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Partial Purification And Characterization Of An Extracellular Protease From *Hypocrea Virens And Trichoderma Koenigii*

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ABSTRACT

The aim of this experimental study was to isolate and partially purify extracellular protease from *Hypocrea virens and Trichoderma koenigii*. The species were inoculated in a protease fermentation medium. The supernatants were collected after 92 hours. The partial purification was realized by applying respectively, ammonium sulfate precipitation, dialysis and DEAE-Cellulose ion exchange chromatography to the supernatant. Effect of pH and temperature on enzyme activity and stability were determined. In addition, the molecular mass of the obtained enzyme was investigated by Sodium Dodecyl Sulphate-Polyacrylamide gel elecrophoresis (SDS-PAGE). The specific activity of partially purified enzyme from *Hypocrea virens and Trichoderma koenigii* were determined to the 63U/mg and 16U/mg respectively. The final enzyme preparation from *Hypocrea virens and Trichoderma koenigii* were 9.3 and 3.1 fold more pure than the crude homogenate respectively. The molecular mass of the partially purified enzyme from *Hypocrea virens and Trichoderma koenigii* were found to be 33kDa and 52kDa respectively by using SDS-PAGE.

Key Words: Protease, *Hypocrea virens and Trichoderma koenigii*, purification, charcterization.

INTRODUCTION

Enzymes are among the most important products obtained for human needs through microbial sources. A large number of industrial processes in the areas of industrial, environmental, clinical, food and pharmaceutical biotechnology utilize enzymes at some stage or the other. Current developments in biotechnology are yielding new applications for enzymes. A large number of microoganisms, including bacteria, yeast and fungi produce different groups of enzymes.

A protease (E.C No.3,4) breaks down proteins. A protease is an enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein. Proteases work best in acidic conditions.

Proteases execute a large variety of functions and have important biotechnological application. They represent one of the three large groups of industrial enzymes and find applications in detergents, leathers, food, pharmaceutical industries and bioremediation processes. (Gupta et al ., 2002).

Fungi elaborate a wide variety of enzymes than do bacteria and protease are among the most important enzymes produced by fungi. Fungi produce a variety of proteolytic enzymes; however, most of these are usually acidic in nature. (Fernandez et al., 1998; Wu and Hang., 2000). Filamentous fungi are used in many industrial processes for the production of enzymes and metabolites. (Adrio et al., 2003), proteases represent an important group of enzymes produced industrially and acoount for 60% of the worldwide sales value of the total industrial enzymes. (Godfrey 1996).

The aim of the present work was to partially purify and investigate the characteristics of the extracellular enzyme protease from *Hypocrea virens and Trichoderma koenigii.*

MATERIALS AND METHODS

Isolation and Screening of protease producing fungi

Proteases producing fungal strains were isolated from marine soil sample. Proteolytic fungi were screened on skim milk agar medium (sharma et al., 2006). Containing skim milk powder 100gm, peptone 5g and Agar 20g per litre at pH 8.0 Fungal isolates showing zone of clearance were picked uip, purified by respected streaking on the same medium and finally transferred to Potato dextrose agar slants and maintained at 4°C. The best porducing strains identified as *Hypocrea virens and Trichoderma koenigii* were isolated from marine soil and were used for further studies.

Protease fermentation media

The protease production medium (Kaey et al., 1970) containing Glucose 250mg, Casein 500mg, yeast extract 50mg, Dipotassium hydrogen phosphate 1gm, Magnesium Sulphate 200mg per litre at pH 7.2 at 37 °C in an incubator (200 rpm). The flask containing 100ml of sterile fermentation medium. The flasks were inoculated at 37 °C for 92h in a rotary shaker regulated at 180 rpm. The culture medium was centrifuged at 5000 rpm for 20 min in a refrigerated centrifuge at 4 °C to remove the fungal mycelia and medium debris the supernatant was used as crude enzyme solution.

Protein assay

Protein was measured by the method of Lowry et al., 1951 with Bovine serum albumin (BSA) as a standard.

Determination of protease activity

Protease activity was determined spectrophotometerically by modified Reese's method. The crude enzyme solution was centrifuged at 10,000 rpm for 30 minutes in a refrigerated centrifuge at 4°C. Enzyme (0.2 ml) was incubated with 1 ml of 2 % casein and 3ml of carbonate-Bicarbonate buffer, pH 9.0 at 37°C min and then the reaction was arrested by the addition of 2.0 ml of 5 % trichloroacetic acid (TCA). The reaction mixture was filtered through what man No.1 filter paper. 1 ml of clear filtrate was mixed with 0.4 M sodium carbonate and 1 ml of 0.5 N Folin - phenol's reagent the solution incubated at 37 °C for 20 minutes. The amount of tyrosine in the solution was measured by reading the absorbance at 660nm. Once unit of protease activity was defined as the amount of enzyme required to liberate 1 µG of tyrosine per minute per ml under essay condition. A tyrosine standard was prepared by dissolving different amounts of tyrosine in TCA solution.

