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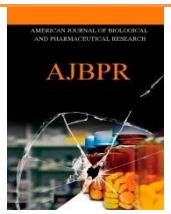

SIMPLE AND RAPID PROTOCOL FOR PECTINASE ZYMOGRAPHY

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ABSTRACT

Differentiation of various subgroups of fungi is one of the major challenges for fungal taxonomist. As morphological and cultural characteristic sometime fails to recognize subgroups in a genera or within a species. Therefore paramorphological criteria have attracted the attention of a large group of researcher's world over. Zymogrpahy of extracellular Pectinase isozyme is now considered to be one of the better tool not only for differentiation of fungi but also for other organisms which are using pectin as sole carbon source. The present study is an attempt to distinguish some morphologically similar but ecologically variant isolates of indigenous *Ganoderma lucidum* by using simple and rapid pectinase zymography.

INTRODUCTION

The analysis of extra-cellular isozyme proved to be very useful for categorization of morphological identical organisms because some time morphological identical organisms behave differently and very difficult to categorize them. Therefore, zymogrpahy of extracellular pectinase is considered to be one of the better tools for differentiation of morphologically similar fungal isolates [1].

In our experience, most of the students find difficulty in performing molecular characterization of different fungal strains because it involves DNA isolation and PCR. Therefore, the present technique is undertaken as it is rapid and simple to characterize different fungal strains based on pectinase isozyme analysis. This analysis determines the diversity of protein in ecologically variant isolates and it is indirectly related to alleles or loci [2]. The alleles or loci codes the proteins (Pectinase enzymes), after few physiological process in the cell act as enzyme and are visualized in gel in the form of discrete bands during the zymography [3]. Indirectly, zymography is interrelated to genetic diversity because it reveals the diversity of mono or polymorphic alleles or loci [4].

Pectinase zymography is a inexpensive and versatile technique which can be used to determine the account of genetic variation among organisms. This procedure is used in population genetic diversity studies of not only to fungi but also for other groups. The banding pattern of specific alleles or loci allows the determination of ratio of alleles expressed in common among the individuals. These ratios are an excellent means of determining phylogenetic relationship among ecologically variant and morphologically identical organisms. In the present study a rapid and easy learning pectinase zymography is discussed for comparison of closely related species and the assumption is made that the same loci are present and are being compared among isolates.

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MATERIALS AND METHODS

Fungal isolates

A total of eight isolates of *Ganoderma lucidum* were isolated from forests of M.P., India by employing standard techniques. Pure cultures were maintained on malt extract slant and stored at 4°C in refrigerator for further use. These cultures have been deposited in Fungal Germplasm Collection Center (FGCC), R.D. University, Jabalpur, India for further references.

Extraction of extra-cellular isozyme

Enzyme extraction was done as per [4] 500mg of pectin was autoclaved with 10ml of basal salt solution (KH_2PO_4,1.5gm; $MgSO_4.7H_2O$, 0.025gm;$CaCl_2$, 0.025gm;$FeSO_4.7H_2O$, ,0.015gm; $ZnSO_4$,0.005gm; D/W,1000ml) in 100ml of Erlenmayer flasks which were seeded with 5mm disc separated from seven days old culture mycelium and incubated at 28^0C for 8 days. Before harvesting, the flasks were kept at 4^0C for 15 min. then 2ml of chilled 50mM Tris buffer (pH 7.4) was added and vortexed properly. Crude enzyme was pipetted out and centrifuged at 10,000 rpm at 4^0C for 4 min. Supernatant was taken as enzyme extract and stored at – 40^0C until used for electrophoresis.

Zymography

The enzymes were separated by SDS polyacrylamide gel electrophoresis in a discontinuous buffer system. The resolving gel contained 10% acrylamide with 2mg pectin per ml of gel and stacking gel contains 5% acrylamide [5]. The reservoir buffer contained 25 mM Tris base and 192 mM glycine with pH 8.3 [6]. 15µl enzyme samples along with 6X gel loading dye (300mM Tris-Cl ,10% SDS, 6% Glycerol and 0.3% Bromophenol Blue-pH6.8) were loaded . The gel was run at 80V and 15mA current at 4^0C until the dye front reaches up to the bottom. The gel was washed twice with 1st washing buffer(50mM Tris buffer, 49ml; Triton-x-100, 1ml) and 2nd washing buffer(50 mM Tris buffer) in shaking condition for ten minutes each, and then it was incubated in substrate buffer (0.1 M malic acid)for 2 hours at room temperature [7].The gel was stained in 0.01% ruthenium red solution overnight at 4^0C then washed for 1 hour in distilled water to remove the excess dye. Zymogram was prepared and relative position of bands were calculated as per [4].

