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SCREENING OF *ASPERGILLUS NIGER* AND *A. FLAVUS* STRAINS FOR EXTRA CELLULAR ALPHA-AMYLASE ACTIVITY

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Abstract

Five fungal strains of each of the two filamentous fungi viz., *Aspergillus niger* Van Tieghem and *A. flavus* Link ex Gray, indigenous to Pakistan, were screened for their alpha-amylase activity. Different selected strains were grown on two growth media viz., Potato Dextrose Agar (PDA) and Enzyme Production Medium (EPM), at three pH levels viz., 4.5, 5.5 and 6.5, in all the possible combinations. Performance of various strains was recorded in terms of hydrolyzing zone formation. Generally test strains exhibited their best performance on EPM at pH 4.5. On the basis of their best performance on solid media, strain 74 and strain 198 of *A. niger* and strain 209 and strain 231 of *A. flavus* were selected for periodic evaluation of their alpha-amylase activity in liquid medium, using shake flask technique. All the test strains exhibited their maximum alpha-amylase activity after 48 h incubation.

Introduction

Amylases are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents (Akpan *et al*., 1999; Pederson & Nielsen, 2000) by degrading 1-4 linkage of starch. This enzyme is extensively used in starch liquefaction, paper industries, food, pharmaceutical and sugar industries (Nigam & Singh, 1995). Enzymes are among the most important products obtained for human needs through microbial sources. In recent years the new potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated renewed interest in the exploration of extra cellular enzymatic activity in several microorganisms (Akpan *et al*., 1999; Buzzini & Martini, 2002). Amylases are a group of enzymes that have been found in several microorganisms including fungi (Fadel, 2000).

Studies on fungal amylase especially in the developing countries have concentrated mainly on *Rhizopus sp.*, and *Aspergillus spp.*, probably because of the ubiquitous nature and non fastidious nutritional requirements of these organisms (Abe *et al.*, 1988). It has been reported that while a strain of *Aspergillus niger* produced 19 types of enzymes, α - amylase was being produced by as many as 28 microbial cultures (Pandey *et a*l., 1999). Thus, the selection of a suitable strain for the required purpose depends upon a number of factors, in particular upon the nature of the substrate and environmental conditions.

Optimization of growth conditions is important for best growth of fungi. The growth requirements for fungi may vary from strain to strain, although cultures of the same species and genera tend to grow best on similar media. Similarly growth responses of fungi also vary from strain to strain though they are grown on same conditions (Smith & Onions, 1994). Fungi grow over a wide range of pH conditions and must thus be able to tailor gene expression to the particular pH of their growth environment. Filamentous fungi vary in pH requirements. Most common fungi grow well over the range pH 3 to 7, although some can grow at pH 2 and below e.g., *Moniliella acetoabutans, Aspergillus niger, Penicillium funicilosum* (Smith & Onions, 1994).

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Though many fungi particularly *Aspergilli* are known to produce different groups of enzymes but the selection of a particular strain, however, remains a tedious task, especially when commercially competent enzyme yields are to be achieved. Therefore, the present study was undertaken to screen the indigenous efficient alpha-amylase producer strains of *Aspergillus niger* and *A. flavus* among the fungal species collection of First Fungal Culture Bank of Pakistan.

Materials and Methods

Fungal isolates: The parental strains of *Aspergillus niger* (isolates FCBP-02, FCBP-74, FCBP-109, FCBP-116, FCBP-198) and *Aspergillus flavus* (isolates FCBP-51, FCBP-52, FCBP-64, FCBP-209, FCBP-231) were obtained from First Fungal Culture Bank of Pakistan (FCBP), University of the Punjab, Lahore, Pakistan. These strains were maintained on Potato Dextrose Agar (PDA), stored in refrigerator at 4^0C and sub-cultured at 3-months interval.

Solid state fermentation

Experimental design: For each of the two *Aspergillus* spp., a 5×3×2 factorial with 5 fungal strains, 3 pH levels viz., 4.5, 5.5 and 6.5 and two growth media viz., Potato Dextrose Agar (PDA) and Enzyme Production Medium (EPM), with 6 replicates was laid down in a completely randomized design.

