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PROTEASE ENZYME PRODUCTION BY DIFFERENT STRAINS OF *ASPERGILLUS FLAVUS* AND *ASPERGILLUS NIGER* USING CASEIN AS A SUBSTRATE

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Abstract

The present study was carried out to evaluate the extent of variation in the production of protease by various strains of aspergilli isolated from patients, soils and air using casein as a substrate. In the case of *A. flavus*, strain FS1 and FS2, isolated from patients, exhibited much lesser protease activity as compared to other strains, while, overall production of protease is much higher in the case of pathogenic strain NS1 (of *A. niger*) as compared to those of *A. flavus* (FS1 and FS2). The proteases produced in the present study are likely to be metalloproteases. While metalloproteases are likely to contribute to the pathogenicity of *A. niger*, in the case of *A. flavus* other proteases might be involved.

Keywords: Protease, Aspergilli, Aspergillosis, *Aspergillus flavus*, *Aspergillus niger*, Casein.

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INTRODUCTION

A number of species of the genus *Aspergillus* cause different forms of diseases in human being, plants, insects and other organisms also [1]. These are generally considered as opportunistic human pathogens. A significant number of *Aspergillius* spp. are known to be the producers of extracellular acid proteases *viz. Aspergillus niger* [2], *A. oryzae* [3], *A. awamori* [4], *A. fumigatus* [5] and *A. saitoi* [6]. The proteases form a complex family of enzymes that possess different catalytic mechanisms with various active sites and divergent substrate specificities [7, 8]. These constitute one of the largest functional groups of proteins involved in many normal and pathological processes. Protease inhibition of pathogenic organisms may aid in the control of several diseases [9]. During last few years, proteases and protease inhibitors have gained additional interests in many health-related fields *e.g.* allergy, asthma and obese-related illness [10]. Proteases can be classified into four major groups *viz.* (i) aspartic; (ii) cysteine; (iii) metallo- and (vi) serine proteases [8]. The present investigation was conducted to evaluate the variations in protease activities in different strains of Aspergilli obtained from patients suffering from aspergillosis and the strains obtained from other sources using casein as substrate.

MATERIALS AND METHODS

COLLECTION AND ISOLATION OF PATHOGENIC FUNGAL SAMPLES

Three clinical specimens (suspected to contain fungi) were collected aseptically, during surgery, from three different patients (two male and one female) suffering from aspergillosis. The specimens were preserved in saline solution (9 gm NaCl/lit D.W.) and brought to the laboratory. All these three specimens were processed for the isolation of fungus on Sabourauds Agar Medium using standard protocol [11]. The plates were incubated in BOD incubator at 25±2°C for 5-6 days. Two of these samples were found positive for *Aspergillus flavus* (designated as FS1 and FS2), while one sample yielded *Aspergillus niger* (designated as NS1).

ISOLATION OF ASPERGILLI FROM SOIL AND AIR SAMPLES FROM SELECTED HOSPITALS

Four different soil samples were collected from ENT waste dumping sites of four different hospitals of Meerut namely Subharati Medical College (Meerut), Military Hospital (Meerut), Pyarelal Sharma Hospital (Meerut) and Cantonment Hospital (Meerut). The samples, collected aseptically in sterile polythene bags, were brought to laboratory. Serial dilution plate method [12] was followed for isolating fungi from the samples using Sabourauds Agar Medium [13, 14]. The

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plates were incubated at 25±2°C for 5-6 days. For collection of samples from air, already prepared plates of Sabourauds Agar Medium were exposed for 15-20 minutes near the ENT waste dumping sections of aforementioned hospitals and were brought to laboratory to be incubated at 25±2°C for 5-6 days.

After incubation, the axenic cultures of concerned fungi were prepared and studied for cultural and microscopic characteristics. In all, eight different strains of *A. flavus* (four from soils and four from air) were obtained and were designated as FSS1, FSS2, FSS3, FSS4 (strains of A. *flavus* obtained from soil samples) and FAS1, FAS2, FAS3 and FAS4 (strains of *A. flavus* obtained from air samples). In the same manner, 8 different strains of *A. niger* (four from soil and four from air) were obtained and designated as NSS1, NSS2, NSS3, NSS4 (strains of *A. niger* obtained from soil samples) and NAS1, NAS2, NAS3 and NAS4 (strains of *A. niger* obtained from air samples). Altogether, nineteen strains of aspergilli (10 strains of *A. flavus* and 9 strains of *A. niger*) were obtained and their pure cultures were prepared for further experiments.

