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DIVERSITY OF SOIL BORN ESTARCH HYDROLYZING FUNGI OF LIVESTOCK FARMS

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ABSTRACT

Soil of livestock farms is rich with mycoflora having diverse biological activities. Fungi isolated from soil (n=145) of livestock farms (n=29) at Lahore district were characterized into pathogenic, toxigenic and phaeoid starch hydrolyzers. Pure fungal isolates (1101) recovered from soil cultured on starch agar and only 112 (10.17%) were positive to starch hydrolysis. Out of 112 starch hydrolyzing fungi, 32 (28.57%) were declared pathogenic. Non-pathogenic starch hydrolyzing fungi further screened for mycotoxin production potential and 27.5 percent were found toxigenic. Starch hydrolyzing non-toxigenic fungi (n=58) were morphologically identified as *Aspergillus, Penicillium, Fusarium, Zygomycete, Actinomycete and Phaeoid*. Highest number of industrially important fungi was of *Aspergillus* (27.58%). *Aspergillus* isolates (n=16) identified at specie level were *A. niger* (03), *A. flavus* (07), *A. versicolor* (01), *A. terrus* (04) and *A. nidulans* (01). These isolates were declared as safe and could be used for mass production of amylases to meet commercial demands.

Keywords: Livestock farms, fungi, starch hydrolysis, non-toxigenic and *Aspergillus species.*

INTRODUCTION

Starch, a complex carbohydrate, is composed of amylose (linear) and amylopectin (branching) molecules (Mojsov, 2012). Starch is a glucose storage form of photosynthetic organisms. Its major sources are plants including rice, wheat, sweet potato, corn and maize. Starch is hydrolyzed into different products by hydrolyzing enzymes. Amylases are major starch hydrolyzing enzymes (Nigam, 2013). Amylases are classified into four groups including exoamylases, endoamylases, debranching enzymes and tranferases (Souza and Magalhaes, 2010). Alpha amylase (1, 4-α glucan glucanohydrolase) is an extracellular (endo-amylase) enzyme (Abdullah *et al*., 2011), which breaks α-1, 4 glycoside bonds in amylose and amylopectin converting these into glucose, maltose and maltotriose. Beta amylases break both types of glycoside bonds in amylose and amylopectin from non reducing end (exo-amylase) producing maltose and dextrin with beta anomeric configuration. Another amylase which causes the hydrolysis at non reducing end (exo-amylase) is glucoamylase which produces glucose molecules. The debranching group (isoamylase and pullanase) breaks α 1-6 glycoside linkage in amylopectin of starch. Tranferases (amylomaltase and cyclodextrin glycosyltranferase) target starch molecules at α, 1-4 glycoside linkages (Souza and Magalhaes, 2010). Microorganisms are the best and cheaper source of starch hydrolyzing enzymes (Kaur *et al*., 2012). Different genera of fungi including *Aspergillus* (Pathak and Narula, 2013), *Thermomyces*, *Penicillium, Cephalosporium, Neurospora, Rhizopus* and *Volvariella* are employed for industrial scale production of amylases (Olufunke and Azeez, 2013). Amylases are being used to increase the availability of nutrients from feed used in poultry industry (Tang *et al*., 2014). Demand for microbial amylases is increasing due to their multiple applications (Nigam, 2013). To meet the industrial needs of amylases, it is imperative to explore newer environments for better fungal strains. Since the ruminants contain enormous mycoflora in their gastrointestinal tract for the digestion of starch based green fodder, concentrates and roughages (Kelleci and Comlekcioglu, 2016). Soil of livestock farms is a better source for the isolation of starch hydrolyzing fungi; it has not previously been explored to its full extent in Pakistan. Therefore, present study was designed to isolate and screen starch hydrolyzing fungi from soil of livestock farms from Lahore, Pakistan. .

MATERIALS AND METHODS

Isolation of fungi: Soil samples (n=145) were randomly collected from livestock farms of ten percent villages (n=29) of District Lahore, Pakistan from October to December, 2013. Samples were taken from depth of three to five cm with sterile spatula. Five samples were taken from each village, one from each of four corners of village (n=4) where animal and human are in close proximity *i.e.* household domestic animal shed and the pathways from where these animals are mostly carried in and out. The fifth sample from each village was taken either from the entrance or exit of the village as control *i.e.* where animal and human interaction was not frequent. Samples were collected in polythene bag and

transported to Mycology Laboratory Department of Microbiology, University of Veterinary and Animal Sciences Lahore (Iram *et al*., 2011). Soil samples were processed for plate dilution method (Waksman, 1992) with minor modification. One mL soil suspension (10%) for each of the collected samples was transferred to sabouraud dextrose agar (SDA) petri plates. All of the inoculated plates were incubated at 25±3℃ under humid conditions for 3 to 5 days. Total numbers of fungal colonies on each plate for every sample were counted.