Partial purification and characterization of protease

Fractionation with ammonium sulfate

The crude enzyme was first saturated upto 20 % with solid ammonium sulfate and then centrifuged at 10000 rpm at 4°C for 10 min. The supernatant obtained was further saturated upto 70 % with solid ammonium sulfate and again centrifuged. The pellets obtained were dissolved in minimum volume of 0.1 M phosphate buffer; pH 6.2 these solutions were dialyzed against 500 ml of the same buffer at 4°C to remove the excess salt. The proteolysis activity of each protein fraction was determined as described by modified Reese's method.

Ion exchange chromatography

The dialyzed solution was applied to a DEAE – cellulose (DE – 52) column (11 x 1 cm) which had been previously balanced with phosphate buffer (50 mm pH 6.0). The enzyme sample was stepwise eluted by using a discontinuous gradient of 150 – 200 mM of Nacl in phosphate buffer (50 mM pH 6.0). The low rate was 0.1 ml min^{-1} and 20 fractions (2 ml each) were collected. Fractions containing the majority of the protease activity were pooled for activity assay. The activity of protease enzyme at the end of the each step was measured by a spectrophotometric method.

Determination of molecular weight by SDS - PAGE

The molecular mass of enzyme was determined by Sodium Dodecyl Sulphate – Polyacrylamide gel electrophoresis (SDS – PAGE). SDS PAGE was carried out as descried by Laemmli, 1970.

Effect of pH on enzyme activity and stability

To assay optimum pH, proteolysis activity was determined at 37°C, at different pH values, using the 0.1 M phosphate buffer solution (pH 4.5 – 8.5). For stability the enzyme was dispersed (1:1) in the 0.1 M phosphate buffer solution (pH 4.5 – 8.5) and maintained at 37°C for 24 hours.
Afterwards residental proteolysis activity was determined under optimum conditons of pH and temperature (pH 6.5 and 37°C repectively).

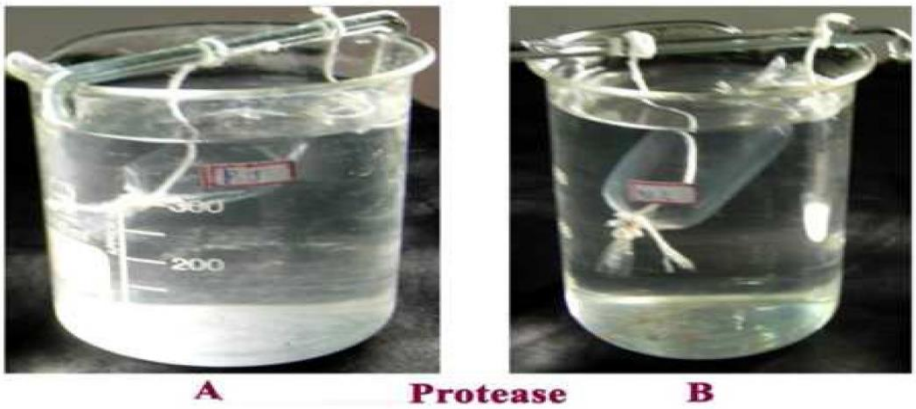
Effect of temperature on enzyme activity and stability

Optimum temperature was determined by incubating the reaction mixture at different temperatures ranging from 30 to 70°C amd assaying the activity at the pH determined as optimum.
Thermal stabiltiy was assayed by incubated the enzyme at different temperature ranging from 30 to 70°C you 1 hour at pH 6.5. Afterwards residual proteolysis activity was determined under optimum conditions of pH and temperature (pH 6.5 and 37°C respectively).

RESULTS AND DICUSSION

The results obtained in this work revealed the ability of *Hypocrea virens and Trichoderma koenigii* to produce extracellular protease.

Figure I : Purification of Extracted Enzymes by Dialysis



A – *Hypocrea virens* **B – *Trichoderma koenigii***

Partial purification nd Characterization of extracellular protease

Protease enzymes from *Hypocrea virens and Trichoderma koenigii.* were partially purified with 70% ammonium sulfate precipitation followed by dialysis (Fig. I). The dialyzed enzyme from *Hypocrea virens and Trichoderma koenigii.* were loaded onto a DEAE – cellulose ionexchange column (Fig. II). Their specific activities and degrees of purification are given is tables 1 and 2.