PRODUCTION OF PROTEASE ENZYME *IN-VITRO* BY DIFFERENT FUNGAL STRAINS

100 ml of mineral medium [$MgSO_4$, 0.52; KCl, 0.52; KH_2PO_4, 1.52; $FeSO_4.7H_2O$, 0.01; $ZnSO_4.7H_2O$, 0.01; sodium caseinate, 5 (g L^{-1})] were dispensed within each of 57 Erlenmeyr flasks. These were divided into nineteen sets of 3 flasks each. Each flask of set 1 was inoculated with 1 ml spore suspension prepared from 5-6 days old culture of FS1. The flasks were incubated at at 28±2°C in BOD shaker incubator, under agitation at 150 rpm, for 5 days. The mycelial mass produced in each flask was harvested separately by filtering the contents of each flask and then dried at 60ºC until it reached a constant weight. The supernatant of each flask was assessed for protease content. The flasks of other 18 sets were also treated similarly− each set receiving inocula of different strain.

PROTEASE ENZYME ACTIVITY ASSAY

Proteolytic activity assay was conducted for supernatant of each of the 57 flasks separately as suggested by Ramakrishna and Pandit (1988), using casein as the substrate, with slight modification as a few drops of 1.25 N NaOH were added in reaction mix to dissolve casein. Enzyme activity was determined by incubating 250 µL of the supernatant with 500 µL 1% (w v^{-1}) casein sodium salt in 50 mM buffer (pH 5.0, 7.0 and 9.0) for two hours at 30ºC. The reaction was stopped by the addition of 375 µL of 20% (w v^{-1}) trichloroacetic acid. These tubes were placed in an ice bath for 30 minutes and then centrifuged at 5000 x g for 15 minutes at 4ºC. Proteolytic

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activity was determined by analyzing the absorbance of the supernatant at 280 nm against an appropriate blank. One unit of protease activity is defined as the amount of enzyme which liberates one micromole of tyrosine per minute per gram dry substrate under experimental conditions. Tyrosine was used as standard.

RESULTS AND DISCUSSION

A comparative assessment of protease production by different strains of both the species of *Aspergillus viz. A. flavus* and *A. niger* revealed marked differences in protease production (Fig 1&2; table 1) as indicated by the amount of tyrosine liberated and the amount of enzyme produced (units/ml). Out of ten strains of *A. flavus*, two strains *viz.* FS1 and FS2 (isolated from patients) exhibited much lesser protease activity *i.e.* 0.077 and 0.066 units/ml enzyme as compared to most of other strains. One of the strains isolated from air *i.e.* FAS2 also displayed very less protease activity (0.050) like pathogenic ones, indicating the possibility of potentially pathogenic nature of the strain FAS2.

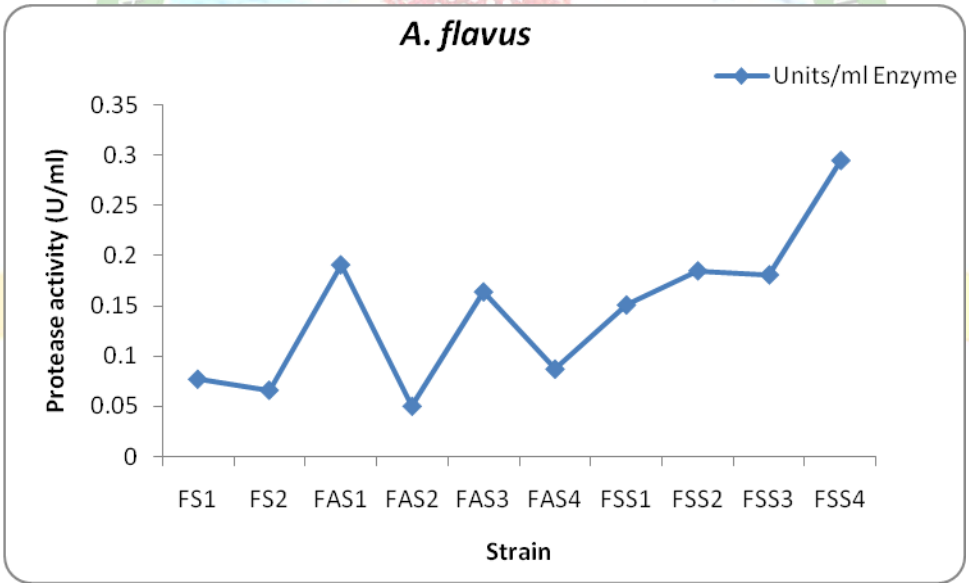

Fig 1: Production of proteases by different strains of *Aspergillus flavus* isolated from soil, air and patients

On the other hand, the findings were completely different in case of *A. niger* where the pathogenic strain NS1 displayed a much higher protease production (0.440 units/ml enzyme) as compared to other strains of not only *A. niger* but *A. flavus* as well.