Screening for starch hydrolysis: The Ability to hydrolyze starch for each discrete fungal colony obtained by primary culture was observed using starch agar (Ominyi *et al*., 2013). The colonies from primary culture were spotted on starch agar plates by spotting. All the plates were wrapped with adhesive tape and incubated at 25±3℃ under humid condition (70%) till growth appeared. Media plates having fungal growth were flooded with lugol's iodine and zone of hydrolysis around colony were observed. Fungal isolates that showed zone of hydrolysis (clear zone, brown or yellow color) were selected for purification and further analysis. The starch hydrolyzing fungi were purified by spot plate technique on SDA.

Identification of Fungi: Fungal genera and species were identified by conventional method based on macroscopic and microscopic characters as described by Buchana and Gibbson (1974). The macroscopic features were recorded from obverse (colony texture, shape, margin and deposition of color in centre and periphery) and reverse side (color, presence or absence of diffusible pigment with its color, pattern of ridges and breakage within medium) of pure growth of fungal isolate on SDA. Microscopic features (type of hyphae, shape of hyphae and type of asexual spores) were observed by slide culture and cellophane tape method (Wijedasa and Liyanapathirana, 2012). The slides were observed at 100X and 400X.

Mycotoxins producing fungi: To detect the toxigenic fungi, isolates were cultured in SD broth and incubated in dark at 28℃ for 45 days. Fungal cultures were autoclaved, homogenized and processed for mycotoxins extraction as described elsewhere by Hussaini *et al*. (2009) with modifications. Briefly, chloroform (45mL), methanol (5mL), NaCl (2.5g) and distilled water (5mL) were added in 12.5g autoclaved fungal culture, incubated at 37ºC for 30 minutes, filtered, dried and crushed to fine powder. This dried powder was dissolved in one mL chloroform and used as mycotoxin preparation Mycotoxins were detected by Thin Layer Chromatography as described by Aidoo *et al*. (2011). Mycotoxin preparation (2uL) was spotted on commercially available silica gel coated aluminum sheets (stationary phase) and placed vertically in toxin free glass tank containing 100mL mobile phase (Chloroform 95mL, Acetone 5mL). All the sheets were observed in wooden UV (ultra violet) lamp (365nm) for absence or presence of fluorescence as indicator of mycotoxin. Aflatoxins was also confirmed and quantified by High performance liquid chromatography (HPLC) as described previously by Salaman *et al*. (2015).

Statistical Analysis: Percentage distribution and frequency distribution for starch hydrolyzing fungi, toxigenic starch hydrolyzing and non-toxigenic starch hydrolyzing fungal isolates was calculated using statistical package for social sciences (SPSS version 16).

RESULTS

Fungi, isolated from soil of livestock farms, were distributed into pathogenic, toxigenic and phaeoid on the basis of morphological characteristics. A total of 1101 colonies were purified from 145 soil samples. The lowest percentage of fungi isolated was from soil samples collected from Paajiyan (2.8%) and highest recorded in Pangali and Pangli (3.7%). Detailed results for percent distribution of fungi in soil of different sampled areas are presented (table 1). On statistical analysis, the highest frequency distribution recorded was at Raiwind Pind, Mota Singh, Mandhiyala, Gujar Colony (Bagrian), Haire, Halokeey, Jiya Baga, Hadria (27.6%). The lowest frequency distribution observed was 3.4 percent in soil samples of Mehdipur, Paajiyan and Bhaseen. The highest starch hydrolyzing percentage of fungal isolates was revealed from soil samples of Hanjarwal (19.44) and the lowest from Raiwind pind and Mandhiyala (table 1). The lowest frequency distribution of starch hydrolyzing fungi in relation to sampled area determined by statistical analysis was 6.9 at Hanjarwal, Jiya Baga, Mehdipur and Maraka.

