace elements and amino acids as well as phosphate. Phytase has both microbial and non-microbial sources. Its non-microbial source includes plants and animals where it is naturally present. Microbial sources include a large number of bacteria and fungi which produce phytase through fermentation using a great variety of substrates. Commercial production of phytase as feed additives is mostly focused on fungi and yeasts, as they are the most prolific extracellular producers of this enzyme (Farhat *et al.,*2008). Most of the naturally occurring phytases having high thermostability and a broad pH range are produced by fungi (Simon and Igbasan,2002). Keeping in view the increasing importance of phytase for food and feed industries, present study was conducted on diversity of phytase producing fungi isolated from soil of livestock farms in Lahore district of Punjab, Pakistan.

MATERIALS AND METHODS

Soil samples (n=29) were randomly collected in polythene bags from livestock farms in ten percent villages of district Lahore. The fungi were isolated by soil dilution method as described by Ocampo *et al*.,(2012). Soil sample (1g) was dissolved in 10mL of 0.85% saline solution in a falcon tube, gently shaken and kept in stand for 10-20 minutes. One mL soil suspension was transferred to 9 mL saline in tube and this procedure was repeated to obtain a dilution of 1/10000 times. One mL of the appropriate dilutions (10^{-2},10^{-3} and10^{-4}) were spread onto sterilized Sabouraud·s dextrose agar plates and incubated at 26°C for 7 days. Pure fungal isolates were inoculated on plates of phytase screening medium (PSM) agar consisting of 0.5% sodium phytate, 0.5% NH_4NO_3, 0.05% KCl, 0.05% $MgSO_4.7H_2O$, 0.03% $MnSO_4.4H_2O$, 0.03%$FeSO_4.7H_2O$, 3% glucose and 2% agar with pH 5.5 (Howson and Davis1983). The plates were incubated at 25°C for 7days. The fungal isolates producing zone of hydrolysis on PSM plates were confirmed by double staining method as described by Bae *et al*., (1999). The PSM plates were flooded with aqueous

cobalt chloride solution (2%). After 5 minutes incubation at room temperature, cobalt chloride solution was replaced with freshly prepared solution containing equal volumes of ammonium molybdate solution (6.25%) and ammonium vanadate solution (0.42%). Following 5 minutes incubation, solution of ammonium molybdate and ammonium vanadate was removed and plates examined for zone of hydrolysis. True phytase producing isolates showing clearing zone after double staining were selected, purified and stored on slants at 4^0C. Phytase positive isolates were screened for mycotoxin production through thin layer chromatography as described by Kofi *et al*., (2011). Selected non-toxigenic isolates were identified by morphological method based on macroscopic and microscopic characters as described by Buchana and Gibson (1974). All inoculated plates of Sabouraud's dextrose agar (SDA) were incubated at 28°C for 7 days and macroscopic characters were recorded. Colonies of each isolate were compared for their diameters, over all colours, colours of conidia, reverse colours, texture, zonation and sporulation. All the isolates were also subjected to microscopic analysis for identification.

Statistical Analysis: The data obtained was analyzed statistically using statistical package for social sciences (SPSS version 20) to calculate percent distribution and frequency distribution for phytase producing fungi, toxigenic and nontoxigenic phytate degrading fungal isolates.

RESULTS

A total of 260 pure fungal isolates were recovered from 29 soil samples by soil dilution technique. The lowest percentage of fungal isolates exists in Motasingh, Pangali and Jallo Pind (2.3%) while highest percentage was recorded in Bhangali (4.61%). Most commonly existing percentage is 3.07% that is found in Hanjarwal, Maraka, Paajiyan, Kahna, Bhaseen, Ali Raza Abad, Jiya Baga and Bhaseen (Table1). Out of 260 fungal isolates, 10.76% fungi were positive for phytase production (Fig. 1). Highest percentage of phytase producing fungi was 33.33% recorded at Bhangali in relation to sampled area where as lowest percentage was found in Gujar colony Bagrian (Table 1). Mycotoxin producing potential of phytate hydrolyzing fungi was determined by Thin Layer Chromatography (TLC). Blue colour observed in most of the mycotoxins indicated potential of aflatoxin B production (Fig. 2). Among the fungal isolates, declared toxigenic or mycotoxin producing based on TLC were *Aspergillus flavus* in nature. The toxigenic phytase producing fungi were 46.42%. The highest phytase producing toxigenic fungi were observed in soil samples collected from livestock farms at Mehdipur, 144 Haloki, Haire and Barki (100%). The toxigenic phytase producing fungi were zero% in soil samples of Maraka, Raiwind pind, Hadiara, Mandhiyala, Bhaseen, Gujar colony Bagrian, Haire and Halokeey. Results for percent distribution of toxigenic phytase producing fungi are presented in table-1. Fifteen isolates (53.58%) were non-toxigenic detected through TLC. Highest percent distribution was recorded at Maraka, Raiwind pind, Hadiara, Mandhiyala, Bhaseen, Gujar colony Bagrian, Haire and Halokeey. Lowest percent distribution was recorded in Mehdipur, 144Haloki, Haire and Barki (Table 1). Non-toxigenic phytase producing fungal isolates were morphologically identified based on their macroscopic and microscopic characters (Table 2). Out of 12 phytase producing isolates selected based on size of zone of hydrolysis, 7 phytase producers were identified as *A. niger* (58.33%) and remaining 5 isolates as *A. flavus* (41.66%). Representative colonies of *A. niger* and *A. flavus* are presented as Fig. 3 (a, b, c and d). Microscopic characters of identified species are shown in Fig. 4. The results showed that native species of non-toxigenic phytase producing fungi found in soil of livestock farms belonged to *Aspergillus*.

