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Comparative Study of Dermatophytic Fungi for Extra Cellular Proteases Efficacy.

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Research Article

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ABSTRACT

Fungi are known to produce proteases of different kind. The dermatphytic fungal strains were isolated from human skin tissues for extra cellular proteases efficacy. The present study deals with purification, estimation and comparison of extracellular proteases from five fungal species. (*Fusarium sp., Curvularia sp. , Fumigatus Sp. , Aspergillus Sp.* and *Mucor Sp.).* All the five fungal strains showed good amount of extra cellular protease activity in terms of unit total protein content. By testing all the crude extracts for enzyme activity, *Fusarium sp.* was found to show the highest activity whereas *Mucor sp.* showed the lowest. The study supports the notion that fungi can be a good source of extracellular enzymes especially proteases.

INTRODUCTION

Fungi are known to produce proteases of different kind. Most of the fungus such as *Alternaria, Fusarium, Absidia, Mucor, Aspergillus, Phytophthora, Penicillium, collectrotricum, Cladosporium, Curvularia, Rhizopus, Trichoderma, Morcella,* and *Dematium* etc. has shown protease activities [1] All enzymes are proteins, used by living cells and responsible for numerous metabolic process of the cell. They can be intracellular or the extracellular. Fungi produce extracellular enzyme(s) in order to break the macromolecules into micro molecules which they can absorb [2]. Fungi require carbon containing substance, such as sugars as source of energy and all need nitrogen containing compound substance to build protein and other essential compound. Each fungus produces different types of enzymes depending upon the chemical nature of its host [3]. Further, a group of enzymes can be secreted simultaneously to target multiple nutrients. Proteases are a group of proteinases and peptidases. Proteinases catalyze the hydrolysis of the protein molecule into smaller fragments. Peptidases hydrolyze polypeptide fragments[4]. Based on the active site moiety, the proteases are of following major types [5]. proteolytic enzymes are required by all organisms for nutrient acquisition. However, pathogenic microorganisms often secrete enzymes of this class that may aid in their virulence by other means, including tissue destruction and modulation of host defense responses. It is therefore important to investigate the production of these enzymes by known pathogens [6]. it was described that, the protease identified and characterized from A*. niger, involved in its proteolytic processing in the secretory pathway,* recognizes dibasic amino acid motifs and removes the propeptide from the newly synthesized proteins. This study also focused on proteins containing only one basic amino acid as cleavage site for propeptide removal [7]. protease producing strain *Aspergillus niger has been isolated* from local soil samples and enzyme production was optimized under submerged conditions.The molecular weight of the enzyme determined by SDS-PAGE was found to be 38 kDa. The enzyme acted optimally at pH 10 and 50°C. It was thermo stable and retained full activity even at the end of 1 hour of incubation at 40°C [8]

MATERIAL AND METHODS [9-22]

Identification and Inoculation of fungal strains

The different fungal cultures were identified on the basis of cultural and microscopic studies. These isolated fungi were *Aspergillus fumigates, Fusarium sp. Aspergillus niger , Curvularia sp. And Mucor sp.* The pure cultures of isolated fungal strains were maintained in PD broths with streptomycin at 28°C during the study.

Biochemical assay for protease activity

Preparation of fungal broths for protease activity

In order to collect the extra cellular proteases secreted by different fungal cultures, the fungus were grown on potato dextrose broth with gelatin. After 72 h of growth the PD broth containing fungal mycelium was filtered to remove fungal spores and the mycelium. All the 5 fungal broth were filtered with the help of syringe filter using Muktell filter paper (Germany). The filtrate was collected in microcentrifuge tubes and kept at 4° C until used. The supematants of these filtrates were used as the enzyme source.

Estimation of total protein

The protein content of each fraction was estimated using the method of Lowry's Method [9] by using Bovine Serum Albumin as standard.

