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Partial characterization of proteases produced by three fungal isolates from the rhizosphere of wild yam *Dioscorea wallichii*

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Abstract

Among the twenty different fungal members isolated from the rhizosphere of wild yam, *Dioscorea wallichii*, three fungal isolates were found to be the best protease producers and identified as *Aspergillus* sp., *Penicillium* sp. and *Trichoderma* sp. The protease production studies of these three isolates were conducted up to 12 days. After nine days there was no significant increase in the enzyme production. Maximum protease activity was observed in the range of pH 6 and temperature 30˚C. Protease from all the three isolates was stable up to 2 h in dettol, tween 20, tween 80 and soap solution. Among the different immobilization techniques used agar block was the most effective. The molecular weight, zymogram analysis and destaining activity of the protease enzyme were also studied.

Key words: Destaining activity, *Dioscorea wallichii*, protease, zymogram

INTRODUCTION

Proteases (EC 3.4.21-24) are enzymes capable of hydrolyzing the peptide bonds between amino acids of proteins and constitute one of the most important groups of industrial enzymes occupying nearly 60% of the enzyme sales [1]. Proteases take a pivotal position with regard to their physiological roles as well as their commercial applications. Since they are physiologically necessary for living organisms, it occurs in a wide diversity of sources such as plants, animals and microbes [2]. The inability of plant and animal proteases to meet current world demand has led to an increased interest in microbial proteases. Microorganisms are the preferred source of proteases owing to their broad biochemical diversity and their susceptibility to genetic manipulation.

Among microbes, fungi as enzyme producers have many advantages, since they are safe and the produced enzymes are extra cellular which makes its easy recovery from fermentation broth [3]. Fungal proteases have a large variety of applications, mainly in the detergent and food industries and also in leather, dairy, baking and pharmaceutical industries. They are important tools in studying the structure of proteins and polypeptides [2, 4]. Because of the potential applications of protease enzyme, the search for new native protease producer is a continuous need. Proteases have been produced in submerged and solid-state fermentations. Each organism has its optimum conditions for maximum enzyme production. Hence, optimization of fermentation conditions is essential for each strain. In this study protease producing indigenous fungal members was isolated from the rhizosphere soil of Dioscorea wallichii, which contains large amount of microorganisms because of the root exudates. The protease production conditions were standardized and the enzyme was partially purified and characterized.

MATERIALS AND METHODS

Culture collection

Twenty fungal members isolated from rhizosphere soil of wild yam *D. wallichii* were collected from microbial culture collection center of Central Tuber Crops Research Institute, Kerala, India. All the fungal cultures were plated on the prepared gelatin agar plates. After incubation at 28˚C for 5 days, the plates were flooded with 15% HgCl2 to screen the protease producing organisms [5]. For the identification of selected isolates different morphological and cultural characteristics were observed and compared with the standard description of a manual of fungi [6]. The screened protease producing fungal cultures were preserved on potato dextrose agar slants.

Protease production

For large scale culturing the screened isolates were inoculated into modified gelatin broth (1.7mM magnesium sulfate, 9.4mM ammonium chloride, 68µM calcium chloride, 6.3 µM ferric chloride, 0.15 µM manganese chloride, 0.124 µM sodium molybdate, 42.8 mM sodium chloride, 27.6 mM potassium phosphate, 3% gelatin, pH 5.6) in Erlenmeyer flasks and incubated at 37˚C up to12 days for protease production.

Partial purification

After the incubation period the broth was filtered by using Whatmann No.1 filter paper to remove the fungal mycelium. To the cell free extract ammonium sulphate was added, stirred continuously by keeping it in ice, until the saturation point is reached. It was then centrifuged at 10,000 rpm for 10 min. The pelleted enzyme was then washed with phosphate buffer (pH 7.2) and was stored at 4˚C [7]. For further purification the protease enzyme fraction was dialyzed against phosphate buffer [8].

Enzyme assay

The protease activity was assayed using 0.5% (w/v) casein in carbonate bicarbonate buffer (pH 10.5) as a substrate. 1 ml of casein and 0.5 ml of enzyme was incubated at 45˚C for 30 min. The reaction was stopped by the addition of 2 ml of 10% trichloro acetic acid. After mixing on a vortex mixture, the tubes were centrifuged at 3000 rpm for 10 min [9]. The tyrosin released was estimated by Lowry's method using a tyrosine standard curve. A unit of enzyme activity was defined as the amount of enzyme that releases 1µg of tyrosine/min/ml under the assay conditions.