Figure II : Purification of Dialysed Enzymes by Ion Exchange Chromatography

Protease

TABLE–1 : Purification of extracellular Protease from *Hypocrea virens*

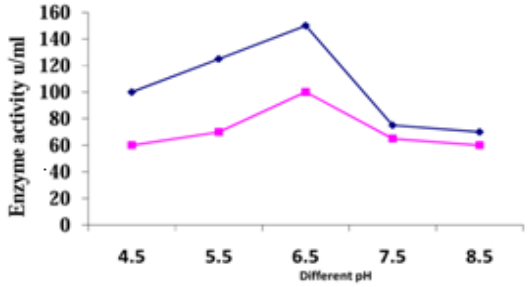
Purification step	Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Yield (1%)	Purification (fold)
Crude enzyme extract	43.26	87.19	49.0	100	1.0

After Ammonium Sulfate frationation	42.05	38.37	1.09	97	2.2
DEAE Cellulose Ion – exchange chromatography	30	21.71	69	69	2.8

TABLE-2 : Purification of extracellular Protease from *Trichoderma koenigii*

Purification step	**Activity (U)**	**Total Protein (mg)**	**Specific Activity (U/mg)**	**Yield (1%)**	**Purification (fold)**
Crude enzyme extract	46.32	89.20	47.0	100	1.0
After Ammonium Sulfate frationation	43.17	37.43	2.01	95	2.7
DEAE Cellulose Ion – exchange hromatography	36	23.62	136.0	8.7	3.2

Fig-IV: Effect of pH on activity of protease from *Hypocrea virens* (◆) and *Trichoderma koenigii* ( )



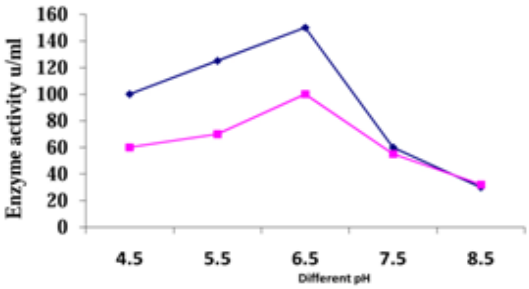


Fig-V: Effect of pH on stability of protease from *Hypocrea virens* (◆) and *Trichoderma koenigii* ( )

Effect of pH on enzyme activity and Stability

The optimum pH of protease from *Hypocrea virens* protease was estimated to be 6.5 and from *Trichoderma koenigii.* protease 6.5, as shown in Fig. IV. The enzyme appeared to be slightly acid, which is expected for protease produced by fungi (Reed & Nagodawithana, 1993) protease from Mucor, described by Maheswari et al., (2000) also exhibited low optimum pH.

The pH stability of protease enzyme *Hypocrea virens and Trichoderma koenigii.* protease was determined to be 4.5-6.5 and 4.5-6.5, respectively Fig. V. After a pH of approx 7.5, a less of activity

was followed by a slight decrease. Proteases from *Pencillium duponti* k 1014 (Hikotaka hashimoto et al., 1973) also exhibited a broad range of stability from pH 2.5 – 6.0.

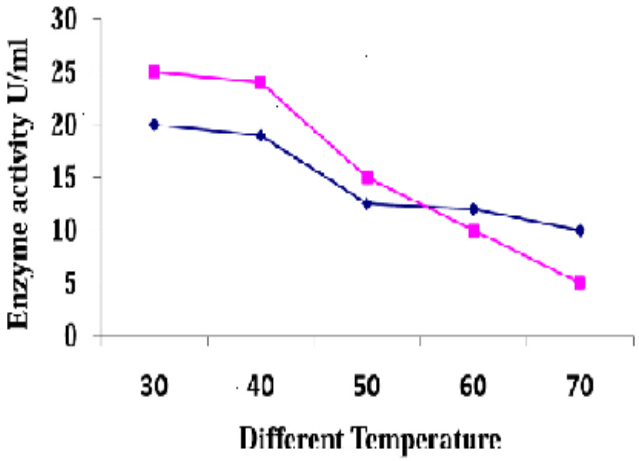


Fig-VI: Effect of Temperature on activity of protease from *Hypocrea virens* (◆) and *Trichoderma koenigii* ( )