Protease activity

For protease activity 1% casein solution prepared in 250 mM Tris/HCl buffer pH 8.5 was chosen as substrate. After centrifugation, 0.5 ml of supernatant was taken out and preceded for protein estimation. The absorbance was recorded and the protease activity of the broth was calculated using the standard curve of BSA. The protease activity of each fungal broth was assayed in the same way. The specific activity was expressed as the number of units of activity per mg of protein.

Partial Purification of enzyme

Ammonium sulfate precipitation

Solid ammonium sulfate was added to the crude extract to 30-70% saturation (Laemmli 1970). The precipitate was collected by centrifugation, dissolved in minimal volume of 250mM Tris-Hcl buffer (pH 8.5) and enzyme activity was estimated in each fraction.

Gel filtration Chromatography

Gel filtration chromatography was performed by using a 35 x 3 cm column made up of silica gel (60-120 mesh SRL chemicals, India). Silica gel was weighed and distilled water was added. Then it was allowed to swell up. Once swelled up, it was poured inside the column and allowed to settle down so that column can be formed. After preparing the column it was equilibrated with 100 ml of chilled buffer to maintain the pH of the column .When a thin layer of buffer appeared above the column,1 ml of sample was loaded and allowed to enter the column. The elution was started with 80 ml of chilled 250 mM Tris/HCl buffer pH 8.5 and 24 fractions each of 3 ml were collected. These fractions were then subjected to protein estimation and enzyme activity and protein content in each fraction was calculated using standard curve. Fractions were kept at 4°C until use.

Characterization of proteases from different fungal source

SDS- PAGE-(Sodium Dodecyl Polyacrylamide gel electrophoresis)

The fungal broth filtrate 35 µl was mixed with 15 µl of sample buffer. The sample buffer consisted of 625 mM Tris base, 2% SDS, 10% glycerol in 100 ml of distilled water and the pH adjusted to 6.8 with 1N HCl. To this buffer few crystals of bromophenol blue was added in order to locate the samples. The samples were centrifuged in a micro centrifuge for 10 min and the supernatant 50 µl was loaded on to the sample well in the gel. In the first well 20 µl of protein marker was also loaded.The gel run was stopped once the bromophenol dye reaches to the bottom of the gel.

Activity staining (Zymography)

After the run, the gel was removed from the gel cassette. The gel was preceded for activity staining according to . For this, the gel was placed in 1% casein solution for 30 min at 4°C without agitation. After 30 min, the gel with casein solution was placed on a platform shaker (Remi, India) for 30 min at RT. The casein solution is then discarded and the gel was washed with distilled water three times. After washing the gel was placed in 10% solution of TCA for 10 min on a shaker. The gel was washed with distilled water and stained for 30 min with Coomassie Brilliant Blue G-250 solution. For this 0.25% of Coomassie Brilliant Blue G-250 ethanol: acetic acid: water (%:1:4). The staining was done under constant stirring. The gel was washed with distilled water and destained with methanol: acetic acid: water (4.2:0.8:5) solution under constant stirring. After destaining, clear bands on a blue background represent protease activity.

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RESULTS AND DISCUSSION

Table 1: Protein content and enzyme activity in crude extracts of fungal spp.

S.NO	Sample	Crude extract		0-30% Ammonium sulfate saturation		30-50% Ammonium sulfate saturation		50-70% Ammonium sulfate saturation	
		Protein (µg)	Activity (µg/ml)	Protein (µg)	Activity (µg/ml)	Protein (µg)	Activity (µg/ml)	Protein (µg)	Activity (µg/ml)
1	*Fusarium sp.*	8.6	0.43	0.55	0.8	2.3	0.35	1.2	0.19
2	*Curvularia sp.*	7.6	0.38	0.86	0.13	1.17	0.17	1.17	0.17
3	*A.niger*	3.2	0.16	0.62	0.09	0.25	0.03	0.64	0.09
4	*Fumigatus sp.*	4.8	0.24	0.57	0.08	0.59	0.08	1.5	0.23
5	*Mucor sp.*	2.0	0.10	0.02	0.03	0.43	0.06	0.45	0.06

In the present study five fungal strains were identified and screened for protein content and activities of proteases. Crude extracts of all the five samples were subjected to “salting in” and 3 different fractions were collected at each step. Protein content and enzyme activity was checked in all the 3 fractions in each strain. **Table 1** summarizes the total protein content as well as the respective protease activity for tall the precipitated fractions for all five fungal cultures. Protease activities are shown in terms of amount of protein degraded per min (in terms of BSA).