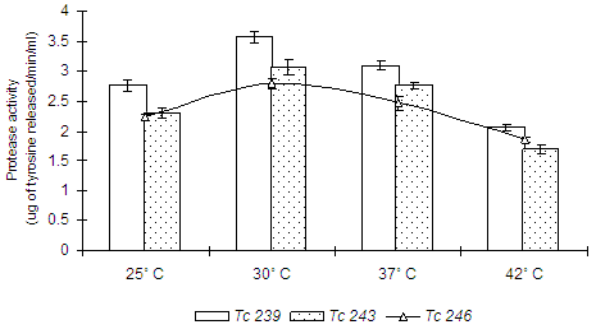

Fig. 1. Protease production by Tc 239, Tc 243 and Tc 246 at different incubation periods.

Optimization of fermentation conditions

An attempt was made to optimize the culture conditions such as temperature, pH and incubation period for maximum protease production.

Effect of incubation time

To determine the optimum incubation period for protease production the cultures were incubated in modified gelatin broth up to 12 days. A portion of media was taken aseptically on 3, 6, 9 and 12th day of incubation and analyzed for protease production

Effect of pH

To standardize the optimum pH, experiments were conducted at different pH ranging from pH 4, 5, 6, 7, 8 and 9 up to the optimum incubation period and the protease activity was assayed.

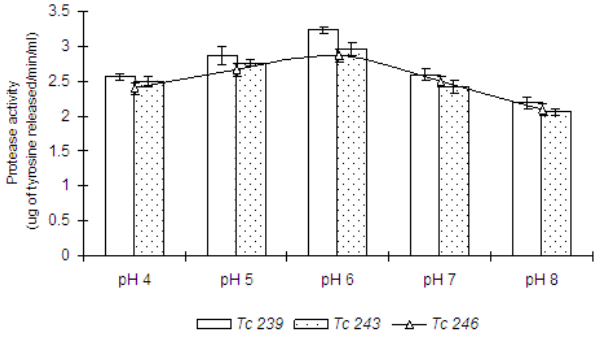

Fig. 2. Influence of initial pH on the protease production by Tc 239, Tc 243 and Tc 246.

Effect of temperature

The culture medium was incubated at 25, 30, 37 and 42°C at optimum pH and incubation time to find out the optimum temperature requirement for protease production. The effect of carbohydrates on protease production was tested by adding 10% glucose, sucrose and lactose to the production medium. The effect of surfactants was tested by the addition of 10% of tween 20 and triton x – 100.

Determination of molecular weight

The molecular weight of the protease enzyme of isolates was determined by SDS-PAGE [10] using 10% acrylamide and the zymogram analysis was carried out [11].

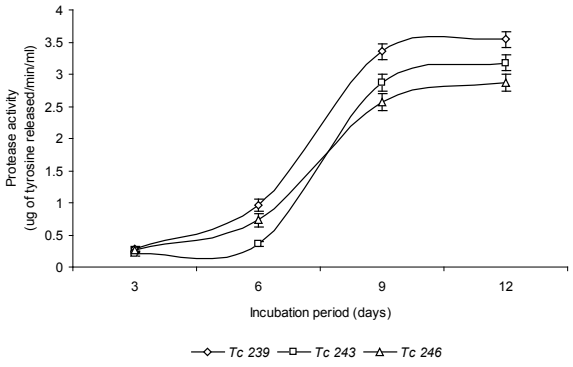

Fig. 3. Effect of temperature on protease production by Tc 239, Tc 243 and Tc 246.

Immobilization

The partially purified protease enzyme was immobilized in a variety of materials such as sodium alginate (3%), talc alginate (3%), clay and alginate (3%) and agar blacks (2%). The immobilized enzyme activity was assayed up to 2h.

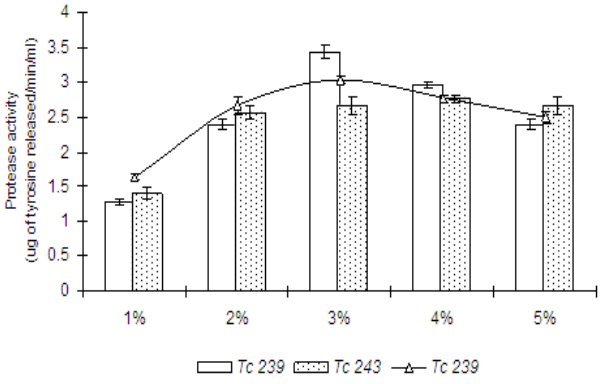

Fig.4. Influence of substrate concentrations on protease production by Tc 239, Tc 243 and Tc 246

Destaining activity

To ensure the destaining capacity of the protease enzyme 2.5 ml of enzyme was added to a mixture of 25