Out of 112 starch hydrolyzing fungal isolates 32 (28.57%) were declared pathogenic. In relation to sampled areas, the highest distribution of pathogenic starch hydrolyzing fungi was recorded at Mandhiyala and Raiwind Pind (100%) whereas zero at Kahna, Berki, Ali Raza Abad and Pangali (table 1). On statistical analysis highest frequency of pathogenic fungi among starch hydrolyzing was determined 62.1 percent in soil sampled from UVAS, Mehdipur, Maraka, Haloki, Raiwind Pind, Paajiyan, Mota singh, Pangali, Hadiara, Mandhiyala, Bhaseen, Gujar Colony Bagrian, Haire, Dholkey, Hadria, Halokeey, Sultankay and Lakhodere.

Among the toxigenic fungi, the isolates having prominent flourescence under UV light by Thin Layer Chromatography (Fig. 3) were selected for mycotoxin quantification by HPLC (High Performance Liquid Chromatography). Fungal isolates which declared mycotoxin producer were *Aspergillus flavus* in nature. Among three *Aspergillus flavus* isolate no. 76 produced highest amount of aflatoxin B_1 (73.72ng/mL). The lowest

quantity of aflatoxin B_2 (2.14ng/mL) was by isolate no. 136 (Fig. 4). Mycotoxin producing fungi among non-pathogenic starch hydrolyzing isolates were 27.5 percent. Significantly higher number of non-toxigenic fungi was recovered from collected soil samples as determined by chi square analysis.

The highest toxigenic fungi among non-pathogenic starch hydrolyzing isolates were observed in soil samples collected from livestock farms at Barki, Gujar colony and Ali Raza Abad (100%). Toxigenic starch hydrolyzing fungi were not detected in soil samples collected from UVAS, Mehdipur, Raiwind pind, Pajiyaan, Mota sing, Mandhiyala, Jallo pind , Gawala colony, Haire, Dholkey, Hadria, Bangali, Sultankay and Bhaseen (table 1).

Out of 80 non-pathogenic starch hydrolyzing fungal isolates; 58 (72.5%) were observed non-toxigenic. Highest percent distribution (100%) recorded was at Mehdipur**,** Jallo Pind, Gawala Colony, Haire, Hadria, UVAS, Paajiyan, Mota sing, Dholkey, Bhangali, Sultankey and Bhaseen whereas lowest at Maraka (table 1). The lowest frequency distribution of non-toxigenic starch hydrolyzing fungi was (17.2) in Ali Raza Abad, Gujar Colony (Bagrian), Barki, Hanjarwal, Halokeey and Lakhodere whereas highest (24.1) at Maraka.

Starch hydrolyzing non-toxigenic fungi (n=58) identified on the basis of macroscopic and microscopic features (Fig. 1 and 2) were *Aspergillus* (16) *Penicillium* (02), *Fusarium* (04), *Zygomycete* (02), *Actinomycete* (01), Phaeoid (15). While (18) isolates remained un-identified. Fungal cultures showed the presence of septate hyphae, foot cell, vesicle and metullae under microscope were placed in genus *Aspergillus*. Colonies showed black color with white periphery granular texture, no color on reverse side were identified as *A. niger* (03). Colonies with yellowish green coloration white periphery and cottony to dusty texture with pale to colorless reverse side of plate were *A. flavus* (07). *Aspergillus* isolates showed color variations were *A. versicolor* (01). *A. terrus* (04) colonies were of cinnamon brown color with white periphery and dusty texture of colony. Colonies with greenish and brownish with nodules were identified as *A. nidulans* (01). *Penecillium sp.* was identified based on bluish green color with velvety texture and yellow pigmentation on reverse side of plate. Colonies with pink color and cottony appearance were identified as *Fusarium spp*. Zygomycetes showed white fluffy growth with no color on reverse side. Fungi showed black pigmentation and dematiaceous hphyae were classified as phaeoids.

Table 1. Percent distribution of fungi isolated from soil samples (n=145) from livestock farms (n=29) in Lahore district.

