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Studies on Extra Cellular Enzyme Keratinase from Dermatophyte *Microsporum gypseum*

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Abstract: Keratinases (E.C. 3.4.4.25) are of particular interest because of their action on insoluble keratin substrates and generally on a broad range of protein substrates. The objective of this study is to isolation, screening, purification and determination of the enzymatic activity of extracellular keratinase from dermatophyte *Microsporum gypseum*. The study clearly indicates the presence of the enzyme keratinases in the dermatophyte *Microsporum gypseum*. One milliliter of the purified sample contain 80 μg of protein, 1.09 μmole mL^{-1} 60 min enzyme activity and 13.6 μ mole mg^{-1} 60 min specific activity with respect to the unpurified one. The purified and unpurified state of the enzyme was judged by SDS/PAGE. Purified enzyme showed a single band of molecular weight of 33 kDa. Characterization studies showed optimum activity at pH 8 and at 35°C. The enzyme kinetics increased with increased concentration of $MgCl_2$ and decreased with increased concentration of $ZnCl_2$. Maximum biomass and keratinase activity were observed from pH 7.0 to 9.0, which agrees with those described for most feather-degrading *Bacillus*. In this study, the optimum conditions for keratinase synthesis by the *Microsporum gypseum* were determined, which will be an essential step for the production of adequate amounts for application in research field and other areas.

Key words: *Microsporum gypseum*, keratinases, Folin Ciocalteau method

INTRODUCTION

Dermatophytes are mycelial and keratinophylic fungi of the mold group, originally saprobial, but have adapted themselves to animal and human parasitism through evolution. *Microsporum gypseum* is a geophilic dermatophyte, frequently transmitted by fungal spores to man-soil contact in rural areas. It grows relatively rapidly and matures in 6 to 10 days. The texture of the colony is powdery to granular and the color is beige to cinnamon brown. From the reverse, it is yellow to brownish red. It produces septate hyphae, macroconidia and microconidia. Macroconidia are abundant, fusiform and symmetrical in shape with rounded ends. The walls of macroconidia are thin and rough and they contain 3-6 cells. Microconidia are moderately numerous in number, club-shaped and located along the hyphae. *Microsporum* usually produces a single inflammatory skin or scalp lesion. Invaded hairs show an ectothrix infection but do not fluoresce under Wood's ultra-violet light. It had been reported as a etiological agent of dermatophytosis in AIDS patients (Giudice *et al*., 1997). Several enzymes produced by dermatophytes, particularly keratinases, are considered to play a role in the virulence of the fungus.

Proteolytic enzymes are largely used in the industry for biotechnological applications involving the hydrolysis of protein substrates. Proteases constitute an important fraction of the global enzyme sales and a relevant part of this market is accounted by bacterial proteases (Rao *et al*., 1998). Bacterial

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keratinases (E.C. 3.4.4.25) are of particular interest because of their action on insoluble keratin substrates and generally on a broad range of protein substrates (Lin *et al*., 1995). These enzymes have been studied for de-hairing processes in the leather industry (Raju *et al*., 1996). Cultivation conditions are essential in successful production of an enzyme and optimization of parameters such as pH, temperature and media composition is important in developing the cultivation process. Despite all the work that has been done on production of proteolytic enzymes, relatively little information is available on keratinases (Wang and Shih, 1999). Keratinolytic fungi are able to attack and destroy keratin, whereas keratinophilic fungi accompany those utilizing only non-keratinous components of keratinous substrata or the products of keratin decomposition. Vanbreuseghem's hair baiting method has been mainly used for examination of keratinolytic and keratinophilic fungi in sewage sludge and other organic waste and waste-contaminated habitats.

The objective of this study was to isolation, screening, purification and determination of the enzymatic activity of extracellular keratinase from dermatophyte *Microsporum gypseum*.

MATERIALS AND METHODS

Collection of Samples

Fifty protein rich soil samples with sandy texture and grey to black in colour were collected from the different part of Bangalore, India during January 2006. The research was carried out at Integrated Biotechnology Extension (IBX) Lab, Bangalore, India

Isolation, Screening, Selection and Identification of Keratinases Producing Bacteria (Vanbreuseghem's Hair Baiting Method)

The isolation of keratinases producing fungi was carried out using hair bait technique. Half filled sterile petriplates with soil samples were taken. Short (2-3 cm)strands of steriliged defatted human hair was spreaded over surface of the soil. Fifteen milliliter of sterile water was added to the soil to facilitate germination of fungal spores. Antibiotic streptromycin (40 mg L^{-1}) was added to prevent bacterial growth. These complete set was incubated at room temperature in a dark for 4 weeks. Inoculum from hairs with fungus growth were taken and placed on plate of Sabouraud's dextrose agar and incubated at 25°C for 7 days. After performing the microscopy, biochemicals, bromocresol purple test, hair perforation test the organism was confirmed as *Microsporum gypseum*.

Mass Production and Extraction of Enzyme

Mass production of enzyme was done by submerged fermentation, *Microsporum gypseum* was inoculated into the medium containing human hair (2.6 g L^{-1}) and incubated at 25°C for 5 days and then in shaker for 7 days. At the end of the growth period, the mycelium and residual hair were removed from the culture fluid by filtration. The filtrate was taken out and precipitated with ammonium sulphate.

Purification of the Enzyme

The purification was carried out by dialysis and ion-exchange chromatography.