Preparation of spore suspension: To stock culture 10 mL of distilled autoclaved water was added and the surface was gently rubbed with sterilized wire loop (Noomrio & Dahot 1992). The stock suspension was serially diluted to prepare a conidial suspension of 5 x 10^5 conidia mL^{-1} concentration with the help of haemacytometer (Neubauer Precidor HBG, Germany).

Growth conditions: The strains were cultured on 2% Potato Dextrose Agar (PDA) medium in one lot while the other lot comprised of EPM containing soluble starch 15g L^{-1}, potato starch 15g L^{-1}, lactose 10g L^{-1}, $(NH_4)_2SO_4$ 5g L^{-1}, $CaCl_2$ 2g L^{-1}, NaCl 2g L^{-1} and 17.5 g L^{-1} agar. Three levels of pH: 4.5, 5.5 and 6.5 were adjusted (pH was adjusted by either 0.5M NaOH or 0.5M HCl) before autoclaving. The medium was then autoclaved at 121°C at 15 lb $inch^{-2}$ for 15 minutes. The sterilized medium was cooled and 250 mg of chloromycetin per 200 mL of medium was used as antibacterial. Each treatment was replicated six times. Three wells in each plate were made and inoculated with test strains suspension of 5 x 10^5 conidia mL^{-1} concentration. The plates were incubated at 30 ± 2^0C for five days to seven days.

The extent of growth of representative strains of both *Aspergillus* spp., was evaluated qualitatively by visual inspection. Then the hydrolyzing zones were determined in three levels of pH.

Shake Flask Fermentation

Fermentation experiment: After 5 to 7 days of incubation, the colonies that had formed large clear zone of starch hydrolysis around the well margins in the Petri plates were picked up as α-amylase producers (Zaghloue *et al*., 1993). Then top two strains among each species were selected for activity assays. The preparation of spore inoculum @ 5 x 10^5 conidia mL^{-1} was carried out as described previously.

Crude enzyme preparation: Fifty mL of the fermentation medium containing soluble starch 15g L^{-1}, potato starch 15g L^{-1}, lactose 10g L^{-1}, $(NH_4)_2SO_4$ 5g L^{-1}, $CaCl_2$ 2g L^{-1}, NaCl 2g L^{-1} in 1000 mL of 0.05 M citrate buffer (pH 4.5) was transferred to 250 mL of cotton plugged conical flasks. The flasks were sterilized in an autoclave and cooled at room temperature. One mL of inoculum was transferred to each flask. The flasks were then placed in the rotary shaker incubator (Technico OS-204, Pakistan) at 200 rpm and 30 ± 2^0C up to 72 hours. All the experimental sets were run in triplicate. At the interval of 12 hours the fermented broth was centrifuged at 5000 rpm for 20 minutes. The supernatant was used for the estimation of α-amylase activity.

Amylase activity assay: Amylase activity was assayed as described by Ramakirshna *et al*., (1982) using a reaction mixture comprising of 1 mL of crude enzyme, 1 mL of 1% (w/v) soluble starch solution in 0.05 M citrate buffer solution (pH 4.5). The reducing sugars liberated were estimated by the 3, 5 Dinitrosalicylic acid (DNS) method (Miller, 1959). The reaction mixture was incubated at 60^0C for 20 minutes and the reaction was terminated by adding 2 mL of DNS in the reaction tube and then immersing the tube in boiling water bath (100°C) for 5 minutes. The absorbance was measured at 540nm with spectrophotometer (UTECH PRODUCTS INC. USA). One unit of amylase activity was defined as the amount of enzyme causing the release of 1 µmole of reducing sugars in one min., under the assay conditions.

Statistical analysis: Data regarding the hydrolyzing zones of the two tested species of *Aspergillus* as affected by pH and growth media were statistically analyzed using three way analysis of variance (ANOVA) followed by LSD method to delineate mean differences. Data regarding amylase activity of the selected strains of two *Aspergillus* spp., were subjected to one-way ANOVA followed by Duncan's Multiple Range Test for mean separation (Steel & Torrie, 1980).

Results

Qualitative analysis of *Aspergillus niger* strains: In solid state fermentation experiments, test fungal strains exhibited significant variation ($p \leq 0.001$) in hydrolyzing ability on solid media. Similarly the effect of pH and growth media was also highly significant. All the bipartite and tripartite interactions of fungal strains, pH and growth media were also highly significant (Table 1).