S. No.	Location	Fungi recovered n (%age)	Starch hydrolyzing n (%age)[a]	Pathogenic n (%age)[b]	Toxigenic n (%age)[c]
1.	UVAS	39 (3.54)	5(10.25)	1(20)	0(0)
2.	Hanjarwal	36(3.26)	7(19.44)	2(28.57)	2(40)
3.	Mehdipur	35(3.17)	4(11.43)	1(25)	0(0)
4.	Maraka	37(3.36)	4(10.81)	1(25)	2(66.67)
5.	144 Haloki	36(3.26)	3(8.57)	1(33.33)	1(50)
6.	Raiwind Pind	40(3.63)	1(2.5)	1(100)	0(0)
7.	Paajiyan	31(2.81)	3(9.68)	1(33.33)	0(0)
8.	Kahna	36(3.26)	3(8.33)	0(0)	1(33.33)
9.	Heir	37(3.36)	5(13.51)	2(40)	1(33.33)
10.	Mota singh	40(3.63)	5(12.50)	1(20)	0(0)
11.	Pangli	41(3.72)	2(4.88)	0(0)	1(50)
12.	Pangali	41(3.72)	3(7.32)	1(33.33)	1(50)
13.	Hadiara	36(3.26)	5(13.88)	1(20)	2(50)
14.	Mandhiyala	40(3.63)	1(2.5)	1(100)	0(0)
15.	Bhaseen	38(3.45)	5(13.16)	1(20)	2(50)
16.	Jallo Pind	37(3.36)	5(13.51)	2(40)	0(0)
17.	Ali Raza Abad	39(3.54)	1(2.56)	0(0)	1(100)
18.	Gawala Colony	37(3.36)	3(8.11)	2(66.67)	0(0)
19.	Gujar Colony	40(3.63)	2(5)	1(50)	1(100)
20.	Haire	40(3.63)	2(5)	1(50)	0(0)
21.	Dholkey	37(3.36)	3(8.11)	1(33.33)	0(0)
22.	Hadria	40(3.63)	2(5)	1(50)	0(0)
23.	Barki	37(3.36)	2(5.41)	0(0)	2(100)
24.	Bhangali	36(3.26)	6(16.67)	2(33.33)	0(0)
25.	Halokeey	40(3.63)	6(15)	1(16.67)	2(40)
26.	Sultankay	39(3.54)	5(12.82)	1(20)	0(0)
27.	Jiya Baga	40(3.63)	7(17.5)	2(28.57)	1(20)
28.	Lakhodere	37(3.36)	6(16.22)	1(16.67)	2(40)
29.	Bhaseen	39(3.54)	6(15.40)	2(33.33)	0(0)
	Total	**1101**	**112(10.17)**	**32(28.57)**	**22(27.5)**

a:starch hydrolyzing fungi from total fungi isolated from a specific farm, b:pathogenic fungi from total starch hydrolyzing fungi from specific farm, c:toxigenic fungi from total non-pathogenic starch hydrolyzing fungi from specific farm.

