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# ENZYMATIC AND AFLATOXIN PRODUCTION POTENTIAL OF ASPERGILLUS FLAVUS

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Fungi especially *Aspergillus* species are potential candidates for production of mycotoxins and industrially important enzymes. *Aspergillus flavus* isolates (129) recovered from soil mixed with animal rations (n=145) had aflatoxins (17.82%) and Enzymes (10.37%) production potential. Quantity of detected Aflatoxins varied for different isolates i.e., 3.25 to 11622.24ng, 21.34 to 194.47ng and 3.36 to 40.12ng per mL of Sabouraud's dextrose broth in case of $AFB_1$, $AFB_2$ and $AFG_1$ as determined by High performance liquid chromatography. Optimization of non-toxigenic starch hydrolyzing *A. flavus* was carried out at different incubation temperatures (22, 30 and 37°C), pH (4.5, 6 and 7.5) and substrates including maize, wheat bran and rice husk (1, 3 and 5% each) for incubation period of 7 days. In optimization experiments for starch hydrolysis, most of the *A. flavus* (86%) produced highest enzyme (IU) at 37°C and pH 6 quantified by Dinitrosalicylic method. Most of the isolates were able to produce enzymes using rice husk followed by maize. The highest quantity of enzyme was produced by *A. flavus* (179.88±1.71IU) using one percent of maize at pH 6 and 37°C. It was concluded that indigenous non-toxigenic *A. flavus* can be used in food industry as biological source of starch hydrolyzing enzymes.
**Keywords:** *Aspergillus flavus*, starch hydrolysis, food industry, aflatoxins, substrates

## INTRODUCTION

*Aspergillus flavus* is saprophytic fungi and common contaminants of agricultural products. It gets entry into crops in pre-harvest, post-harvesting and during harvesting period. Under favorable growth conditions, *A. flavus* produce toxic secondary metabolites called aflatoxins (Carry *et al.*, 2018). Four major naturally occurring aflatoxins are $AFB_1$, $AFB_2$, $AFG_1$, and AFG2. However, other important aflatoxins are $AFM_1$ and $AFM_2$ secreted in milk and meat of animals (Kumar, 2018). Aflatoxins are major safety concern in food industry because these suppress immune system and predispose to diseases, affect different organs (liver, kidneys and brain) and molecules (Protein and DNA) of living body (Wu *et al.*, 2016).

Fungal species are also source of industrially important enzymes that hydrolyze carbohydrates, protein and lipids (Kumari *et al.*, 2017). *Aspergillus* is a well-known genus for amylases production and has higher production potential among fungi (Souza and Magalhaes, 2010; Champreda *et al.*, 2007). *Aspergillus niger* is the most important specie capable of producing amylases so far. *Aspergillus flavus* is also a good amylase producer (Shafique *et al.*, 2010). Microbial (bacteria and fungi) sources of amylases are preferred over other sources (plants and animals) on industrial level because of their cost effectiveness, better quality of amylases and easy manipulation of microorganisms (Shafique *et al.*, 2010; Bhanwar *et al.*, 2014).

Amylases are amylolytic enzymes (Abe *et al.*, 2015). Amylases (α-Amylase E.C.3.2.1.1, β-Amylase EC 3.2.1.2 and γ-Amylase EC 3.2.1.3) have wide spectrum of applications in various industries including paper, textile and pharmaceutical industry (Mehta and Satyanarayana, 2016). Amylases are also utilized in fermentation, brewing, detergents, baking (Silva *et al.*, 2013) and distilling industry (Khalid-Bin-Ferdaus *et al.*, 2018). Amylases application in food industry ranges from clearing fruit juices, starch syrups preparation, making cakes, bread making (dough preparation and anti-stalling agent), to sweetening agent (conversion of glucose to fructose (Singh *et al.*, 2014; Mehta and Satyanarayana, 2016). Amylases are being used as feed supplement to improve the digestibility of starch based diets in birds (Onderci *et al.*, 2006).