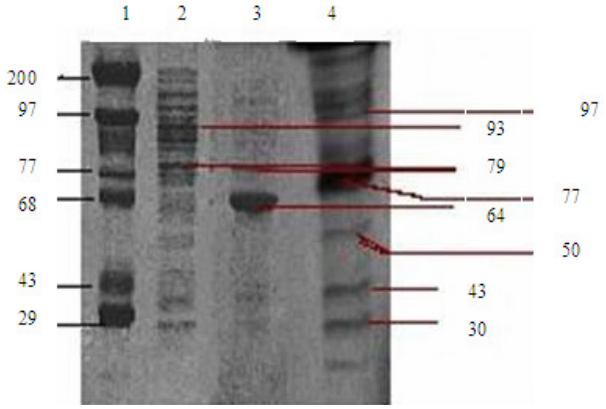

Fig.5. SDS-PAGE of partially purified proteases from Tc 239 (lane 4), Tc 243 (lane 3) and Tc 246 (lane 2).

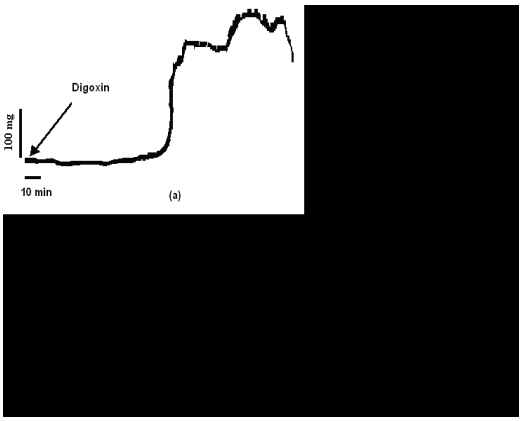

Fig. 6. Clear zone in gelatin zymography corresponding to the protease enzymes produced by Tc239, Tc243 and Tc246.

ml of distilled water and 25 ml of soap solution. The egg stained and blood stained cloths were immersed in these solutions. One piece of cloth was immersed in soap and water mixture without enzyme, as a control. It was then incubated for 2 h and washed with tap water. The cloths were visually examined for the effect of protease enzyme in removal of stains.

RESULTS AND DISCUSSION

All the twenty fungal isolates from rhizosphere soil of *D. wallichii* were grown in gelatin agar media and compared for the protease production by adding mercuric chloride test reagent [12]. The clear zone around the colonies indicated protease production. Out of the twenty fungal isolates only twelve were found to be positive for protease production. Among the protease producers three fungal isolates (Tc 246, Tc 243 and Tc 239) were selected as efficient producers because of a clear zone with large diameter around them. These fungal isolates were stained with lacto phenol cotton blue and identified as *Aspergillus* sp., *Penicillium* sp. and *Trichoderma* sp. respectively.

Effect of incubation time

Tc 246, Tc 243 and Tc 239 were inoculated into modified gelatin broth and their protease activity was estimated at 3, 6, 9 and 12th day of incubation. During the time course study, protease activity was increased continuously in the culture supernatant from the third day till the twelfth day (Fig. 1). Among the three organisms Tc 239 showed maximum protease activity followed by Tc 246 and Tc 243. After 9 days the increase in protease activity was insignificant. This is probably due to limited availability or depletion of available nutrient sources. Maximum protease production was observed for *A. oryzae* at 72 h [3]. However, for *Streptomyces* sp. and [13] *A. fumiculosus* maximum production was observed after 5 days of incubation [5].

Influence of pH

The pH of the surrounding environment strongly affects any enzymatic processes and transport of various components across the cell membrane [14]. Production of protease was greatly influenced by difference in initial pH of the medium. Experiments were conducted with gelatin media of different pH and incubated for nine days. Enzyme production increased gradually with increase in pH from 4 to 6. Maximum production was observed at pH 6 for Tc 246, Tc 243 and Tc 239 (Fig. 2). But protease production decreased in pH above 6. It is reported that in *A. oryzae* maximum protease production was observed at pH 7.5 [3]. The optimum pH for protease activity by *A. nidulans* HA-10 was found at 8 but the enzyme was more stable at pH 6-10 for 1h [15]. However, for *A. tamari* the highest protease activity was observed between pH 6 and 10 [2].

Effect of temperature

To ascertain the optimum incubation temperature for maximum production of protease the three isolates were incubated at 25, 30, 37 and 42˚C for 9 days. The optimum temperature for protease production was 30˚C for all the three isolates (Fig. 3). Maximum protease activity was exhibited by Tc 239 followed by Tc 243 and Tc 246. The optimum temperature for protease production by A. oryzae is 30˚C instead of is [3]. But it is also reported that 35˚C was the optimum temperature for *A. tamari* [15] and slightly higher temperature for *A. terreus* and *A. fumigatus* [16,17].