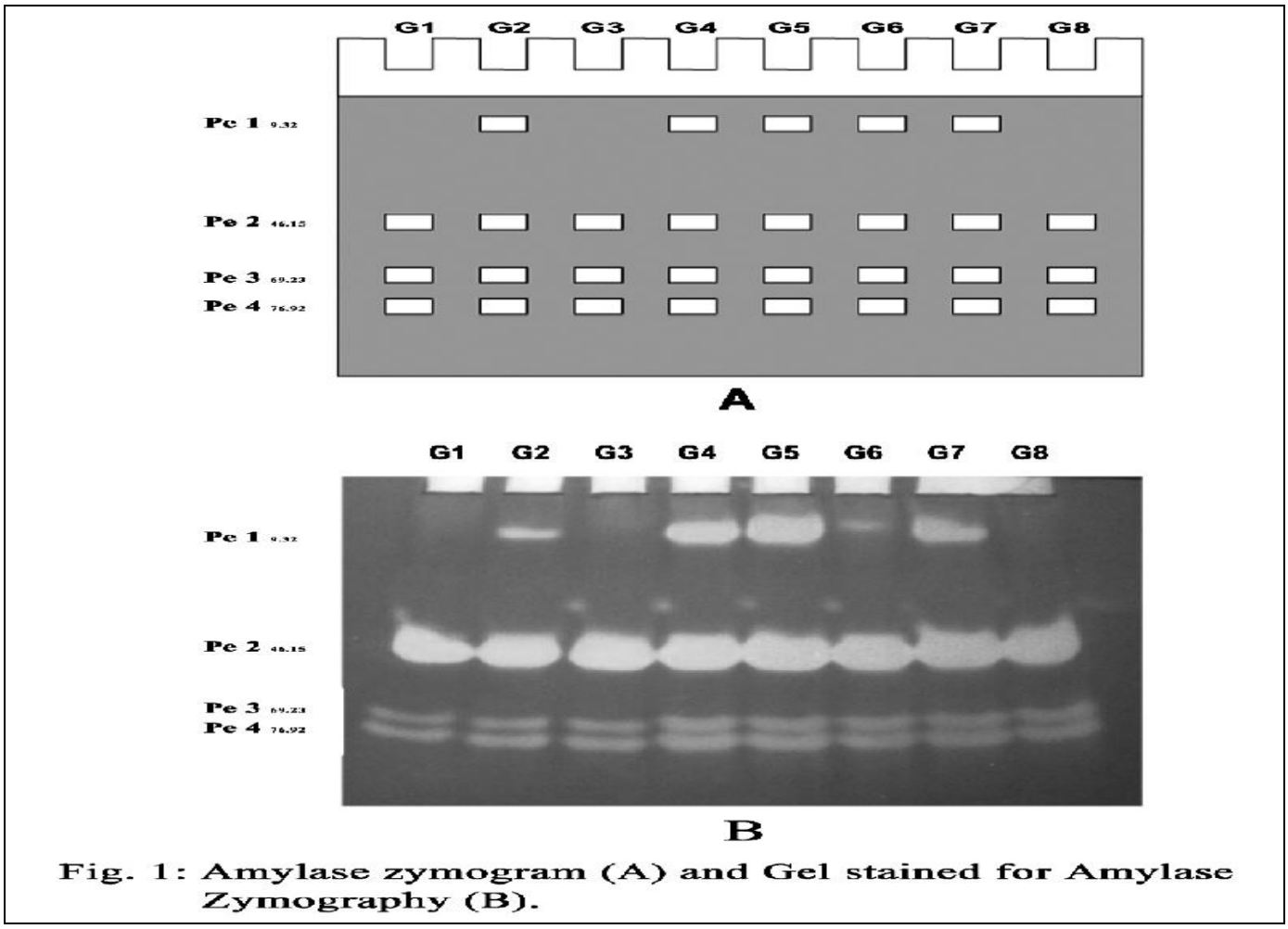

Fig. 1: Amylase zymogram (A) and Gel stained for Amylase Zymography (B).