In *Fusarium species* fractions 7-10 and 12-14 found to have the maximum protein content and also showed highest enzyme activity. Table 2 represents the gel filtration profile of *Fusarium sp.* which is further explained by Fig.1.

In *Curvularia sp.*, fractions 6-7, 10-14 and 15-17 showed maximum protein content and also the highest enzyme activity. These fractions were pooled together and headed for SDS-PAGE. Table 3 represents all gel filtration profile of *Curvularia sp.* and fig.2 shows it graphically.

Fumigatus sp. unlike the above two strains, showed different pattern in gel filtration profile. As seen by table 4 and fig.3 fractions 1-4 show considerable protein content and enzyme activities but these fractions couldn’t be used for further studies as it might contain some residues of the previous fractions which could be due to improper washing of the column before use. Rather fractions 11-13, 16-17 and 22-23 were subjected to further study.

According to the gel filtration profile of *A.niger.*, it was observed that fractions 7-9, 11-13 and 14-16 had maximum protein content and highest enzyme activity as shown by the fig.4 and table 5.

In *Mucor sp.,* fractions 7-8 and 19-21 showed maximum protein content and highest enzyme activity. Table 6 and fig.5 clarified the above fact and thus these fractions were pooled together and subjected to further studies.

SUMMARY AND CONCLUSION

All the five fungal strains showed good amount of extra cellular protease activity in terms of unit total protein content. By testing all the crude extracts for enzyme activity, *Fusarium sp.* was found to show the highest activity whereas *Mucor sp.* showed the lowest. However, the amount of broths was different after the growth and the inoculum size was also different, the protease activity of the crude extracts could not be correlated. However, once the broths were precipitated using the ammonium sulfate, and the precipitates were resuspended in known amount of buffer, the protease activities could be compared.

It was found that there was no correlation of elution patterns between the five fungal samples. Further, protease activities appeared in one or more fractions that were distant with each other. This shows the presence of more than one protease with a difference in their molecular weights.

This interpretation was strengthened by the findings of zymography, where more than one band appeared for all the given samples. Further, the pattern of bands differed from each other showing that no fungal strain was producing similar kinds of proteases in the given condition.

The study supports the notion that fungi can be a good source of extracellular enzymes especially proteases. However, further research is needed in this direction for the characterization of such proteases before such products can be used commercially.

Table 2: Gel filtration profile for *Fusarium sp.*

	Activity (mg of protein degraded min^{-1})	
Crude extract	0.43	
	Salting In:	
fraction 1:	0.08	
fraction 2:	0.35	
fraction 3:	0.19	
Gel filtration	*Protein (mgml^{-1})*	*Activity(mg of protein degraded min^{-1})*
fraction 1	0.06	0.002
fraction 2	0.09	0.004
fraction 3	0.09	0.003
fraction 4	0.11	0.001
fraction 5	0.11	0.005
fraction 6	0.11	0.002
fraction 7	0.16	0.006
fraction 8	0.18	0.007
fraction 9	0.22	0.003
fraction 10	0.22	0.017
fraction 11	0.2	0.017
fraction 12	0.18	0.013
fraction 13	0.16	0.001
fraction 14	0.13	0.003
fraction 15	0.09	0.003
fraction 16	0.13	0.006
fraction 17	0.09	0.003
fraction 18	0.13	0.004
fraction 19	0.11	0.002
fraction 20	0.06	0.002
fraction 21	0.06	0.003
fraction 22	0.09	0.005
fraction 23	0.06	0.001
fraction 24	0.06	0.002

