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DIFFERENTIATIONS OF *ASPERGILLUS* SP. REVEALED BY STARCH ZYMOGRAPHY OF MADHYA PRADESH INDIA

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ABSTRACT

Sixteen isolates of *Aspergillus* belonging to six species viz. *A. flavus, A. fumigates, A. nidulens, A. niger, A. terreus* and *A. ustus* were grown on sterilized wheat grains. This resulted in the release of extracellular amylase enzymes for the utilization of substrate by them. The enzymes were detected by using the starch plate assay in which appreciable amylase activity was observed. The various extracellular amylases released by different isolates were separated by vertical SDS-PAGE conditions (created by SDS). It showed 14 polymorphic bands categorized in 6 zymogram groups (starch) indicating genetic variability in Aspergilli. Amylase designated, as $A1^{24.28}$ was common in all the zymogram groups except some isolates and there were few unique bands (amylases) that were characteristic of particular isolates. The results showed that the species of *Aspergillus* studied were producing different amylase enzymes when they were given a common substrate for growth and reveals that at least 14 different amylase enzymes are present in these six species of Aspergilli, although more number of enzymes could be present because production of individual enzyme largely depends on the particular substrate available since some enzymes are inductive in nature.

(A= Amylase band, 1= Band number, 24.28= Relative mobility)

KEYWORDS: *Aspergillus* sp, Zymography, Enzymes Etc

INTRODUCTION

Differentiation of various subgroups in fungi is one of the major challenges for mycologists/fungal taxonomists. Morphological and cultural characteristic sometime fail to recognize subgroup in a genus or within a species. Paramorphological criteria have now a days attracted the attention of a large group of workers world over (Frisvad, 1983; Micales *et al.,* 1986; Neate *et al.,* 1988; Christensen *et al.,* 1999). Zymography of extracellular amylase enzyme can also be one of the better tools for differentiation of fungi (Cruickshank and Pitt, 1987) and the present study is an attempt to distinguish some *Aspergillus* sp. by amylase zymography. This work deals with the evaluation of enzyme activity of industrially important amylase producing Aspergilli and determination of their genetic variation on the basis of relative mobility (Rf) of various amylase enzymes on starch containing polyacrylamide gel. The number and amount of amylase enzyme produced depends on the present or absence of genes/loci coding for amylase and if present their number and frequency.

MATERIALS AND METHODS

Isolation and Taxonomic Description of Isolates

Apergillus sp. were isolated from litter and soil collected from different forests of Madhya Pradesh. Standard isolation techniques were used to isolate of various species of *Aspergillus* (Agarwal and Hasija, 1986) which were

identified on the basis of colony and micro-morphological characteristics (Thom and Raper, 1945) showed that all the 16 isolates belong to six species viz., *A. niger, A. fumigates, A. flavus, A. nidulans* and *A. ustus.*

Maintenance of Culture

Isolated pure culture of Aspergilli were maintained on PDA slants and stored at 4^0C in refrigerator and also preserved its in lyophilized ampoules (Smith and Onions, 1994) for further use.

Enzyme Production

According to Cruickshank and Pitt (1987), 5 Wheat grains (approx. 100mg) with 2ml basal salt solution were autoclaved in 25ml culture tubes, inoculums of different *Aspergillus* isolates were inoculated aseptically in individual tube and incubated in above at 28^0C for a week. Before harvesting the enzyme, the culture tubes were kept at -4^0C for 60min. and then 1ml of precooled 50mM tris buffer (pH 7.4) was added and the same amount of crude enzyme extract was pipette out in fresh eppendrof tube kept in ice bath and then centrifuged at 8000rpm in cooled condition and supernatant was taken as crude enzyme extract (CEE) and stored at -20^0C for further use.

Determination of Enzyme Activity

Plate Well Assay: According to Mann, 1962, solid agar containing 0.2% starch was poured in sterilized petriplate then 7mm diameter well were prepared and 100µl was added CEE and tha above plates were incubated for 1 hr at 28^0C. After incubation the plates were flooded with iodine reagent and clear zone was measured and enzyme activity was determined by following formula (Upadhyay *et al.*, 2002).