All test strains showed best hydrolyzing zone formation potential at pH 4.5, EPM further enhanced their activity significantly ($p \leq 0.05$) (Fig. 1). The pH above 4.5 resulted in a parallel decrease in diameter of hydrolyzing zones. Among the five test strains, performance of strain 198 was the best in hydrolyzing activity followed by strain 74 and strain 2. Strains 109 and 116 were comparatively less efficient (Fig. 1).

On the basis of their best hydrolyzing activity on solid media, *A. niger* strains 198 and strain 74 were selected for quantitative analysis of alpha-amylase activity in liquid medium. Both these strains displayed their best activity at 48 h incubation period that was significantly greater than activity at other incubation periods (Fig. 3A).

Table 1. Three-way ANOVA for hydrolyzing zones of different strains of *Aspergillus niger* under three pH levels and on two growth media.

Sources of variation	df	SS	MS	F-values
Treatments	29	3946	136	85***
Strains (S)	4	1671	418	262***
pH	2	1718	859	539***
Growth media (G)	1	96	96	60***
S × pH	8	266	33	21***
S × G	4	84	21	13***
pH × G	2	33	16	10***
S × pH × G	8	79	10	6***
Error	150	239	1.6	
Total	**179**	**4185**		

Note: Numbers represent F-values *** = p≤ 0.001.

Qualitative analysis of *Aspergillus flavus* strains: Test strains of *A. flavus* did not show any significant difference for their hydrolyzing zone formation on solid media (Table 2). Among the five strains, strain 231 showed maximum activity followed by strain 209 (Fig. 2). Effect of growth media and pH on the studied parameter was significant (Table 2). Generally maximum activity was recorded at pH 4.5. Increase in pH exhibited a decrease in the diameter of hydrolyzing zones. Effect was more pronounced and significant in strains 51 and 52. Except in strain 51, EPM enhanced the diameter of hydrolyzing zone by the test strains. Effect was significant in strains 231 and 209 (Fig. 2).

On the basis of their better performance, strains 231 and 209 were selected for evaluation of their alpha-amylase activity in liquid medium. Both the test strains exhibited their best performance after 48 h of incubation, which was significantly greater than their activity after other incubation periods (Fig. 3 B).

Discussion

The result of this conceptual study clearly reflect that pH value of medium has the ability to affect the mycelial growth rate and consequently on proliferation of these fungi. The relative intensity of this effect however varies with the species involved. A wide body of evidence indicates that optimum fungal growth occurs in acidic media; however, the range of pH that will permit growth varies with the species and the composition of the culture medium (Wolf & Wolf, 1947).

It is evident from the present study that the extent of zones of starch digestion was significantly enhanced by lowering the pH up to 4.5. In similar investigations Kim *et al*., (2005) have reported the pH range 3-6 for *Sphaeropsis pyriputrescens* and optimum pH between 3-4 which is comparable to other such studies on fungi.

It is enumerated from the spectrum of microbial cultures employed for enzyme production in solid state fermentation that four isolates among the tested species gave the highest detectable quantities of starch hydrolysis. These findings are in line with the work conducted by Omemu *et al*., (2005) where the selection of potent species was made by plate method. However, zonation can not in any way be correlated quantitatively with the amount of enzyme produced. Therefore, the isolation of enzyme producers using starch plates can only be partially selected. So, the selection of more efficient amylolytic strains was made on biochemical basis (Elander, 1982).