Table 3: Gel filtration profile of *Curvularia sp.*

	Activity((mg of protein degraded min^{-1})	
Crude extract	0.38	
	Salting In	
fraction 1	0.13	
fraction 2	0.17	
fraction 3	0.18	
Gel filtration	*Protein (mgml^{-1})*	*Activity(mg of protein degraded min^{-1})*
fraction 1	0.16	0.001
fraction 2	0.25	0.004
fraction 3	0.16	0.003
fraction 4	0.41	0.005
fraction 5	0.13	0.003
fraction 6	0.27	0.01
fraction 7	0.22	0.04
fraction 8	0.18	0.006
fraction 9	0.2	0.003
fraction 10	0.27	0.06
fraction 11	0.29	0.06
fraction 12	0.25	0.02
fraction 13	0.25	0.01
fraction 14	0.41	0.01
fraction 15	0.27	0.05
fraction 16	0.34	0.03
fraction 17	0.18	0.03
fraction 18	0.16	0.003
fraction 19	0.2	0.003
fraction 20	0.18	0.002
fraction 21	0.2	0.001
fraction 22	0.13	0.004
fraction 23	0.25	0.002
fraction 24	0.13	0.001

Table 4: Gel filtration profile of *Fumigatus sp.*

	Activity(mg of protein degraded min^{-1})	
Crude extract	0.24	
	Salting In	
fraction 1:	0.08	
fraction 2:	0.08	
fraction 3:	0.23	
Gel filtration	*Protein ($mgml^{-1}$)*	*Activity(mg of protein degraded min^{-1})*
fraction 1	0.91	0.002
fraction 2	0.68	0.003
fraction 3	0.43	0.004
fraction 4	0.43	0.005
fraction 5	0.27	0.001
fraction 6	0.13	0.009
fraction 7	0.18	0.003
fraction 8	0.11	0.001
fraction 9	0.11	0.002
fraction 10	0.27	0.008
fraction 11	0.36	0.003
fraction 12	0.43	0.05
fraction 13	0.41	0.002
fraction 14	0.18	0.003
fraction 15	0.18	0.004
fraction 16	0.25	0.005
fraction 17	0.29	0.005
fraction 18	0.2	0.002
fraction 19	0.45	0.007
fraction 20	0.13	0.001
fraction 21	0.13	0.002
fraction 22	0.32	0.07
fraction 23	0.32	0.004
fraction 24	0.16	0.003

Table 5: Gel filtration profile of *A.niger*

	Activity(mg of protein degraded min^{-1})	
Crude extract	0.17	
	Salting In:	
fraction 1:	0.09	
fraction 2:	0.03	
fraction 3:	0.09	
Gel filtration	*Protein ($mgml^{-1}$)*	*Activity(mg of protein degraded min^{-1})*
fraction 1	0.52	0.003
fraction 2	0.2	0.005
fraction 3	0.29	0.002
fraction 4	0.29	0.001
fraction 5	0.41	0.004
fraction 6	0.41	0.002
fraction 7	0.45	0.003
fraction 8	0.5	0.04
fraction 9	0.8	0.001
fraction 10	0.41	0.002
fraction 11	0.59	0.004
fraction 12	0.5	0.04
fraction 13	0.41	0.005
fraction 14	0.45	0.004
fraction 15	0.64	0.002
fraction 16	0.41	0.003
fraction 17	0.55	0.001
fraction 18	0.48	0.005
fraction 19	0.45	0.007
fraction 20	0.52	0.003
fraction 21	0.48	0.002
fraction 22	0.59	0.001
fraction 23	0.45	0.004
fraction 24	0.36	0.002