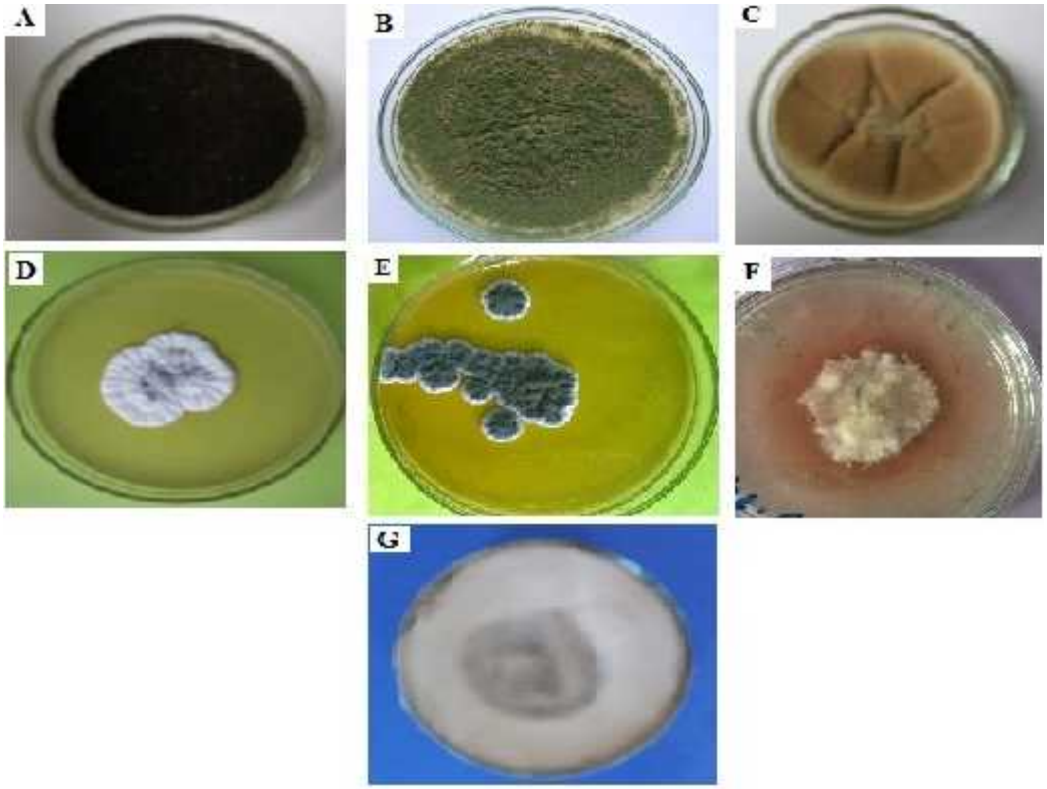


Figure 1: Colonies of non-toxigenic starch hydrolyzing fungi from soil of livestock farms
A: *Aspergillus niger*, B: *Aspergillus flavus*, C: *Aspergillus terreus*, D: *Aspergillus nidulans*, E: *Penecillium sp*. *F: Fusarium sp.and G: zygomycetes*

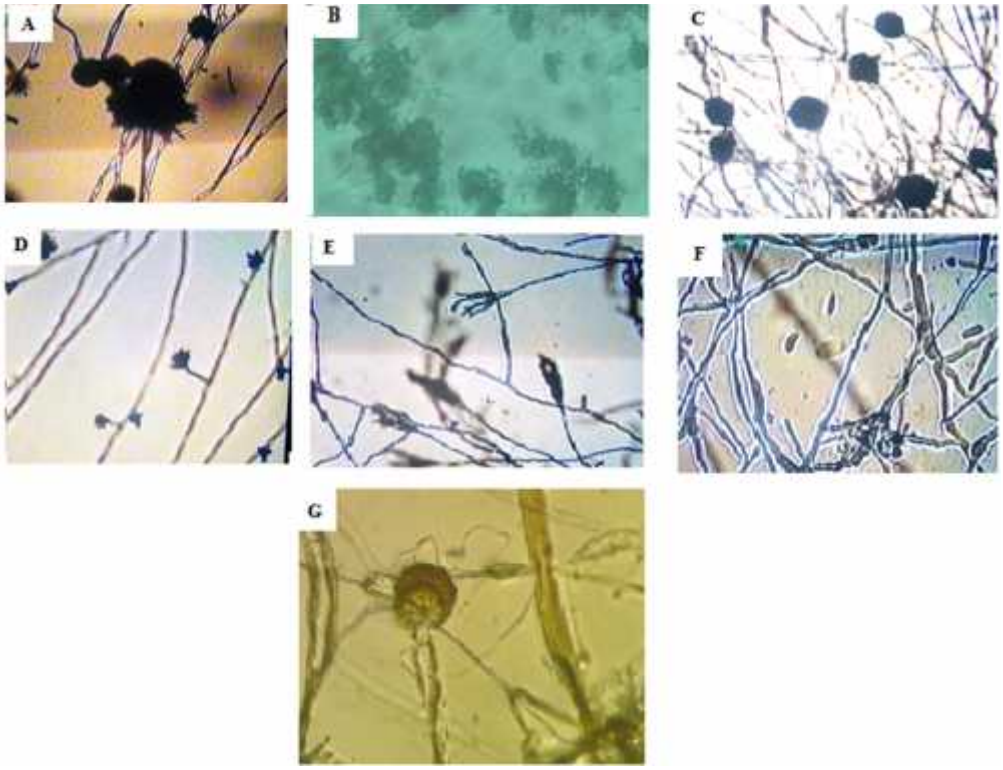


Figure 2: Microscopic view of non-toxigenic starch hydrolyzing fungi from soil of livestock farm
A: *Aspergillus niger*, B: *Aspergillus flavus*, C: *Aspergillus terreus*, D: *Aspergillus nidulans*, E: *Penecillium sp*. *F: Fusarium sp.and G: zygomycetes*