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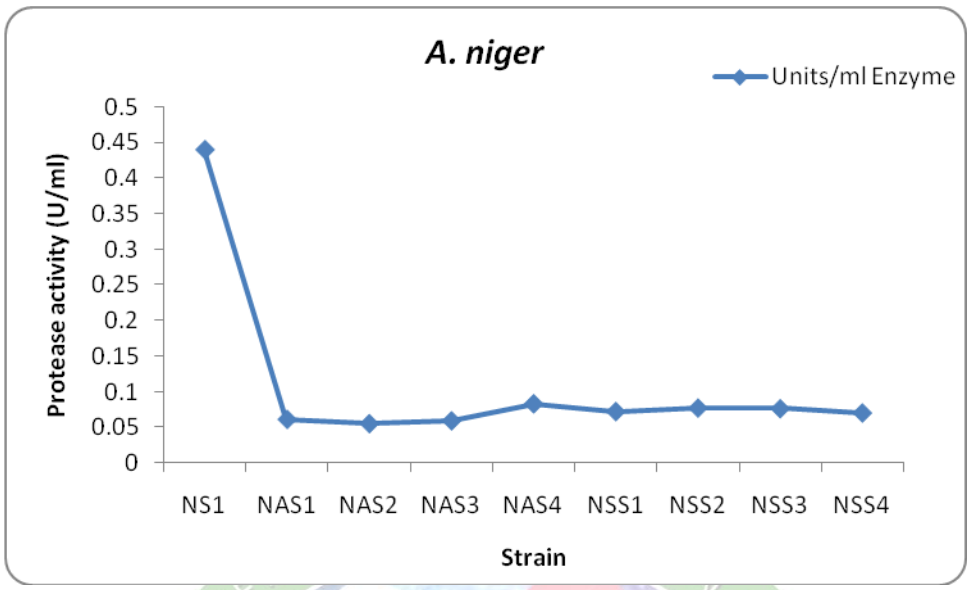

Fig. 2: Production of proteases by different strains of *Aspergillus niger* isolated from soil, air and patients

Proteases are believed to play an important role in pathogenesis [16, 17]. The present study extends support to the contentions of some earlier workers who believe that in addition to metallo-proteases (produced on casein substrate), serine proteases and cysteine proteases (produced on elastin substrate) might be involved in invasion of human tissues by *A. flavus* and *A. niger*. The production of elastinolyic proteases by *Aspergillus* species *in vitro* contribute to fungal pathogenicity. Elastin degradation may facilitate tissue invasion by these organisms [18, 19]. Once the fungus is in its mycelial form, it is capable of producing a number of proteases that facilitate persistence of the fungus within the airway including serine proteases, phospholipases, metalloproteases and superoxide dimutases [20]. Fungal proteases cause epithelial cell detachment from respiratory epithelium [21].

The results of the present study indicate that overall production of protease is much higher in the case of pathogenic strain NS1 (of *A. niger*) as compared to those of *A. flavus* (FS1 and FS2). Budak *et al*. (2014) also reported that greater protease abundance was found in *A. niger* than *A. flavus* [22]. Zhu *et al*. (1990) reported that when grown on minimal medium with milk protein as a nitrogen source, *A. flavus* primarily produced a metalloprotease, but when grown with insoluble collagen or elastin, serine proteases as well as metalloprotease are produced. The protease produced in the present study are likely to be metalloproteases [23]. Thus, production of metalloproteases might be assumed to be associated with pathogencity of *A. niger*. This observation differs from that of Budak *et al*. (2014) who could detect no metalloprotease activity

in *A. niger*− rather they identified serine protease in the supernatant of *A. niger*. The strains of *A. flavus* isolated from air and soil (with exception of FAS2), exhibited greater metalloprotease activity than not only those of *A. niger*, but the pathogenic strains of *A. flavus* also. The lesser metalloprotease activity of pathogenic strains of *A. flavus* indicates the possible involvement of other class of proteases (other than metalloprotease) in the pathogenesis by the fungus. Budak *et al*. (2014) reported that (i) the broadest set of proteases was found in *A. flavus*; (ii) in *A. flavus*, the most abundant protease belongs to aminoprotease group.

It would be worthwhile to examine the production of proteases by fungal strains under study, on more substrates like collagen and elastin to obtain a more complete picture in this context.

Table 1: Production of protease enzyme (units/ml) by different strains of *Aspergillus flavus* and *Aspergillus niger* (pathogenic and non-pathogenic) using casein as a substrate

Fungal species	Strain	Amount of Tyrosine produced (mg/ml)	Units/ml enzyme
Aspergillus flavus	FS1	0.635	0.077
	FS2	0.547	0.066
	FAS1	1.565	0.191
	FAS2	0.413	0.050
	FAS3	1.343	0.164
	FAS4	0.715	0.087
	FSS1	1.236	0.151
	FSS2	1.516	0.185
	FSS3	1.485	0.181
	FSS4	2.419	0.295
Aspergillus niger	NS1	3.603	0.440
	NAS1	0.502	0.061
	NAS2	0.458	0.055
	NAS3	0.484	0.059
	NAS4	0.684	0.083
	NSS1	0.596	0.072
	NSS2	0.636	0.077
	NSS3	0.623	0.076
	NSS4	0.578	0.070

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