Amylases production potential of non-mycotoxin producing indigenous *A. flavus* soil isolates was determined and optimized under different physical and nutritional parameters. The non-toxigenic amylase producing *A. flavus* may be better candidate for amylases to be used on industrial level.

## MATERIALS AND METHODS

***Collection of soil samples*:** Soil samples having animal rations (n=145) were randomly collected from livestock farms of 29 villages (one sample from each farm, selected 5

farms/village) located in and around District Lahore, Pakistan in sterilized plastic zipper bags. Soil suspensions (10%) for each sample were prepared in sterile normal saline, mixed using vortex and placed in vertical fashion at room temperature undisturbed till settling of soil debris (Henderson, 1961).

***Isolation and identification of Aspergillus flavus*:** Soil suspensions (1 mL each) were poured on independent properly labeled growth free Sabouraud's dextrose agar (SDA) petri plates under sterile conditions. Spread plate technique was followed for inoculation of samples and incubated at 25±3℃ for 3 to 5 days (Zafar *et al.*, 2007). Plates were observed daily for fungal growth and photographed by digital camera (Samsung ES-80). The macroscopic features including fungal colony texture, shape, margins and deposition of colors in central and periphery were recorded. Slide cultures were prepared by placing a drop of melted agar on sterile glass slide, inoculation of spores from purified culture, covered with cover slip and incubated at 25±3℃ by providing humidity. On appearance of visible growth cultured slides were observed under bright field compound microscope (Meiji Techno) at 100X and 400X magnifications. Microscopic characters observed and recorded were type of hyphae, presence of foot cell and types of asexual spores (Tsuneo, 2010).

***Mycotoxins production*:** Isolates of *A. flavus* were screened for mycotoxins production as described by Gonzalez *et al.* (2005) with minor modifications. Sabouraud's dextrose broth culture flasks were wrapped with brown papers and incubated at 28℃ temperature for 45 days. Extraction was carried out by mixing fungal culture (12.5g) with chloroform (45mL), methanol (5mL), NaCl (2.5g) and distilled water (5mL) in round bottom flasks and placed at 37℃ for 30 minutes on shaking incubator. The mixtures were filtered through Whatman filter paper (0.4µ) and extracts were dried by placing beakers at 50℃. Mycotoxins produced were detected qualitatively using Thin Layer Chromatography (TLC). The isolates showed fluorescence were considered mycotoxins producers and excluded from remaining experiments. Mycotoxins producing isolates were further confirmed by High Performance Liquid Chromatography (HPLC) using the procedure of Sobolev (2007). The type and concentration of mycotoxins was determined with the help of standards chromatogram.

***Amylases production*:** *A. flavus* non-toxigenic isolates were screened for amylase production on starch agar as described by Kim *et al*. (2011). Fungal spores were inoculated on agar and incubated at 25℃ for 5 days. The amylase production was detected by pouring iodine solution onto the plate. The colonies showed zone of hydrolysis were considered as positive.

Optimization of amylase production by *A. flavus* was carried out using one factor one-time method. Standard inoculums of *A. flavus* spores ($10^6$ per mL) were cultured at three different temperatures, pH levels, and substrate with varying concentrations. Selected *A. flavus* isolates were cultured in basal medium having one percent of each selected substrates (rice husk, maize and wheat bran) at pH 6.0 and incubated at 22, 28 and 37°C temperatures for one week. Optimum temperature was selected for each fungus isolate at which highest quantity of enzymes produced. In next experiment fungal isolates were cultured at 4.5, 6 and 7.5 pH levels for each substrate using optimized temperature for 7 days. Suitable pH level for each fungus was recorded in relation to highest amylases production. Similarly, at optimum temperature and pH values, each of the fungal isolate was cultured using 1, 3 and 5 percent concentration of each substrate for amylases production (Sethi *et al.*, 2016). Qualitatively amylases production was detected by iodine test using method described by Yoo *et al.* (1987). Iodine solution (20 µL) was added to one mL of filtrate having crude enzyme and mixed well. The reaction color was observed to detect the degree of starch hydrolysis.