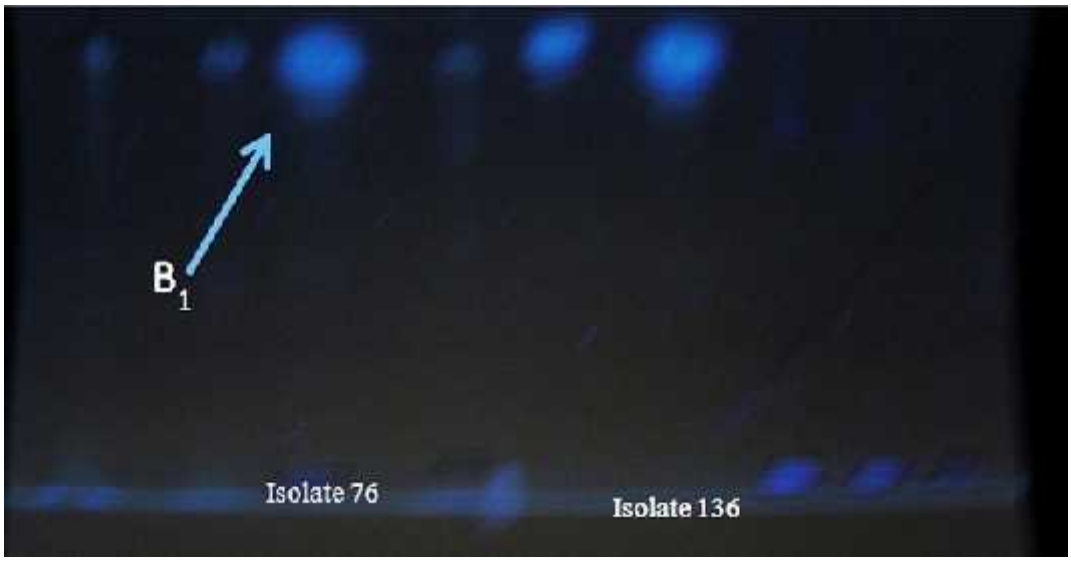
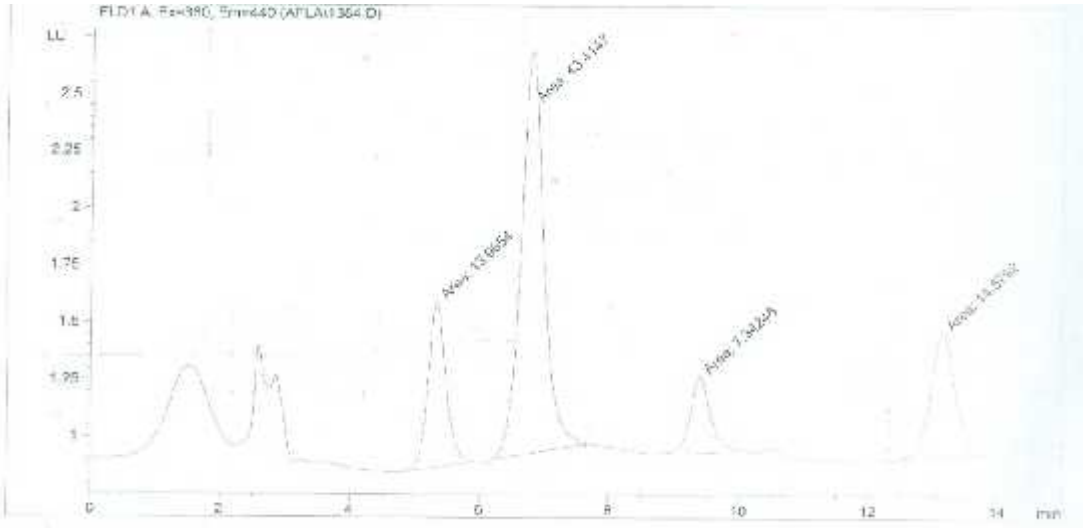
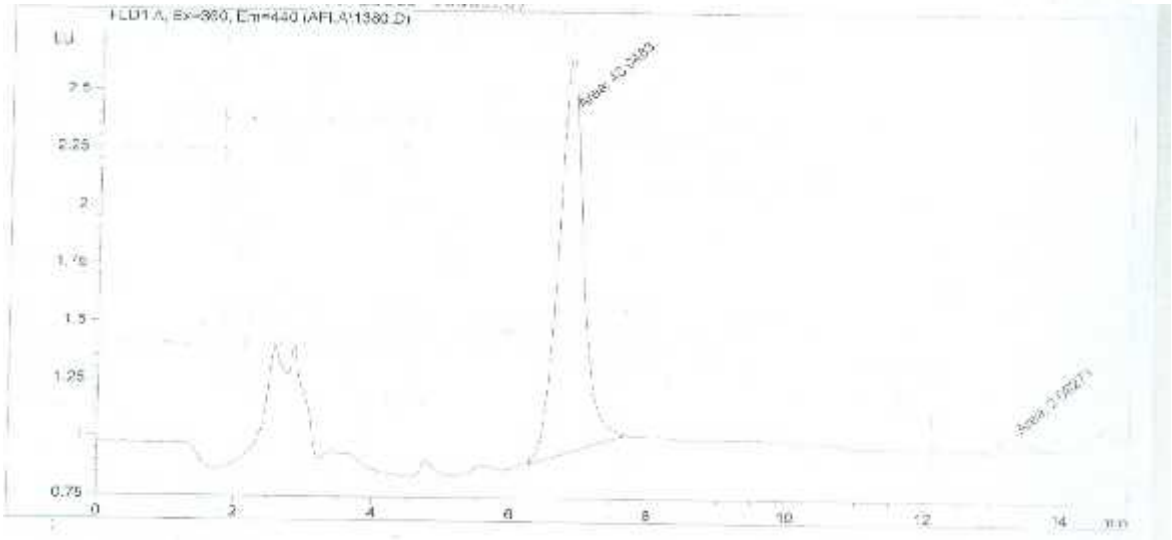