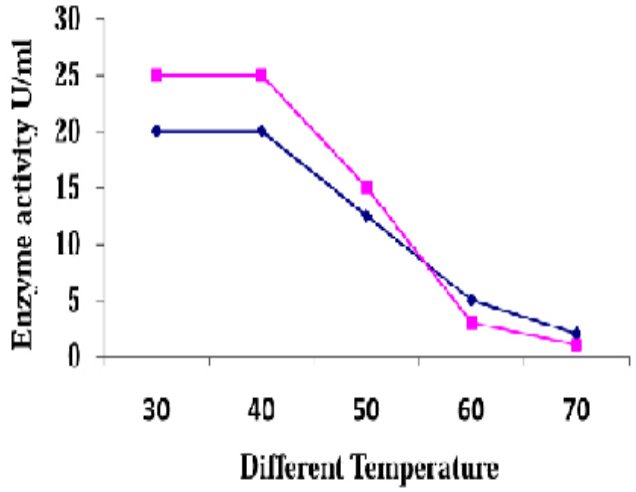


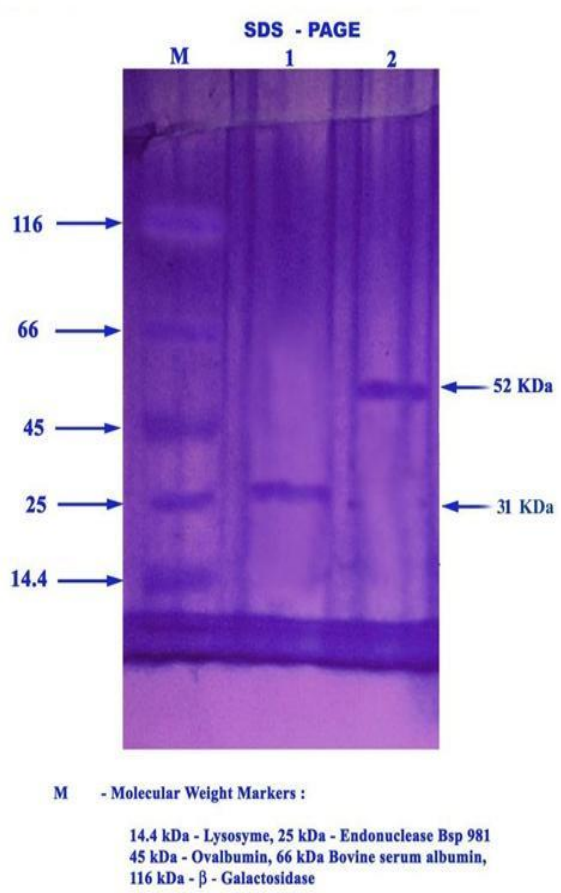
Fig-VII: Thermostability of protease from *Hypocrea virens* (◆) and *Trichoderma koenigii* ( )

Effect of Temperature on Enzyme Activity & Stability

The optimum temperature of activity ranged between 30 to 40°C. Yet, proteases from the genera *Aspergillus* (coral et al., 2003, Tunga et al., 2003) and from *Penicillium* sp. (Ggermano et al., 2003) showed optimum activities at lower temperatures 40°C and 45°C respectively.

The thermal stability of protease from *Penicillium jnathinellum* and *Neurospora crassa* was determined to be 30 – 40°C and 30 – 40°C respectively Fig. VII. *Aspergillus Parasiticus,* which maintained 100% of activity at only 40°C for 1 hour (Tunga et al., 2003)

Fig-III: Determination of molecular mass of purified protease by SDS-PAGE Molecular Weight Determination of Protease by



Lane 1 – Purified fraction of protease from *Hypocrea virens*
Lane 2 – Purified fraction of protease from *Trichoderma koenigii*
Determination of Molecular Weight by SDS – PAGE

The molecular mass of the partially purified protease from P.J. and N.C. were estimated to be approximately 52 kDa and 52 kDa as measured on SDS – PAGE Fig. III. The native enzyme is thought to be a monomer, and which is composed of only one subunit. This result is very similar for protease purified from *Aspergillus oryzae* AWT 20 has reported 33 kDa (Sharma et al., 2006).

Boer et al., 2000 & Hossain et al., 2006 have reported molecular mass approximately 48 kDa for protease from *Aspergillus* species.

CONCLUSION

In the present study, an extracellular protease were partially purified and characterized from *Hypocrea virens and Trichoderma koenigii.*. The isolated strain of *Hypocrea virens* was found to be a potential producer of extracellular protease than *Trichoderma koenigii*. The enzyme yields were more in *Hypocrea virens* than that of *Trichoderma koenigii.*

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