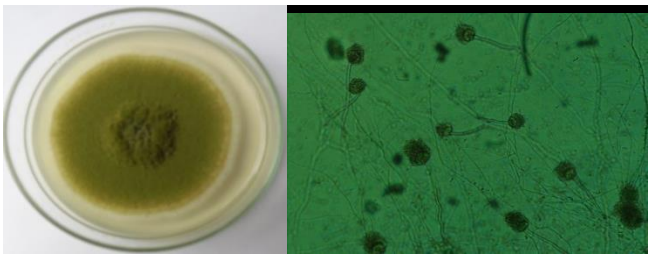
Amylases were quantified by dinitro salicylic method using protocol described by Mitidieri *et al.* (2006). Soluble starch (1%; pH 6) was mixed with filtrate in equal volume (1:1) and incubated at 37℃ for 30 minutes. It was boiled for ten minutes by adding 3mL of DNS (3-5, Dinitro salicylic acid) reagent. Absorbance was recorded at 570nm wavelength. Amylases were measured in term of Unit activity. One unit activity was defined as amount of enzyme which liberated one µmol of reducing sugars from substrate under assay conditions. For data analysis one-way ANOVA followed by Duncan's multiple range posthoc tests applied using Statistical Package for Social Sciences (SPSS version 16).

## RESULTS

Fungal isolates purified from soil samples were identified based on macroscopic and microscopic characters. Colonies of *A. flavus* were initially white filamentous which converted to granular, dusty and green on maturation (Fig. 1a). There was no color on reverse side of colony. Microscopic characters observed at 400X magnification were hyaline septate hyphae, presence of vesicle, foot cell and phialospores in chains (Fig. 1b). Total fungi isolated from soil samples were 1101 and out of which 129 were *A. flavus*. Among these 23 *A. flavus* isolates were detected as aflatoxin producers. The aflatoxins detected and quantified were $AFB_1$, $AFB_2$ and $AFG_1$ in range from 3.25 to 11622.24ng, 21.34 to 194.47ng and 3.36 to 40.12ng per mL of Sabouraud's dextrose broth respectively. From total toxin producing isolates 18.4 percent were $AFB_1$, 4.6 percent were $AFB_2$ and 9.2 percent were $AFG_1$ producers. From remaining 106 non-toxigenic isolates, 11 isolates (10.37%) were able to hydrolyze starch (Fig. 3).

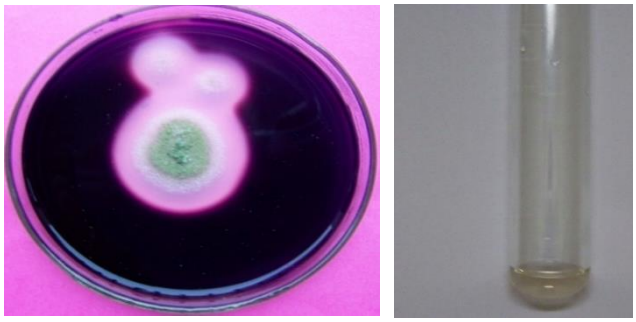
Seven non-toxigenic isolates of *A. flavus* (AFL-01, AFL-02, AFL-03, AFL-04, AFL-05, AFL-06 and AFL-07) showed complete hydrolysis were selected for optimization

experiments. Amylase production was optimized with different substrates (maize, wheat bran and rice husk) at varying concentrations (1, 3 and 5%) at different incubation temperatures (22, 30 and 37°C) and pH levels (4.5, 6 and 7.5). Degree of starch hydrolysis was analyzed with refrence to dipicted colors. AFL-05 showed partial hydrolysis at pH 6 using maize (1%) at 37℃ (Fig. 2).