Figure 3: Thin layer chromatograph for *Aspergillus flavus* showing B_1

a



b



c

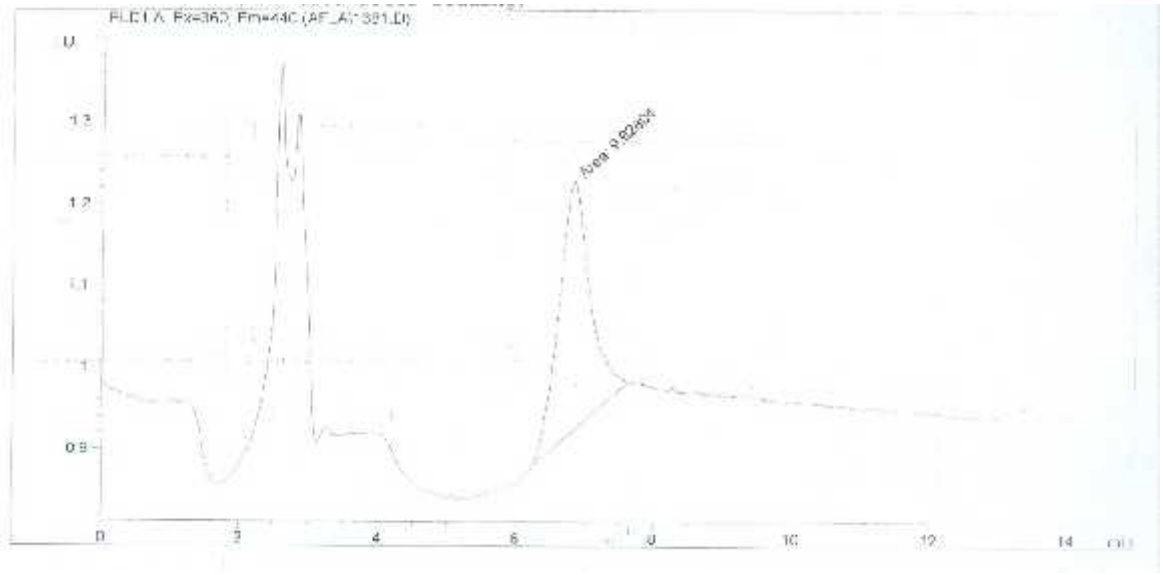


Figure 4: High Performance Liquid Chromatogram for Aflatoxin standards and Aflatoxins produced by *Aspergillus flavus*

a: aflatoxins standards, b: Chromatogram for isolate 76 showing B_1, c: Chromatogram for isolate 136 showing B_1

DISCUSSION

Soil is an excellent habitat of all microorganisms especially fungi. Soil fungi provide several benefits including recycling of nutrients, production of antibiotics (Makut and Owolewa, 2011), organic acids and hydrolytic enzymes (Sohail *et al*., 2009). Present study was designed to explore the diversity of starch hydrolyzing fungi in soil of livestock farms. Various methods used for the isolation of fungi from soil include soil dilution method (Waksman, 1992), soil plate method, direct isolation and soil washing method (Zakaria *et al*., 2011). Soil dilution method was used in present study which is preferred for fungal isolation due to presence of spores in inactive form in soil.