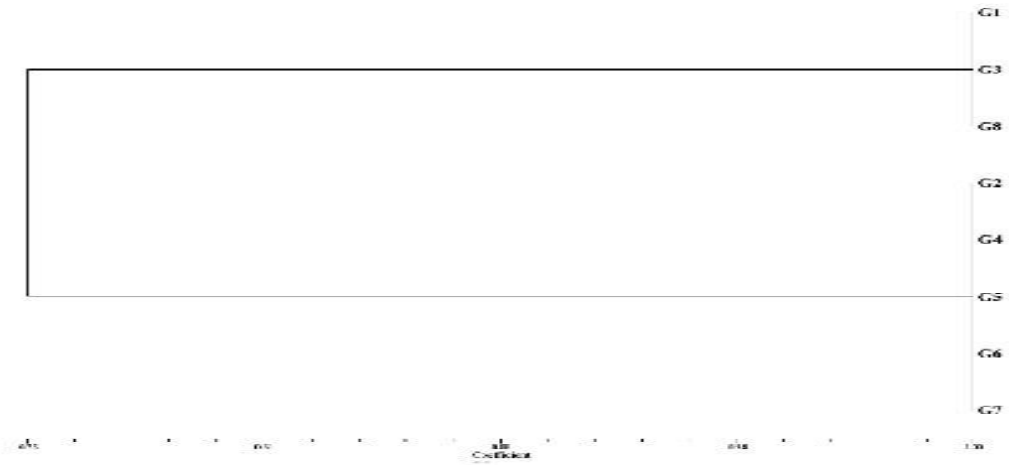

Fig.2 : Dendogram based on eucladian distance analysis of Pectinase isozymes of *Ganoderma lucidum*

RESULT AND DISCUSSION

Figure 1B shows that the band visualized in pectinase zymography is very clear because the substrate buffer used in this method contains 0.1M malic acid which activate and stabilize the pectinase enzymes. The complete hydrolysis of the pectin requires 2 hours incubation at 28°C in gel but some research groups use only 90 min. incubation at 25°C for fungal pectinase zymography which could not give proper and distinguish bands. [8]

The pectinase zymogram (Fig1 A&B) shows three monomorphic($Pe2_{46.15}$, $Pe3_{69.23}$ and $Pe4_{76.92}$) and one polymorphic($Pe1_{9.32}$) bands. The various extracellular pectinases released by different isolates were separated by vertical SDS-PAGE and in which polyacrylamide gel contained 0.02% pectin under slightly denaturing conditions (created by SDS). It showed 3 monomorphic and 1polymorphic bands categorized in 2 zymogram groups (pectin) indicating genetic variability in *Ganoderma lucidum*. Pectinase designated as $Pe2_{46.15}$, $Pe3_{69.23}$ and $Pe4_{76.92}$ were common in all the zymogram groups whereas band $Pe1_{9.32}$ was present in most of the isolates except G1, G3 and G7. The results showed that the species of *Ganoderma ludium* studied were producing different pectinase enzymes when they were given a common substrate for growth and reveals that 4 different pectinase enzymes are present in these eight species of *Ganoderma*, each pectinase designated band represent an active gene and on the basis of which differentiation of *Ganoderma* was done.

The dendrogram (Fig-2) was obtained with eucladian distance and UPGMA cluster analysis. A total of two clusters were obtained by 8 isolates of *G. lucidium.* The genetic distance between the isolates of group 1 viz. G1, G3 and G8 were more closely related to each other whereas. G2, G4, G5, G6 and G7 are placed on other line of cluster . The grouping of *G. lucidum* derived from numerical analysis of the electrophoretic data demonstrate a high degree of concordance with intraspecific classification established by conventional classification. Similarly [9] distinguished ecologically variant 134 isolates of *Ganoderma* species on the basis of isozyme analysis and stated that data matrix including morphological and isozyme character may clarify the subject and allowing to proposed natural classification of the species within the *Ganoderma*.

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