a. Colony of *Aspergillus flavus* b. Microscopic view of *Aspergillus flavus*

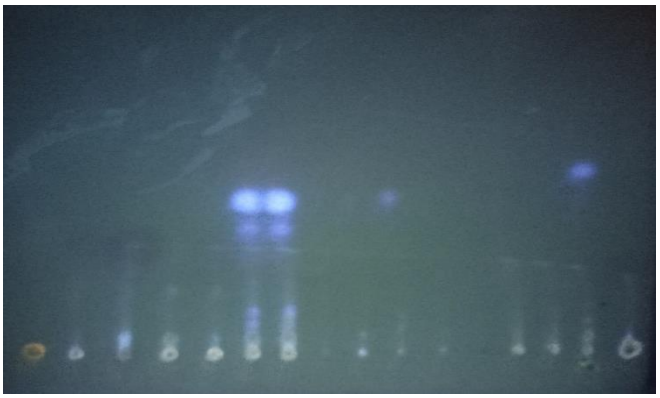
**Figure 1. Macroscopic and microscopic characters of *Aspergillus flavus* isolate.**



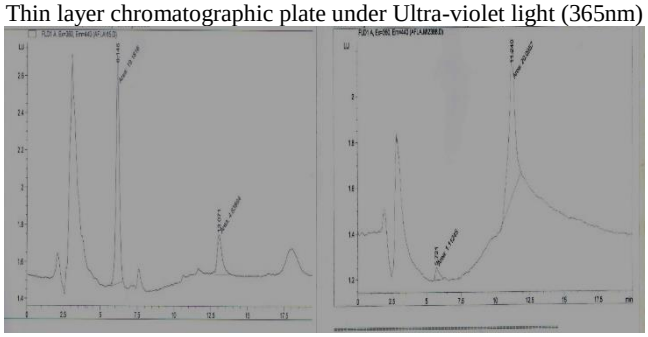
Starch hydrolysis on starch agar Starch hydrolysis in broth

**Figure 2. Qualitative detection of amylases produced by *Aspergillus flavus* isolates.**

All other six isolates (AFL-01, AFL-02, AFL-03, AFL-04, AFL-06 and AFL-07) showed complete hydrolysis represented by colorless solution. Among seven isolates of *A. flavus*, six isolates (AFL-01, AFL-02, AFL-03, AFL-04, AFL-05 and AFL-06) showed the highest enzyme production at 37℃ and pH 6 for amylase (Table 1). Rice husk proved best substrate for three isolates (AFL-03, AFL-04 and AFL-05) with concentration of one percent for AFL-04, AFL-05 and five percent for AFL-03 with amylase activities 128.60±8.90, 99.17±2.49 and 104.72±.71 IU respectively at 37℃ and pH 6. For AFL-06 maize (1%) was optimum for highest enzyme yield (179.88±1.71 IU). AFL-01 the highest amylase activity was 154.50±3.66 at three percent wheat bran. The AFL-02 and AFL-07 showed highest enzyme yield at five percent concentrations of wheat bran which were 114.72±.99 and 119.01±1.42 IU, respectively (Table 2 and 3). The highest producer of amylase was AFL-06 (179.88±1.71 IU). The optimum temperature for AFL-07 was 22℃, pH 7.5 and best substrate wheat bran with 5% concentration.



Thin layer chromatographic plate under Ultra-violet light (365nm)



Representative picture of High Performance Liquid Chromatography of aflatoxins

**Figure 3. Qualitative and quantitative detection of aflatoxins produced by *Aspergillus flavus* isolates.**

**Table 1. Growth optimization of *Aspergillus flavus* for amylases production at different temperatures using one percent substrate and pH 6.**