Table 6: Gel filtration profile of *Mucor sp.*

	Activity(mg of protein degraded min^{-1})	
Crude extract	0.1	
	Salting In:	
fraction 1:	0.03	
fraction 2:	0.06	
fraction 3:	0.06	
Gel filtration	*Protein ($mgml^{-1}$)*	*Activity(mg of protein degraded min^{-1})*
fraction 1	0.11	0.002
fraction 2	0.09	0.002
fraction 3	0.13	0.001
fraction 4	0.09	0.003
fraction 5	0.11	0.004
fraction 6	0.11	0.005
fraction 7	0.18	0.002
fraction 8	0.16	0.001
fraction 9	0.16	0.03
fraction 10	0.18	0.005
fraction 11	0.18	0.001
fraction 12	0.11	0.002
fraction 13	0.16	0.006
fraction 14	0.18	0.003
fraction 15	0.13	0.001
fraction 16	0.11	0.003
fraction 17	0.11	0.005
fraction 18	0.11	0.007
fraction 19	0.22	0.003
fraction 20	0.25	0.1
fraction 21	0.13	0.003
fraction 22	0.09	0.002
fraction 23	0.16	0.003
fraction 24	0.13	0.001

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Figure 1: Gel filtration profile of *Fusarium sp*

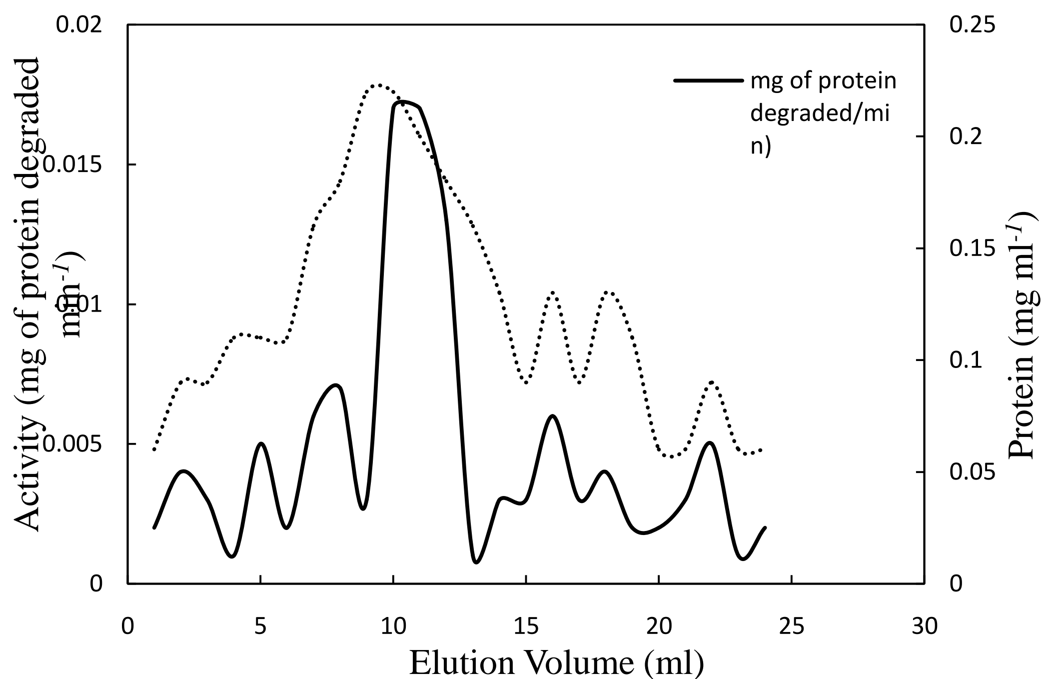

Figure 2: Gel filtration profile of *Curvularia sp.*

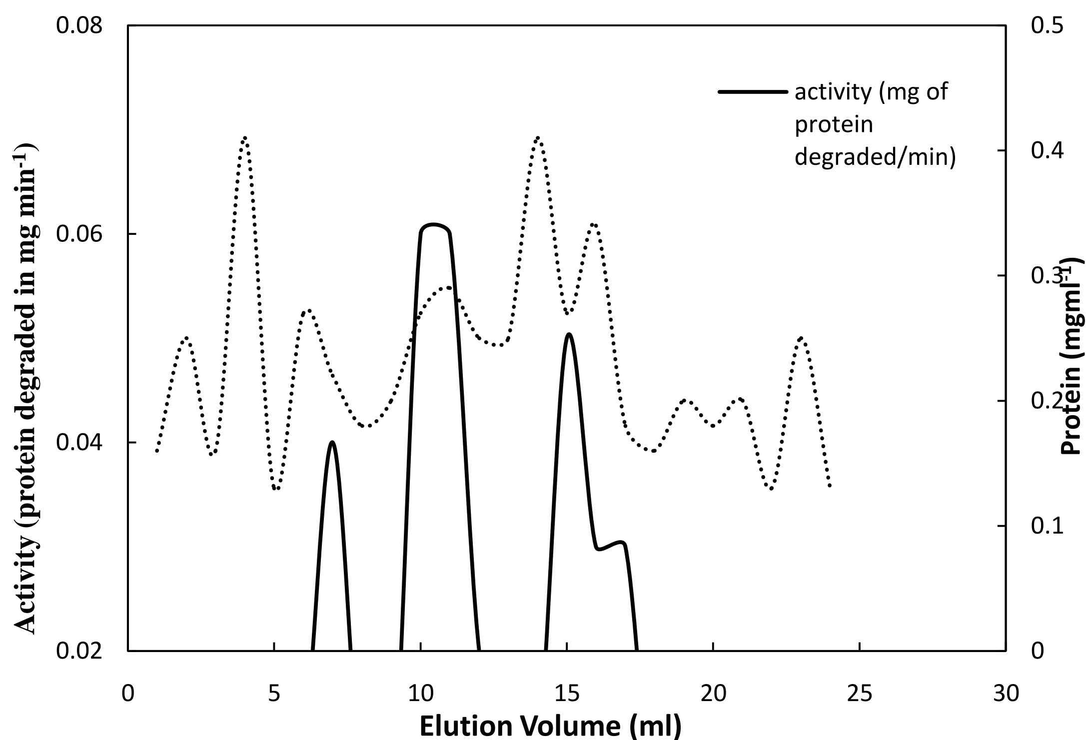

Figure 3: Gel filtration profile of *Fumigatus sp*

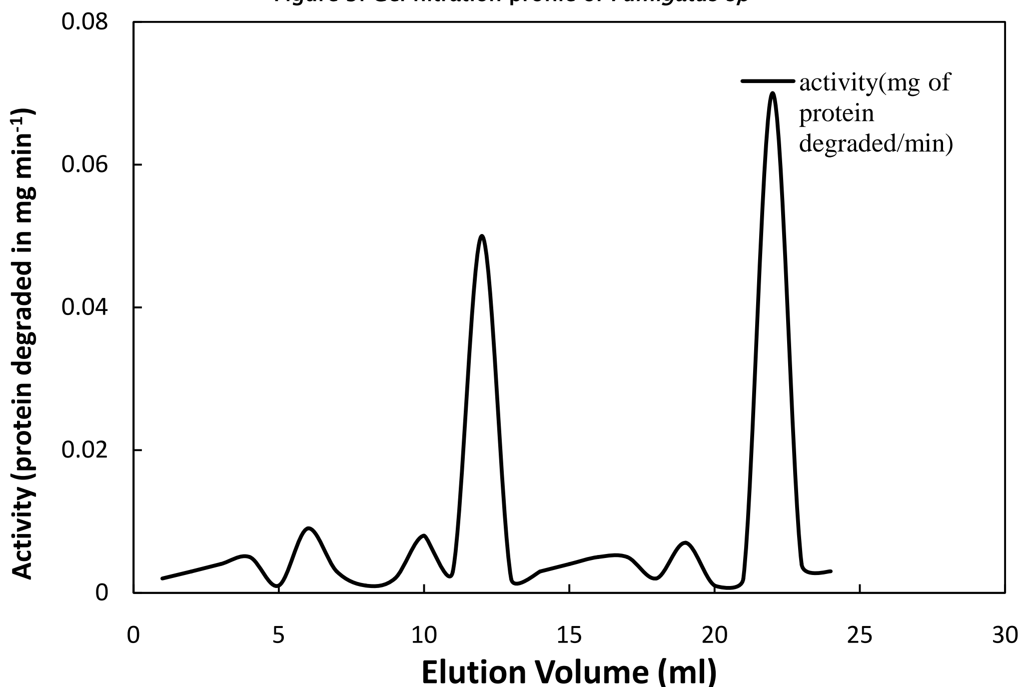

Figure 4: Gel filtration profile of *A.niger*

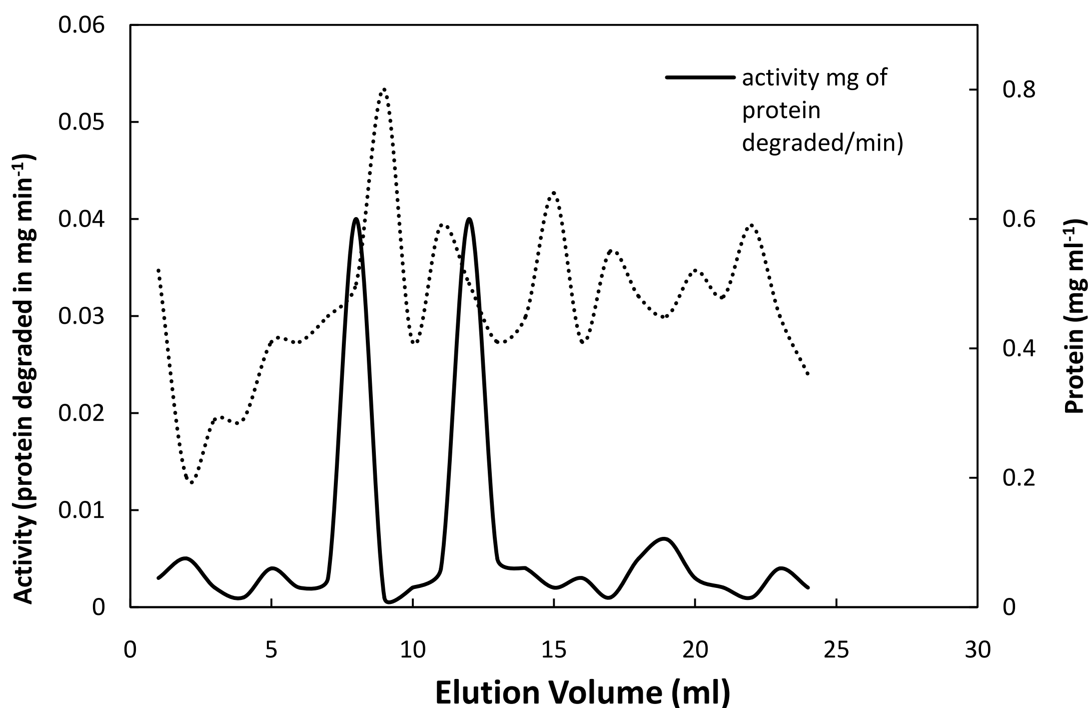

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Figure 5: Gel filtration profile of *Mucor sp*