Estimation of Total Protein by Lowry's Method

Different dilutions of Bovine Serum Albumin (BSA) solutions were prepared by mixing stock BSA solution and water in the test tube. The final volume in each of the test tubes is 1 mL. The BSA range is 0 to 200 μg mL^{-1}. Five milliliter of alkaline copper sulphate reagent (analytical reagent) was added and mixed well. This solution was incubated at room temperature for 10 min. Then 0.5 mL of reagent Folin Ciocalteau solution (reagent solutions) was added to each tube and incubated for 30 min

in dark. The absorbance was taken in the spectrophotometer at 660 nm using blank. The absorbance against protein concentration was plotted to get a standard calibration curve. The absorbance of purified and unpurified sample was taken and the concentration of the unknown sample was calculated.

Determination of Activity of Keratinases by Folin Ciocalteau (FC) Method

The standard solution of amino acid, tyrosine was dispersed in a series of test tubes in the range of 0 to 2 mL and the volume of each tube was made up to 2 mL with water and 6 mL of alkaline copper sulphate reagent was added and incubated at room temperature for 15 min. 0.5 mL of Folin Ciocalteau solution was added and incubated at room temperature for 30 min. The optical density was taken using 660 nm filter using spectrophotometer. Fifty milligram of substrate (hair), 6 mL of 28 mM phosphate buffer, 1 mL of enzyme sample was incubated at room temperature. 2.0 mL of 10% TCA was added to stop the reaction. Centrifuged at 3000 rpm for 10 min. 1.0 mL of supernatant, 2.5 mL of 0.5 N NaOH and 0.5 mL of Folin Ciocalteau solution were added and incubated for 30 minutes at room temperature. Amino acids released were estimated by taking optical density at 660 nm. the control was prepared without incubation. The absorbance against tyrosine concentration was plotted to get a standard calibration curve. The absorbance of purified and unpurified sample was taken and the concentration of the unknown sample was calculated.

Determination of Keratinases Activity

The effect of pH, temperature, activator ($MgCl_2$), inhibitor ($ZnCl_2$) was also studied.

Calculation

Enzyme Activity

Micro molar of PNP Liberated/Molecular weight of PNP (139)

$\mu mole\ mL^{-1}$ 60 min at 37°C

Specific Activity

Enzyme activity/Protein concentration

$\mu mole\ mg^{-1}$ 60 min at 37°C

RESULTS

Table 1 shows the protein concentration, enzyme activity and the specific activity of the 1 mL of sample at 37°C.

From the obtained result the total protein content of the purified enzyme was 1.33 fold. The enzyme activity was 2 fold and the specific activity was 1.49 fold.

The charecterisation studies were carried out with effect of pH, temperature, activator ($MgCl_2$), inhibitor ($ZnCl_2$) and tabulated in Table 2.

Maximum enzyme activity and enzyme yields were observed at temperature range 25-45°C and pH ranging from 7.0 to 9.0. Maximum enzyme activity was obtained with more alkaline pH values (Table 2). Although the production pattern of keratinase was similar for the different pH values tested, lower activities were observed at pH 5.0 and 15°C.

Table 1: Total protein concentration, enzyme activity and the specific activity of keratinases at 37°C

Sample	Total protein (μg)	Enzyme activity ($\mu mole\ mL^{-1}$ 60 min^{-1})	Specific activity ($\mu mole\ mg^{-1}$ 60 min^{-1})
Unpurified	60	0.545	9.09
Purified	80	1.090	13.60

Table 2: Characterization of keratinases

Characters	Minimum value	Optimum value	Maximum value
pH	7	8	9
Temperature	25°C	35°C	45°C
Activator ($MgCl_2$)	Enzyme activity increases		
Inhibitor ($ZnCl_2$)	Enzyme activity decreases		

DISCUSSION

The study clearly indicates the presence of the enzyme keratinases in the dermatophyte *Microsporum gypseum*. One milliliter of the purified sample contain 80 μg of protein, 1.09 μ mole mL^{-1} 60 min enzyme activity and 13.6 μ mole mg^{-1} 60 min specific activity which was higher with respect to the unpurified one. The purified and unpurified state of the enzyme was judged by SDS/PAGE. Purified enzyme showed a single band of molecular weight of 33 kDa. Characterization studies showed optimum activity at alkaline pH and at 35°C. The enzyme kinetics increased with increased concentration of $MgCl_2$ and decreased with increased concentration of $ZnCl_2$.

Maximum biomass and keratinase activity were observed from pH 7.0 to 9.0, which agrees with those described for most feather-degrading *Bacillus* (Wang and Shih, 1999; Suntornsuk and Sutornsuk, 2003). For production of keratinase by *B. licheniformis* and a recombinant *B. subtilis*, uncontrolled pH operation was more favorable than the controlled pH operations (Wang and Shih, 1999). The same was observed for alkaline protease production by *B. licheniformis* (Çalik *et al*., 2002).

It is thought that the abundance of keratinous debris favors the growth of keratinolytic and keratinophilic fungi in sludge, *Microsporum gypseum* in particular. A keratinase was produced by *Microsporum gypseum* under different growth conditions. This enzyme has been shown to be useful for biotechnological purposes such as hydrolysis of poultry feathers (Riffel *et al*., 2003a) and de-hairing of bovine pelts (Riffel *et al*., 2003b).

Although the production of proteases in complex growth media often promotes exuberant growth and high enzyme yields (Johnvesly and Naik, 2001; Joo *et al*., 2002), their expensive cost makes them unsuitable for a large-scale production. It seems more adequate to use raw materials like some wastes from the food industry as a basis of the culture.

The recent finding that *B. licheniformis* PWD-1 keratinase cause enzymatic breakdown of prion protein PrP^{Sc} (Langeveld *et al*., 2003) leave open a novel relevant application for broad range of keratinase. In this study, the optimum conditions for keratinase synthesis by the *Microsporum gypseum* were determined, which is an essential step for the production of adequate amounts for application in research field and other industrial areas.

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