| Substrate | Isolate # | 22°C | 28°C | 37°C |
|---|---|---|---|---|
| Maize | AFL-01 | 23.79±2.48b | 9.92±0.17a | 61.42±0.46c |
| | AFL-02 | 3.39±0.27a | 9.33±0.40b | 13.84±1.50c |
| | AFL-03 | 4.41±0.41a | 9.40±0.24b | 18.10±0.17c |
| | AFL-04 | 6.28±0.58a | 10.75±0.18b | 89.88±0.89c |
| | AFL-05 | 0.83±0.02b | 11.22±0.17c | 0.08±0.00a |
| | AFL-06 | 3.02±0.25a | 11.47±0.34b | 179.88±1.71c |
| | AFL-07 | 14.60±1.62c | 8.42±0.00b | 2.48±0.09a |
| Wheat bran | AFL-01 | 5.68±0.16a | 5.84±0.16a | 29.73±0.17b |
| | AFL-02 | 4.27±0.09b | 4.70±0.16c | 1.78±0.16a |
| | AFL-03 | 6.98±0.16c | 3.78±0.09a | 5.65±0.17b |
| | AFL-04 | 2.99±0.08a | 4.14±0.08b | 11.11±0.30c |
| | AFL-05 | 2.73±0.04a | 4.27±0.09b | 7.02±0.14c |
| | AFL-06 | 6.16±0.16b | 3.41±0.16a | 18.16±0.24c |
| | AFL-07 | 8.86±0.08c | 4.28±0.08b | 0.13±0.01a |
| Ricehusk | AFL-01 | 24.89±0.93b | 6.08±0.08a | 152.52±1.17c |
| | AFL-02 | 6.55±0.08a | 4.48±0.08a | 15.63±3.10b |
| | AFL-03 | 7.25±0.09b | 3.89±0.16a | 57.61±1.78c |
| | AFL-04 | 10.12±0.10a | 5.23±0.17a | 128.60±8.90b |
| | AFL-05 | 6.20±0.22a | 4.26±0.09a | 99.17±2.49b |
| | AFL-06 | 2.75±0.16a | 8.40±0.21b | 156.68±1.24c |
| | AFL-07 | 7.72±0.08c | 3.52±0.09b | 1.40±0.15a |

Means carrying same superscript vary non-significantly, whereas means with different superscript differ significantly in rows.

**Table 2. Growth optimization of *Aspergillus flavus* for amylases production at different pH using 1% substrate and temperature 37°C.**

| Substrate | Isolate # | 4.5 | 6 | 7.5 |
|---|---|---|---|---|
| Maize | AFL-01 | 3.65±0.08a | 61.42±0.46c | 4.61±0.08b |
| | AFL-02 | 4.13±0.08a | 13.85±1.49b | 5.75±0.08a |
| | AFL-03 | 3.98±0.08a | 18.10±0.17c | 5.09±0.08b |
| | AFL-04 | 3.50±0.08a | 89.88±0.89c | 6.26±0.08b |
| | AFL-05 | 3.81±0.08b | 0.0842±0.00a | 4.05±0.00c |
| | AFL-06 | 3.63±0.19a | 179.88±1.71b | 4.59±0.09a |
| | AFL-07 | 3.99±0.08b | 2.48±0.09a | 4.64±0.08c |
| Wheat bran | AFL-01 | 7.11±0.31a | 29.73±0.17c | 7.96±0.16b |
| | AFL-02 | 3.84±0.09b | 1.78±0.16a | 4.92±0.09c |
| | AFL-03 | 3.73±0.16a | 5.65±0.17b | 9.71±0.15c |
| | AFL-04 | 3.59±0.13a | 11.11±0.30b | 3.77±0.17a |
| | AFL-05 | 3.75±0.13a | 7.01±0.14b | 8.77±0.15c |
| | AFL-06 | 3.39±0.02a | 18.16±0.24c | 4.58±0.17b |
| | AFL-07 | 3.89±0.16b | 0.12±0.01a | 4.46±0.08c |
| Rice husk | AFL-01 | 3.93±0.17a | 80.22±0.61c | 6.05±0.17b |
| | AFL-02 | 9.86±0.01b | 15.63±3.10c | 5.24±0.23a |
| | AFL-03 | 3.66±0.15a | 57.61±1.78b | 4.75±0.17a |
| | AFL-04 | 4.04±0.01a | 128.60±8.90b | 5.47±0.09a |
| | AFL-05 | 3.59±0.12a | 99.17±2.49b | 4.42±0.17a |
| | AFL-06 | 3.36±0.10a | 156.68±1.24b | 4.38±0.00a |
| | AFL-07 | 3.96±0.12b | 1.40±0.15a | 5.82±0.13c |

Means carrying same superscript vary non-significantly, whereas means with different superscript differ significantly in rows.