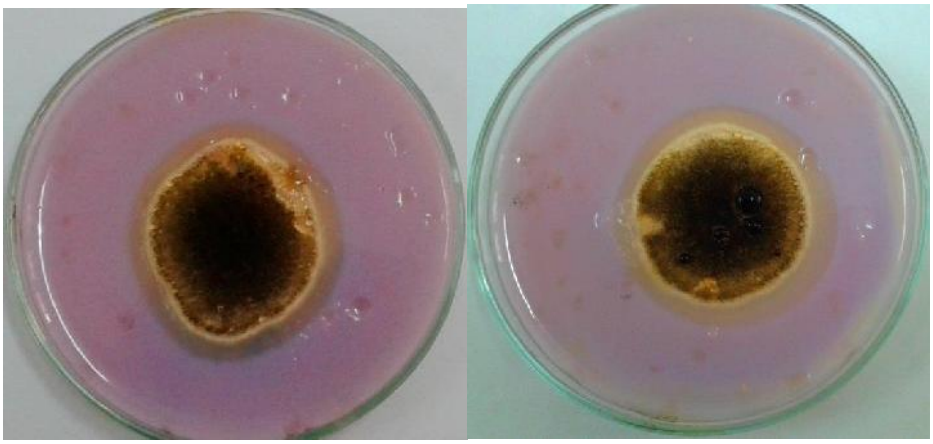


Fig. 1. Zone of Hydrolysis on PSM agar plate.