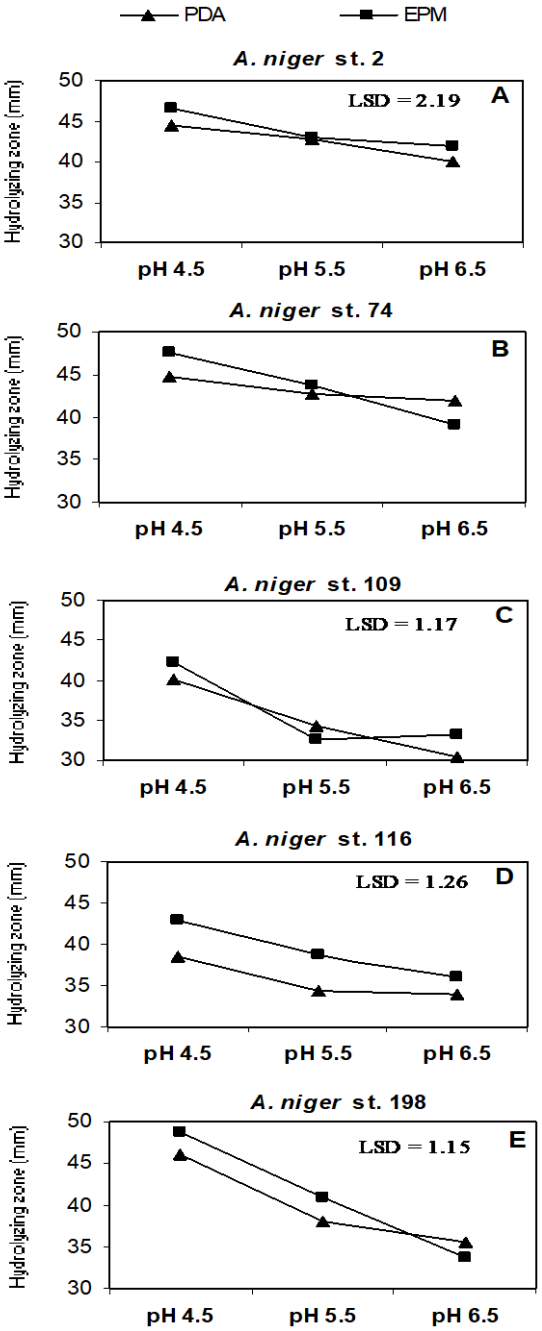

Fig. 1. Effect of pH and growth media on hydrolyzing zones of different strains of *Aspergillus niger* LSD at p≤0.05.

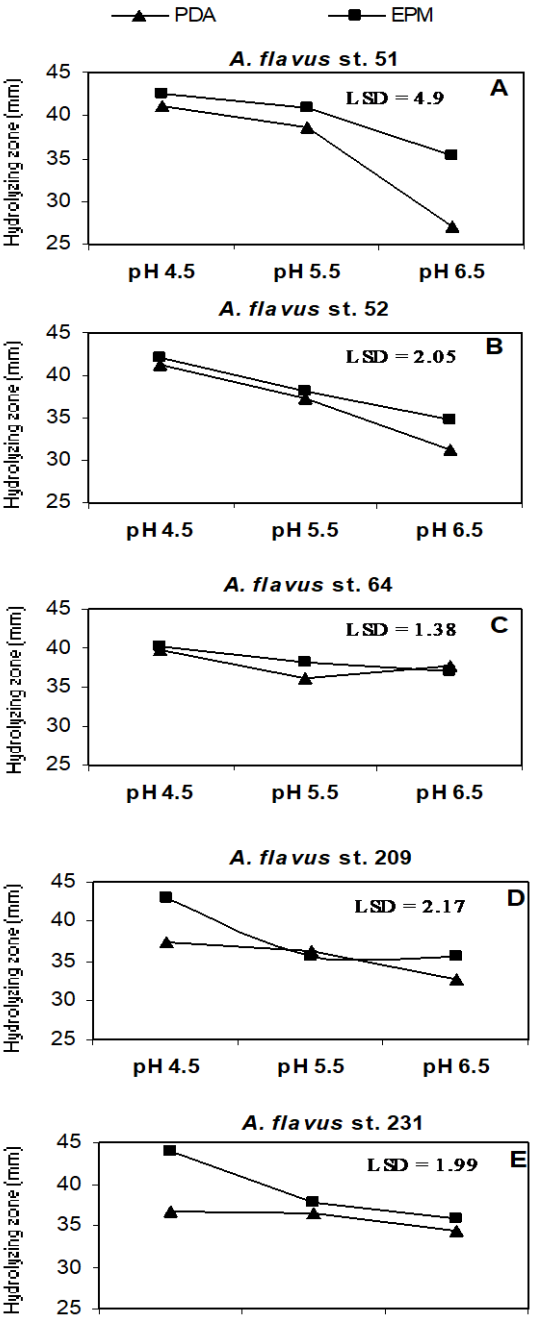

Fig. 2. Effect of pH and growth media on hydrolyzing zones of different strains of *Aspergillus flavus* LSD at p≤0.05.