**Table 3. Growth optimization of *Aspergillus flavus* for amylases production at different substrate concentrations at pH 6 and temperature 37°C**

| Substrate | Isolate # | 1% | 3% | 5% |
|---|---|---|---|---|
| Maize | AFL-01 | 61.42±0.46a | 127.17±4.68c | 85.28±2.92b |
| | AFL-02 | 13.85±1.49a | 75.76±3.74b | 76.41±0.37b |
| | AFL-03 | 18.10±0.17a | 79.00±6.75b | 103.42±0.71c |
| | AFL-04 | 89.88±0.89b | 92.43±7.00b | 69.61±3.14a |
| | AFL-05 | 22.13±0.89a | 68.35±2.72c | 52.31±0.33b |
| | AFL-06 | 179.88±1.71c | 47.49±0.78a | 98.70±3.95b |
| | AFL-07 | 2.48±0.09a | 36.79±1.87c | 31.32±0.70b |
| Wheat bran | AFL-01 | 29.73±0.17a | 154.50±3.66c | 99.14±1.63b |
| | AFL-02 | 1.78±0.16a | 62.77±0.99b | 114.72±0.99c |
| | AFL-03 | 5.65±0.17a | 28.68±2.47b | 86.36±1.12c |
| | AFL-04 | 11.11±0.30a | 60.82±0.99b | 123.51±1.31c |
| | AFL-05 | 7.01±0.14a | 73.72±0.97b | 95.02±0.99c |
| | AFL-06 | 18.16±0.24a | 34.02±0.93b | 96.97±0.74c |
| | AFL-07 | 0.12±0.01a | 55.95±0.81b | 119.01±1.42c |
| Rice husk | AFL-01 | 152.52±1.17b | 102.00±4.16a | 98.40±1.72a |
| | AFL-02 | 15.63±3.10a | 40.91±1.29b | 96.36±3.74c |
| | AFL-03 | 57.61±1.78b | 42.53±0.32a | 104.72±0.71c |
| | AFL-04 | 128.60±8.90c | 79.00±0.99a | 91.86±1.99b |
| | AFL-05 | 99.17±2.49c | 80.30±1.35b | 30.69±1.56a |
| | AFL-06 | 156.68±1.24c | 32.77±0.32a | 93.29±2.08b |
| | AFL-07 | 1.40±0.16a | 48.27±1.35c | 17.20±0.85b |

Means carrying same superscript vary non-significantly, whereas means with different superscript differ significantly in rows.

## DISCUSSION

*Aspergillus flavus* is a ubiquitous fungal species and found in soil and other substrates as contaminant. Soil is the main reservoir of aflatoxins producing *A. flavus* and a source of contamination to various food and feed materials. These contaminants grow and produce aflatoxins under favorable growth conditions and transmit these toxins to human and animal via food chain (Zhang *et al.*, 2017). *A. flavus* was isolated from various types of samples and its aflatoxin production potential was detected. Zhang *et al.* (2017) isolated 94.2% *A. flavus* from field soil. This specie produced $AFB_1$ ranged from 16501 to 82083 ng/mL is higher than present study. Fakruddin *et al.* (2015) recovered *A. flavus* from feed samples and 90 percent of these isolates were $AFB_1$ producer in range from 7-22ug/g of solid medium. El-Hamaky *et al.* (2016) detected toxin producing aspergilli from feed samples and from 44 isolates of *A. flavus*, 33 contaminants were found as toxin producers. Raju *et al*. (2016) isolated toxigenic species of *A. flavus* along with other fungi from animal feed. Results of aflatoxin production of *A. flavus* isolates in present study are in contrast with Carranza *et al.* (2014). Carranza isolated 87·5 percent *A. flavus* out of which eight percent were toxigenic. The quantity of $AFB_1$ was in range of 18·0-84·3ng/mL. However, $AFB_1$ quantity is close to results of present study (73.73ng/mL). The results of toxigenic *A. flavus* (27.5%) are differed from Razzaghi-Abyaneh (2006). Razzaghi-Abyaneh reported 87.9 percent of toxigenic *A. flavus*. The differences in results of toxigenic *A.flavus* prevalence in present study may be attributed to the presence of animal rations in soil samples from where the fungi were isolated.