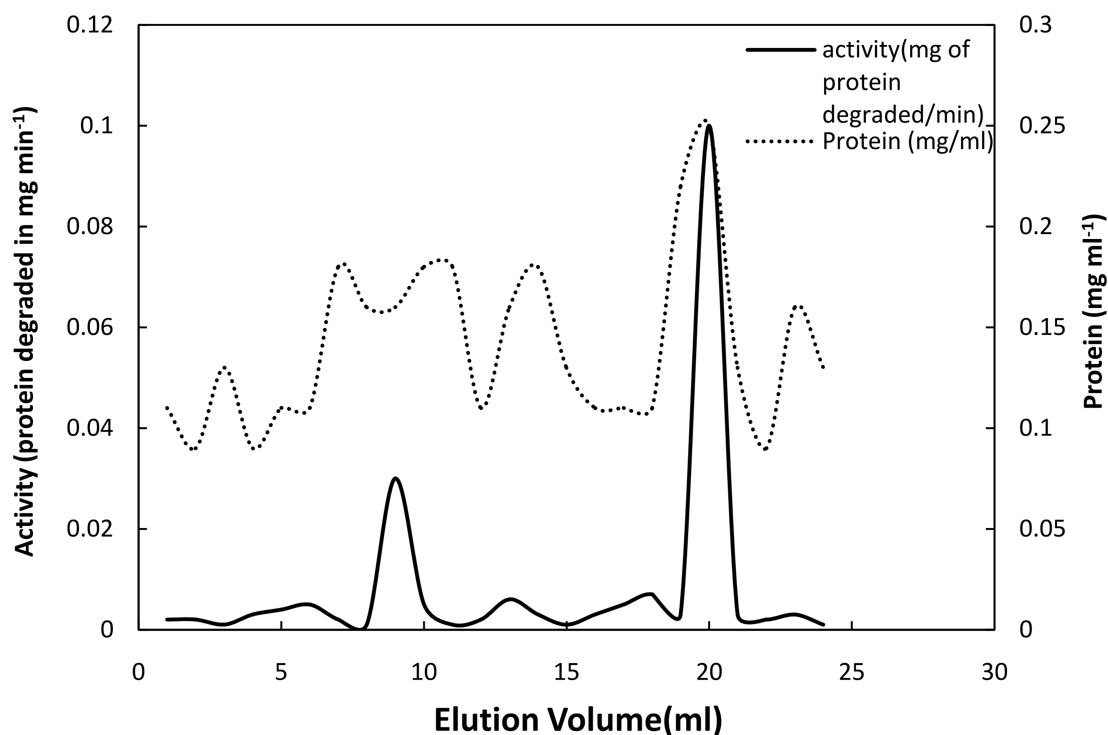

Figure 6: Zymography of proteases

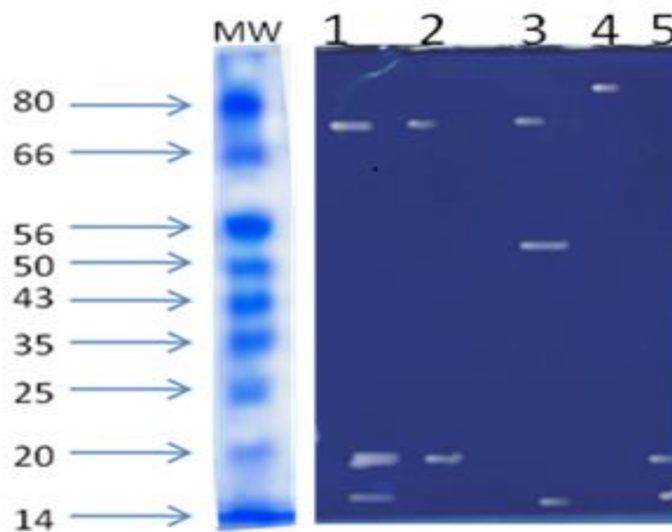

Fig.6 shows the activity staining of selected fungal cultures. Clear zones indicate the proteolytic activity. The molecular weights were compared with protein markers, loaded on the same concentration of gel. The lanes are-

1. *Fusarium sp.*
2. *Fumigatus sp*
3. *Curvularia sp.*
4. *Mucor sp.*
5. *Aspergillus niger*

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