EA= D-d

Where,

D= diameter of clear zone

D= diameter of well

Quantitative Estimation: According to Gogoi *et al.*, (1995), 0.5ml of 1% starch and 0.3ml of 0.1M acetate buffer (pH 5.5) and 1ml distilled water was warmed at 55^0C for 10 min then 0.5ml CEE was added in above cooled mixer, then allow to proceed for 60min at 28^0C. The reaction was stopped by adding 0.5ml 1M HCl and cooled at room temperature and then 0.2 ml of reaction was diluted by distilled water to 15ml including 0.1 ml 1 M HCl then 0.1 ml of iodine reagent was added and the absorbance was measured at 610nm. 1unit of amylase was defined as the amount of enzyme which connected absorbance 0.05 under assay condition.

Enzyme Electrophoresis

According to Cruickshank (1987), 105 polyacrylamide gel containing 0.2% soluble starch was used for enzyme separation and then 15µl enzyme sample was loaded on to each well. After complete electrophoresis gel was washed with 1^{st} and 2^{nd} washing buffer respectively for 2 round of 10 min. and gel was incubated in substrate buffer for 3 hrs at 30^0C. Gel was stained with iodine reagent for 5min. and zymogram was observed (Figure 2) and relative positions of bands were calculated as per Gottlied *et al.*, (1998).

$$Rf = \frac{d}{f} \times 100$$

Where,

d = Migration distance from the origin of each band

f = Migration distance from the origin of dye marker

RESULT AND DISCUSSIONS

Data recorded in Table-1 clearly indicates that fungal isolates varied greatly in their amylotytic activity on starch containing plates. Out of 16 isolates of 6 *Aspergillus* species screened, 4 isolates (FGCC# 203, FGCC# 245, FGCC# 255 and FGCC# 256) showed maximum activity where as the rest of the isolates showed significant but comparatively lower activity. Zymography of these extracellular amylase enzymes resulted in 6 zymogram pattern (Zg1 to Zg6) as shown in Figure 1C, 1D and 2. There were 13 polymorophic bonds and a total of 42 bands obtained in the isolates except *A. terrus* (L1 and L2) and *A. ustus* (L14) indicated that it represents one of the key enzymes in the amylolytic machinery of Aspergilli.

There was wide variation in the presence or absence of bands in different isolates. Few bands are shared in individual Zg viz, A432.85 in Zg 1, A851.428 in Zg4, A227.14 in Zg5 and A1071.43 were shared in Zg6. Due to this, however we could not deduce any conclusion on the taxonomic aspect but we could obtain a fairly clear cluster based on the amylase zymogram pattern as indicated Figure 3. We strongly believe that the amylase produced extracellularly by various fungi indicate a clear line of adaptation towards a widely common plant substrate the starch. These fungi including the single celled yeast are potential producers of amylases and are being exploited commercially (Gupta and Gautam, 1993). The present study indicates that there has been numerous forms of amylase enzyme present even in a single genus *Aspergillus.* So there is possibility of finding a large number in other genera too. This again give us an opportunity to explore the amylase gene pool for commercial exploitation in various amylolytic or amylase producing fungi.

Table 1: Amylase activity of *Aspergillus* spp

Isolate No.	Name of Fungi	EA (D-d)	Enzyme unit **
FGCC-203	*Aspergillus niger*	0.7	26.14
FGCC-255	*A. niger*	0.8	26.44
FGCC-201	*A. niger*	0.6	25.58
FGCC-246	*A. terreus*	0.6	20.38
FGCC-282	*A. terreus*	0.6	21.88
FGCC-285	*A. fumigatus*	0.8	34.46
FGCC-245	*A. fumigatus*	0.9	35.43
FGCC-199	*A. fumigatus*	0.9	32.33
FGCC-202	*A. flavus*	0.6	27.66
FGCC-200	*A. flavus*	0.4	20.11
FGCC-263	*A. flavus*	0.5	20.04
FGCC-281	*A. nidulans*	0.3	21.04
FGCC--262	*A. nidulans*	0.5	21.09
FGCC-271	*A. ustus*	0.5	20.12

FGCC-256	*A. ustus*	0.6	20.11
FGCC-272	*A. ustus*	0.6	22.00

FGCC- Fungal germplasm collection centre, Jabalpur (M.P.)

** 1 unit of amylase was defined as the amount of enzyme which connected absorbance 0.05 under assay condition

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