Amylase is a valuable enzyme in starch-based industries having 30% share in worldwide enzyme production. It can be obtained from plants and animals, but microorganisms are better choice for amylase production. Among microbes, *Bacillus* and *Aspergillus* are potential candidates for amylase production on industrial level (Wang *et al.,* 2016). Filamentous fungi are famous for extracellular enzyme production. *Aspergillus* species; *A. oryza* and *A. niger* are well known for industrial amylases (Mathew *et al.,* 2016). In a study conducted by Pathak and Narula (2013) starch hydrolyzing genera were *Aspergillus, Fusarium* and *Rhizopus*. *A. flavus* and *A. niger* were major species. Sohail *et al.* (2005) found 16.15 percent amylase producing fungi and *A. flavus* was 13.64 percent. Several physical and chemical factors such as temperature, pH, carbon and nitrogen influence the amylase biosynthesis (Sethi *et al*., 2016).

Fungal growth is affected by nutritional and environmental conditions. The growth conditions for beneficial and industrially important fungal isolates can be optimized for enhanced production of desired products (Shafique *et al.*, 2010). In present study, amylolytic *A. flavus* isolates were optimized under different physicho-chemical conditions. The production of amylases was quantified by detecting reducing sugar using DNS (Dinitro Salicylic Acid) method. The

amount of reducing sugar is directly proportional to amylase activity (IU/mL). Several studies have been conducted in order to optimize the growth conditions for amylases production. The findings of optimization study of Bakri *et al.* (2009) and Shafique *et al.* (2009) differed from present study. The starch hydrolyzing isolates by Bakri *et al.* (2009) belonged to genus *Aspergillus* and showed the best result at alkaline pH. Kim *et al.* (2005) found optimal pH for *Sphaeropsis pyriputrescens* between 3 to 6 and the highest amylolytic activity in pH 3-4. According to Pathak and Narula (2013), the optimum pH for *A. flavus* (10.3 IU/Ml), *A. niger* (18 IU/mL), *Fusarium* (6.66 IU/mL) and *Rhizopus* (15 IU/mL) was 9 in contrast to present study where only one isolate AFL-07 showed the best activity at alkaline pH 7.5 and rests at pH 6.

Ragunathan and Swaminathan (2005) carried out optimization study using solid state fermentation on amylase producing *A. oryzae* isolated from litter soil. The maximum specific activity (380U/mg) was found at temperature 35°C and pH 5. A linear relation was found between specific activity and addition of starch (1%) to growth medium. The results are in corroboration to present study findings.

Effect of agriculture waste was evaluated on amylases production by Varalakshmi *et al.* (2009). *A. niger* produced higher amylase units when growing on wheat bran at 22°C and 7.5 pH. The results strengthened the findings of present study as similar results were obtained by AFL-07. Oladapo (2013) used maize sorghum, cassava peel and soluble starch as substrates to grow starch hydrolyzing *A. flavus*. Oladapo concluded that amylase activity increased with increase in substrate concentration up to two percent. Higher percentage negatively affected amylase production of *A. flavus*.

Optimum conditions for higher amylase production vary with the source from which that fungal specie has been isolated. Both physical and nutritional factors required to be optimized for better production of enzymes. It was concluded that indigenous non-toxigenic *A. flavus* isolates are better and safe producers of amylases. Agriculture waste products can be used for cost effective production of amylases.

***Conclusion*:** Indigenous *A. flavus* isolates are potential candidates for aflatoxins and enzyme production under optimized conditions.

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