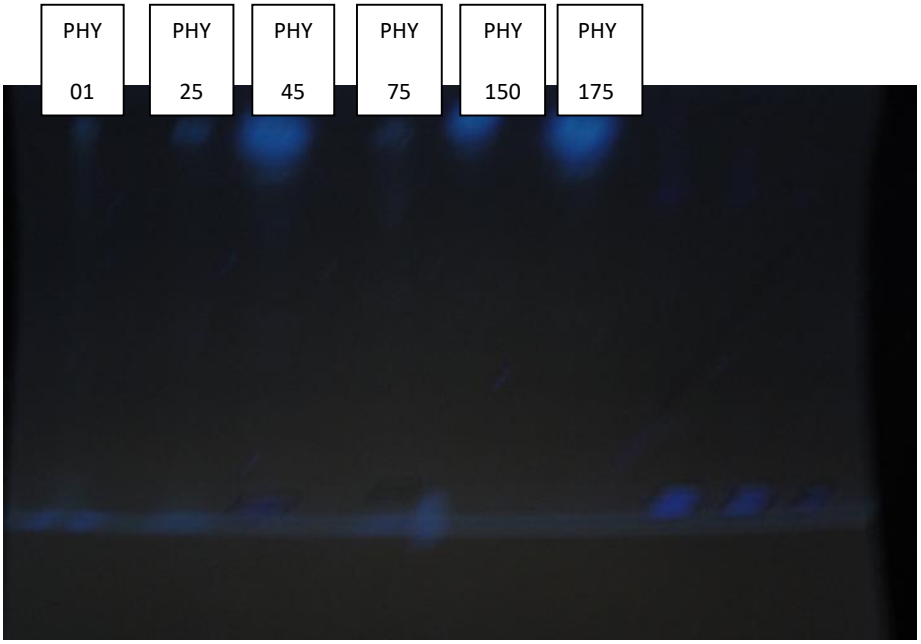
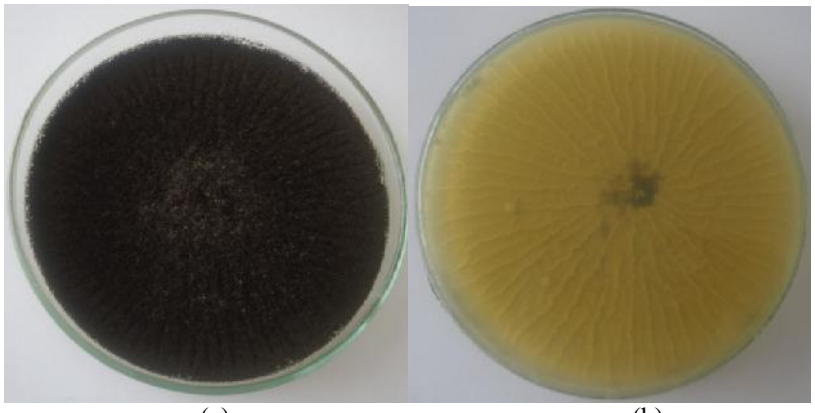
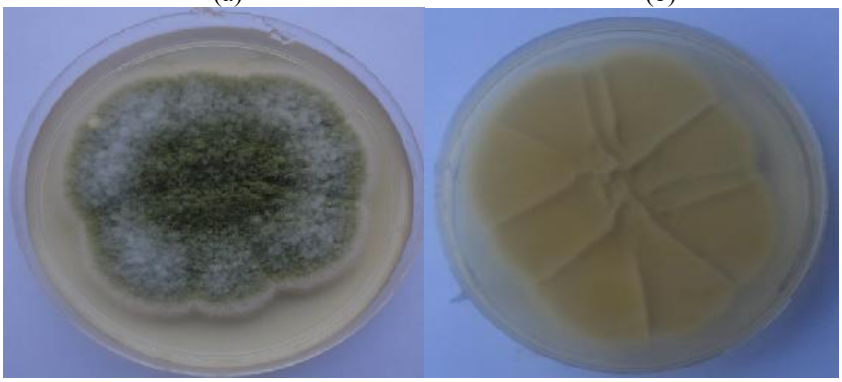


Fig. 2. Thin layer chromatogram of aflatoxins produced by toxigenic phytase producing isolates



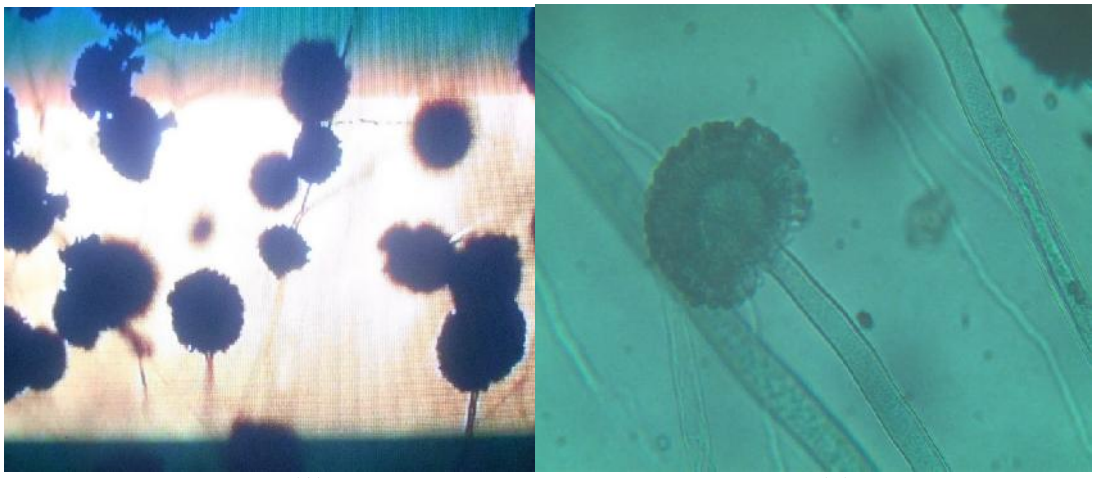
(a) (b)



(b) (d)

Fig. 3 : Representative pure colonies of phytase producing fungal isolates
a: Obverse view of *Aspergillus niger*, b: Reverse view of *Aspergillus niger,*

c: Obverse view of *Aspergillus flavus*, d: Reverse view of *Aspergillus flavus*



(A) **(B)**

Fig. 4 : Microscopic view of non-toxigenic phytase producing fungi from soil of livestock farms
A: *Aspergillus niger*, B: *Aspergillus flavus*

Table 1. Distribution of fungi isolated from soil of livestock farms in Lahore district