Present study showed 10.17 percent starch hydrolyzing fungi from *Aspergillus, Penecillium, Fusarium and zygomycetes spp*. Among starch hydrolyzing soil isolates 28.57 percent were pathogenic and 27.5 percent were toxigenic. Pthak and Narula (2013) isolated starch hydrolyzing fungi from soil samples of Jabalpur region. In this study *Aspergillus, Rhizopus* and *Fusarium* were the most common genera. The results are in agreement to present study being *Aspergillus spp.* as abundant starch hydrolyzing fungi. Fungi were screened for starch hydrolyzing ability and comparatively higher recovery percentage was reported (21.67%) in Nigeria (Ominyi *et al*., 2013). On other hand in Ethopia reported few fungi (4.04%) with the ability of starch hydrolysis from soil and water samples (Haki and Gezmu, 2012). Chandel *et al.* (2013) isolated 44.4 percent starch hydrolyzing fungi isolated from agricultural soil in India. The percentage of starch hydrolyzing fungi is in contrast to present study. Occurrence of starch hydrolyzing fungi varies with type of soil, geographical area, environmental influences, use of fertilizers and isolation method.

In present study starch hydrolyzing, non-toxigenic fungi were identified as *Aspergillus, Penicillium, Fusarium, Zygomycete, Actinomycete,* Phaeoids and some remained unidentified. *Aspergillus* species identified were *A. niger*, *A. flavus*, *A. versicolor*, *A. terrus* and *A. nidulans*. In another study starch hydrolyzing fungi from 50 soil samples were isolated (Chukwudi and Bamidele, 2013) and most abundant was *A. niger* (33.33%) followed by *P. chrysogenum* (25.49%), *A. flavus* (23.53%). In present study distribution of *A. flavus* is the highest followed by *A. niger, A. terreus and Fusarium* and the lowest of both *Penicillium, A. nidulans.* In contrast, another researcher observed starch hydrolyzing ability of fungi and found *P. chrysogenum, A. candidus* and *A. fumigates* as suitable isolates (Mukunda *et al*., 2012). Results of present study are somewhat similar to Pathak and Narula (2013). Starch hydrolyzing fungi isolated by Pathak and Narula (2013) belongs to genera *Aspergillus, Fusarium* and *Rhizopus.* Similarly genus *Aspergillus* was abundantly isolated in present study along with *Fusarium* and Zygomycetes. One other study conducted by Sohail *et al*. (2009) found *Aspergillus* as abundant starch hydrolyzing genus similar to present study. These studies support the results of present study in which *Aspergillus* have highest contribution in hydrolyzing starch.

Percent distribution of dermatophytes and dimorphic fungi was 28.57, significantly lower than non-pathogenic isolates. Shrama and Choudhary (2015) isolated pathogenic fungi belongs to *Trichophyton* and

Microsporum genera from 22 soil samples of Saharanpur, India. The prevalence of pathogenic fungal species was also reported by Jain and Sharma (2011).

These studies indicate that soil is a natural habitat of all types of fungi including the pathogenic fungal species. Presence of pathogenic fungi in soil of different locations clearly indicates the relation of soil with the transfer of fungal infection in human as well as animal populations in vicinity.

The commonly used method for mycotoxin detection is thin layer chromatography (Gautam *et al*., 2012) however aflatoxin producing *Aspergillus* can be screened with help of ammonia vapors (Saadullah, 2012). The methods which are used for quantification of mycotoxins are HPLC and LC-MS/MS (Ediage *et al*., 2015). The other methods include ELISA (immunological assay), capillary electrophoresis and biosensors (Roseanu *et al*., 2010). In present study, toxigenic fungi from starch hydrolyzing non-pathogenic isolates detected by TLC were 27.5 percent; significantly lower than non-toxigenic. Much higher percentage (72%) for mycotoxin producing fungi belongs to *A. flavus* (Gautam *et al*., 2012). In a study conducted by Okun *et al.* (2015) isolated 45.9 percent toxigenic aspergilli from soil samples which is opposite to results of present study by thin layer chromatography. In spite of detection of mycotoxins, production potential of isolated fungal species in present study was determined by high performance liquid chromatography. In present study *A. flavus* was found to produce B1 as a level of (73.72ng/mL). In several studies mycotxons were quantified by HPLC. Rani and Singh (1990) found 1580.0–3000.0 µg/kg of B1 aflatoxin in spice sample contaminated with aflatoxin producing fungi. Similarly Roy and Chourasia (1990) and Mahgubi *et al*. (2013) found B1 in different edible seeds and spices respectively. It is concluded that mycoflora of livestock farms highly diverse which contain industrially useful starch hydrolyzing fungi as well. Furthermore, presence of pathogenic and toxigenic fungi insinuates for further screening and analysis to control the fungal diseases and problems of mycotoxins in poultry and large animals.

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