Influence of substrates and surfactants

3% gelatin concentration was found to be optimum for all the three isolates (Fig. 4). The addition of surfactants such as tween 20 and triton x-100 caused reduction in protease yield at about 80% and 75% respectively. The addition of glucose, sucrose and lactose resulted in strong inhibition of protease synthesis (data not shown). The decreases in protease production at higher concentration of carbohydrates suggest that, at least in part, the protease synthesis is regulated by carbon catabolic suppression. [18, 19].

Zymogram analysis

Only one band of clearing was visible in the gelatin zymogram for the three organisms after the incubation

Table 1. Stability of protease enzymes of Tc 239, Tc 243 and Tc 246 in dettol, soap solution, tween 20 and tween 80 after 2 h of incubation.

		Tc 239	Tc 243	Tc 246
Dettol	0h	2.277± 0.033	1.952 ± 0.027	2.067 ± 0.047
	1h	2.243± 0.042	1.937 ± 0.016	2.032 ± 0.093
	2h	2.199 ± 0.015	1.942 ± 0.015	2.072± 0.051
Soap	0h	2.063 ± 0.097	2.103 ± 0.074	2.057 ± 0.123
	1h	1.99 ± 0.012	2.02 ± 0.107	1.993 ± 0.073
	2h	1.957 ± 0.042	2.01 ± 0.093	1.925 ± 0.114
Tween 20	0h	2.266 ± 0.009	1.97 ± 0.022	1.84 ± 0.016
	1h	2.187± 0.012	1.973 ± 0.012	1.827 ± 0.033
	2h	2.177 ± 0.012	1.956 ± 1.956	1.82 ± 0.032
Tween 80	0h	1.902 ± 0.006	2.38 ± 0.016	1.947 ± 0.029
	1h	1.857 ± 0.017	2.3 ± 0.081	1.93 ± 0.008
	2h	1.897 ± 0.025	2.326 ± 0.044	1.904 ± 0.014

period. The molecular weight of the corresponding protease band in SDS-PAGE was found to be 93 KDa for Tc 246, 64 KDa for Tc243 and 77 KDa for Tc 239 (Fig. 5 & 6). For *A. tamari* [2] and *A. nidulans* HA-10 [16] one major band was obtained on zymogram. Alkaline proteases with different molecular weight were obtained from other fungal species such as 124 KDa serine proteases from *A. fumigatus* [17], 33KDa from *A. fumigatus* [20], 35 KDa from *A. clavatus* [21], 37KDa from *A. terreus* [16] and 23KDa from *A. parasiticus* [22].

Stability of protease

The protease enzyme showed good stability and compatibility in dettol, soap solution, tween 20 and tween 80 and retained its activity up to 2h in these solutions (Table 1). Protease produced by Tc239 was more active and stable in dettol and tween 20 up to 2h. But in tween 80 and soap solution protease produced by Tc 243 was more stable than the others. But overall, the protease of all the three isolates retains their maximum activity in these solutions up to 2 h. It is already reported that protease showed decreased activity in some of the commercially available detergent powders such as 16% activity in Revel, 11.4% activity in Aerial and 6.6% Activity in Wheel [23]. Compared with these results, the protease enzymes of this study was more stable.

Immobilization

The enzyme was immobilized on sodium alginate, talc and alginate mixture, clay and alginate mixture and on agar blocks. The activity of immobilized enzyme was little bit less when compared to the free enzyme but stable till the second hour of incubation with substrate (Fig. 7). Among the different immobilization techniques, agar block technique was found to be the best closely followed by clay alginate mixture. The percent-entrapped activity was high at 3% sodium alginate concentration. The enzyme lost its activity in higher or lower concentration of alginate. But contradictory results were also reported. The amount of entrapped activity was comparatively low at 2.5-3.5 % (w/v) sodium alginate concentration [24].

Destaining activity

The supplementation of the protease in detergent improved the cleansing of blood and egg stains. After 2 h of incubation of egg stained and blood stained cloths in soap solution along with enzyme, stain on the cloths were completely removed on washing with tap water. The stain was not completely removed on washing from the cloths dipped in soap solution without enzyme [8]. This shows the contribution of the enzyme in improving the washing performance of the detergent. The supplementation of the enzyme preparation in detergent could considerably improve the cleansing of the egg and blood stains.

CONCLUSION

The proteases isolated from *Aspergillus* sp., *Penicillium* sp. and *Trichoderma* sp. of rhizosphere of wild yam *Dioscorea wallichii* have almost similar characteristics. This might be due to their same habitat which influences these organisms in a similar way. The optimum incubation for protease production is 9 days,

temperature 30˚C, pH 6 and gelatin concentration in the medium is 3%. These protease enzymes showed good stability and compatibility in the presence of soap solution, tween 20, tween 80 and dettol. In the presence of commercial detergents its cleansing property indicates the possibilities to utilize these proteases in the detergent industry. This enzyme can be exploited commercially.

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