Table 2. Three-way ANOVA for hydrolyzing zones of different strains of *Aspergillus flavus* under three pH levels and on two growth media.

Sources of variation	df	SS	MS	F-values
Treatments	29	2326	80	14^{***}
Strains (S)	4	41	10	1.8^{ns}
pH	2	1330	665	117^{***}
Growth media (G)	1	58	58	10.3^{**}
S × pH	8	357	45	7.8^{***}
S × G	4	181	45	8^{***}
pH × G	2	65	33	5.7^{**}
S × pH × G	8	294	37	6.5^{***}
Error	150	851	5.7	
Total	**179**	**3178**		

Note: Numbers represent F-values *** = p≤0.001, ** = p≤0.01, ns = Non-significant.

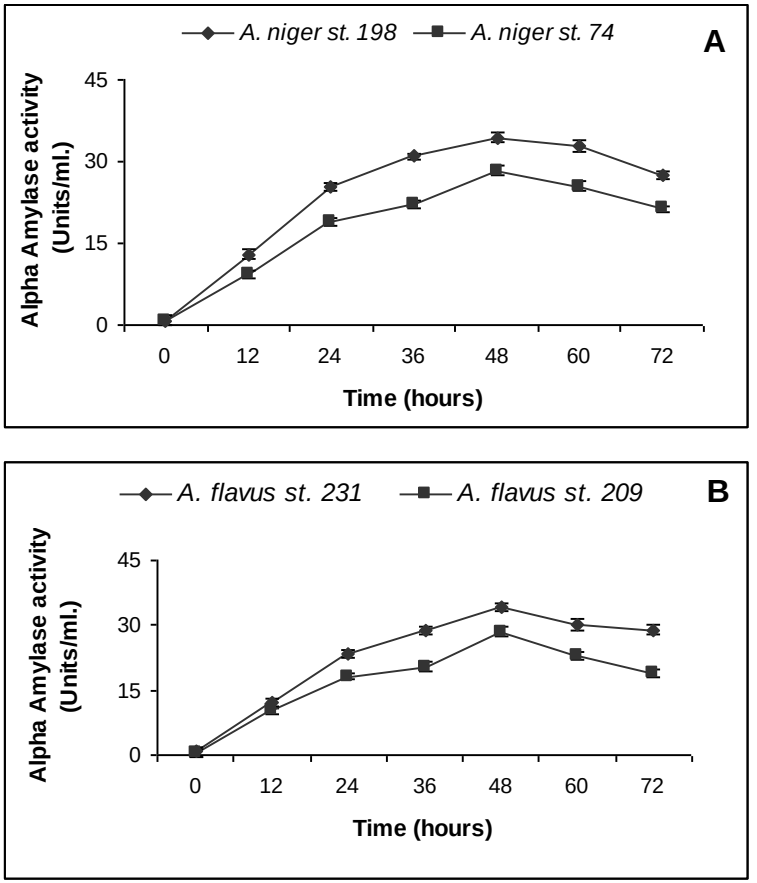

Fig. 3. Alpha amylase activity of selected *Aspergillus niger* and *A. flavus* strains. Vertival bars show standard error of means of three replicates.

The temperature of the medium was kept at 30 ± 2ºC with shaking at 200 rpm, which is in accordance with the previous work conducted by Haq *et al*., (2002). Similarly, Smith & Wood (1991) observed that the optimum temperature was 30ºC and 35ºC for the production of extra cellular xylanases and xylosidase by *Aspergillus awamori.*

The relative enzyme activities of the four tested isolates are shown in Fig. 3, which reached its maximum at 48 hours after inoculation in the entire medium. Further increase in the incubation period resulted in declined activity of alpha-amylases. It might be due to the depletion of nutrients, lag phase of fungi and production of proteases in the fermentation medium (Haq *et al*., 2002).

Another important feature of this study was the significant differences of amylolytic activities associated with different fungal isolates. These findings are parallel with the work carried out by Alazard & Raimbault (1981) in which they compared the amylolytic enzyme production potential of *Aspergillus niger* in liquid and solid state cultivation. In another study by Mikiashvili *et al*., (2004) significant difference among enzyme activities showed that extensive differences in enzyme production may exist among fungal species and even among strains of same species as was demonstrated with species of genera *Ganoderma, Omphalotus* and *Pleurotus* etc.

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