Location	Fungi Recovered	Phytase positive isolates	Toxigenic isolates	Non-toxigenic isolates
UVAS	11 (4.20)	2 (18.18)	1 (50)	1 (50)
Hanjarwal	08 (3.07)	0 (0)	0 (0)	0 (0)
Mehdipur	09 (3.46)	1 (11.11)	1 (100)	0 (0)
Maraka	08 (3.07)	1 (12.5)	0 (0)	1 (100)
144Haloki	09 (3.46)	1 (11.11)	1 (100)	11.11
RaiwindPind	10 (3.84)	1 (10.0)	0 (0)	1 (100)
Paajiyan	08 (3.07)	0 (0)	0 (0)	0
Kahna	08 (3.07)	0 (0)	0 (0)	0
Heir	11 (4.20)	3 (27.27)	1(33.33)	2(66.66)
Mota Singh	07 (2.69)	0 (0)	0 (0)	0 (0)
Pangli	07 (2.69)	0 (0)	0 (0)	0 (0)
Pangali	06 (2.30)	0 (0)	0 (0)	0 (0)
Hadiara	10 (3.84)	1 (10.0)	0 (0)	1 (100)
Mandhiyala	11 (4.20)	1 (9.09)	0 (0)	1 (100)
Bhaseen	08 (3.07)	1 (12.5)	0 (0)	1 (100)
JalloPind	05 (1.92)	0 (0)	0 (0)	0 (0)
AliRazaAbad	08 (3.07)	0 (0)	0 (0)	0 (0)
GawalaColony	09 (3.46)	1 (11.11)	1 (100)	11.11
GujarColony,Bagrian	11 (4.20)	1 (9.09)	0 (0)	1 (100)
Haire	09 (3.46)	1 (11.11)	0 (0)	1 (100)
Dholkey	11 (4.20)	2 (18.18)	2 (100)	0 (0)
Hadria	10 (3.84)	2 (20.0)	1 (50)	1 (50)
Barki	11 (4.20)	2 (18.18)	2 (100)	0 (0)
Bhangali	12 (4.61)	4 (33.33)	2 (50)	2 (50)
Halokeey	10 (3.84)	1 (10.0)	0 (0)	1 (100)
Sultankay	08 (3.07)	2 (25.0)	1 (50)	1 (50)
JiyaBaga	07 (2.69)	0 (0)	0 (0)	0 (0)
Lakhodere	08 (3.07)	0 (0)	0 (0)	0 (0)
Bhaseen	08 (3.07)	0 (0)	0 (0)	0 (0)

Total	**260**	**28 (10.76)**	**13 (46.4)**	**15 (53.6)**

The values in parentheses are percentages

Table2. Morphological identification of non-toxigenic phytase producing fungi.

Sr. No	Isolate No.	Macroscopic Characters	Microscopic Characters	Identification
1	PHY50	**Obverse side** Black colony with white periphery and radially furrowed zonation. Powdery texture with black spores, **Reverse side** Pale to colorless having ridges	Septate & hyaline hyphae, Vesicle present on conidiophore, Conidial head biseriate and radiate. Circular black conidia attached in chains.	*Aspergillus niger*
2	PHY80			
3	PHY82			
4	PHY105			
5	PHY120			
6	PHY125			
7	PHY215			
8	PHY05	**Obverse side** Cottony, periphery white, then slight yellowish with granular texture, central fluffy green **Reverse side** Pale	Hyphae are septate & hyaline. Conidiophores are hyaline and coarsely roughened. Phialides arise circumferentially from the globose vesicle.	*Aspergillus flavus*
9	PHY35			
10	PHY160			
11	PHY168			
12	PHY190			

DISCUSSION

Fungal diversity of soil depends on a large number of factors of the soil such as pH, organic content, and moisture (Alexander, 1977; Rangaswami and Bagyaraj, 1998.). Soil is habitat of microorganisms including fungi. Soil fungi provide many benefits including recycling of nutrients (Hoorman, 2011), a potential source of antibiotics (Makut and Owelewa, 2011), organic acids (Scervino *et al*., 2011) and hydrolytic enzymes (Sohail *et al*., 2009). In the present study soil samples collected from livestock farms in ten percent villages of Lahore district of Punjab were processed by soil dilution method to isolate fungi having potential of phytase production to meet needs of food and feed industries. Various methods used for isolation of fungi from soil include soil dilution method (Waksman, 1922), soil plate method (Warcup, 1950), direct isolation (Zakaria *et al*., 2011), soil washing method (Gams and Domsch, 1967). Soil dilution method was preferred for isolation of fungi in present study due to presence of spores in inactive form in the soil.

Present study showed that 10.76 percent fungal isolates were positive for phytase production. Phytase producing fungal isolates were confirmed by double staining technique to avoid selection of false positive phytase producers. Our results of counterstaining showed that all isolates produced phytase to hydrolyze sodium phytate in PSM agar leading to formation of zone of hydrolysis which did not disappear after counterstaining of PSM plate. Chelius and Wodzinski (1994) and Bae *et al*. (1999) described that certain bacteria and fungi do not produce zone of hydrolysis on phytase screening medium. Such microbes produce various acids during the metabolic pathway which solubilise sodium or calcium phytase present in the medium which is mistaken as phytase positive microbe. True zone of hydrolysis on plate of phytase screening medium did not disappear after double staining where as in case of false positive result, zone of hydrolysis disappeared. The phytase is widely distributed in plants, microorganisms and in some animal tissues (Konietzny and Greiner 2002; Vohra and Satyanarayana 2003). Due to increasing importance of phytase for food and feed industries, researchers isolated phytase producing fungi in samples collected from different habitats of world such as soil of livestock farms, cattle sheds, poultry farms, birds and animals excreta, poultry waste, straw, compost, vermicompost, agricultural fields, stored grains, fish pond, rotten wood-logs, rotten fruits and vegetables (Vats and Bannerjee, 2005; Gunashree and Venakateswaran, 2008; Javed *et al*., 2010).

Fungal species produce secondary metabolites known as mycotoxins which are toxic for human and animals. Thin layer chromatography (TLC) was used for detection of toxigenic isolates among phytase producing fungi. In present study, toxigenic fungi were 46.4 percent, significantly lower than non-toxigenic isolates. Gautam *et al*. (2012) observed a high percentage (72) of mycotoxin producing fungi and most of the identified species were *Aspergillus flavus*. Bugno *et al*. (2006) detected mycotoxin producing ability of *Aspergillus* and *Penicillium* isolates and found that 21.97 percent isolates produced mycotoxins such as aflatoxins, ochratoxins and citrinine. Percentage declared was lower than present

findings. The mycotoxin production was detected by TLC in five species including *A. flavus*, *A. niger*, *A. ochraceus*, *A. parasiticus* and *Penicillium citrinum* were found to be toxigenic where as in present study only one *Aspergillus* species was found toxigenic. Among many fungi isolated from soil, *Aspergillus* was proved to be the most abundant and frequently recovered toxigenic fungus. The important mycotoxin producing genera of fungi include *Aspergillus*, *Penicillium* and *Fusarium* (Soliman, 2003), which are frequently isolated from soil (Alghamirian and Ghaisian 2013). Toxigenic fungi found in soil contaminate agricultural products and produce mycotoxins during improper storage conditions of these products. The consumption of such products by humans and animals results in health related issues. Thus, it was necessary to exclude toxigenic fungi having potential of mycotoxin production.

Selected non-toxigenic fungi were identified using morphological method. The species of identified isolates were *A. niger* (PHY50, PHY82, PHY105, PHY120, PHY125, PHY80 and PHY215) and *A. flavus* (PHY05, PHY35, PHY160, PHY168 and PHY190). Our results are in agreement with those of Tahir *et al*. (2010) who isolated phytase producing *Aspergillus niger* ST-6 from decaying organic soil samples of Kasur, Punjab. In a similar study, Ocampo *et al*. (2012) isolated and characterize *A. niger* and *A. flavus* in samples collected from soil, fruits and cereals in Antioquia (Colombia). Most of the phytate hydrolysing fungal species belong to genus *Aspergillus* (*A. niger, A. flavus, A. ficcum, A. fumigatus* and *A. oryzae.* There are several published reports of phytase production by species of the Aspergillus genus: *A. niger*, *A. flavus, A. terrus, A. carneus, A. oryzae* and *A. fumigatus* (Shieh and Ware, 1968; Shieh *et al.,* 1969; Oh *et al.,* 2004; Gunashree and Venkateawaran, 2008; Soni and Khire, 2007; Gaind and Singh, 2015). However, the most widely used phytases are isolated from *Aspergillus niger* (Xiong *et al.,* 2005), *A. fumigatus* (Pasamontes *et al.,* 1997) and *A. ficcum* (Mitchell *et al.,* 1997). All these findings indicate that *Aspergillus* species are widely distributed in soil and have great potential of enzyme production.

Conclusion: It is the first systematic study on isolation of phytase producing fungi from soil of livestock farms in Lahore, Punjab. The indigenous non-toxigenic phytase producing fungi can be used for phytase production on industrial level from inexpensive agricultural by-products.

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