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Partial Purification and Characterization of an Extracellular Protease from Penicillium janthinellum and Neurospora crassa

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ABSTRACT

The aim of this experimental study was to isolate and partially purify extracellular protease from Penicillium janthinellum and Neurospora crassa.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 electrophoresis (SDS-PAGE).The specific activity of partially purified enzyme from Penicillium janthinellum and Neurospora crassa were determined to be 63U/mg and 16U/mg respectively. The final enzyme preparation from Penicillium janthinellum and Neurospora crassa were 9.3 and 3.1 fold more pure than the crude homogenate respectively. The molecular mass of the partially purified enzyme from Penicillium janthinellum and Neurospora crassa were found to be 33kDa and 45kDa respectively by using SDS-PAGE.

Key Words: Protease, *Penicillium janthinellum*, *Neurospora crassa*, purification, characterization.

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 microorganisms, 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 applications. They represent one of the three largest groups of industrial enzymes and find applications in detergents, leather, 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 account 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 *Penicillium janthinellum* and *Neurospora crassa.*

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 100 gm, peptone 5g and Agar 20g per litre at pH 8.0 Fungal isolates showing zone of clearance were picked up, purified by repeated streaking on the same medium and finally transferred to Potato dextrose agar slants and maintained at 4^{o}C. The best producing strains identified as *penicillium janthinellum* and *Neurospora crassa* 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 500 mg, yeast extract 50mg, Dipotassium hydrogen phosphate 1gm, Magnesium Sulphate 200mg per litre at pH 7.2 at 37^{o}C in an incubator (200 rpm). The flask containing 100 ml of sterile fermentation medium. The flasks were inoculated at 37^{o}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^{o}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^oC. Enzyme (0.2 ml) was incubated with 1 ml of 2 % casein and 3 ml of carbonate – Bicarbonate buffer, pH 9.0 at 37^oC 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^o C for 20 minutes. The amount of tyrosine in the solution was measured by reading the absorbance at 660 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µG of tyrosine per minute per ml under assay 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^oC 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^OC 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 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^o C, at different pH values, using the 0.1M phosphate buffer solution (pH 4.5 – 8.5). For pH stability the enzyme was dispersed (1: 1) in the 0. 1 M phosphate buffer solution (pH 4.5 – 8.5) and maintained at 37^o C for 24 hours.

Afterwards residual proteolysis activity was determined under optimum conditions of pH and temperature (pH 6.5 and 37^{o} C respectively). .

Effect of temperature on enzyme activity and stability

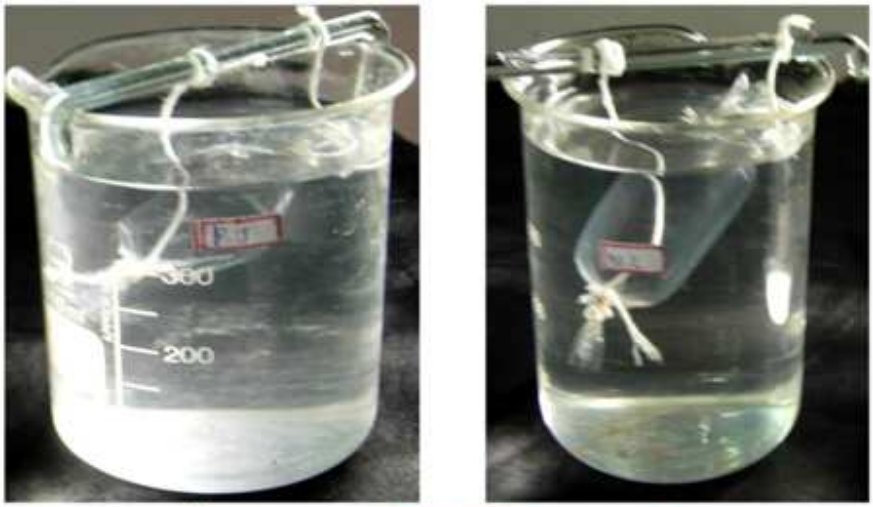
Optimum temperature was determined by incubating the reaction mixture at different temperatures ranging from 30 to 70^{o} C and assaying the activity at the pH determined as optimum.

Thermal stability was assayed by incubated the enzyme at different temperatures ranging from 30 to 70^{o}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^{o}C respectively).

RESULTS AND DISCUSSION

The results obtained in this work revealed the ability of *Penicillium janthinellum* and *Neurospora crassa* to produce extracellular protease.

Figure I : Purification of Extracted Enzymes by Dialysis



A - *Penicillium janthinellum*
B - *Neurospora crassa*

Partial purification and Characterization of extracellular Protease

Protease enzymes from *Penicillium janthinellum* and *Neurospora crassa* were partially purified with 70 % ammonium sulfate precipitation followed by dialysis (Fig I).The dialyzed enzyme from *Penicillium janthinellum* and *Neurospora crassa* were loaded onto a DEAE -cellulose ion - exchange 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

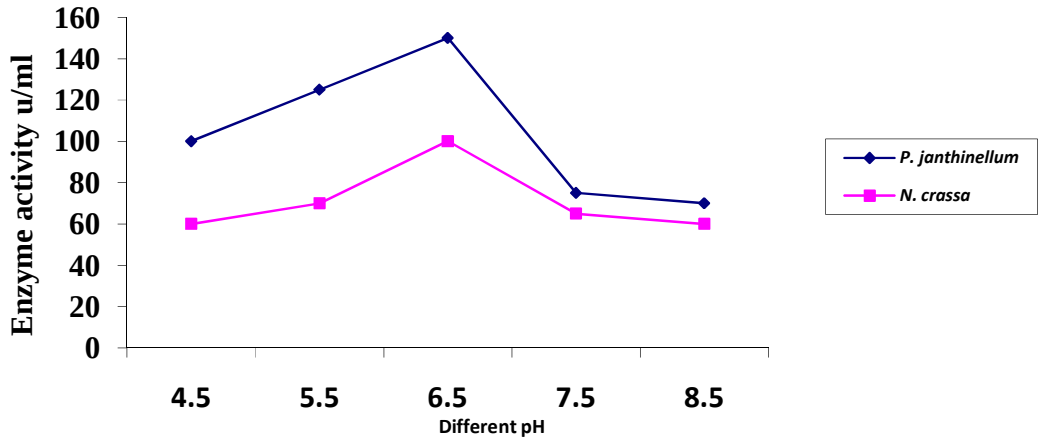
TABLE-1: Purification of extracellular Protease from *Penicillium janthinellum*

Purification step	Activity (U)	Total protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme extract	2032	302	7	100	1.0
After Ammonium Sulfate fractionation	1600	71	23	79	3.3
DEAE Cellulose Ion -exchange chromatography	565	9	63	28	9.3

TABLE-2: Purification of extracellular Protease from *Neurospora crassa*

Purification step	Activity (U)	Total protein (mg)	Specific Activity (U/mg)	Yield (%)	Purification (fold)
Crude enzyme extract	1254	253	5	100	1.0
After Ammonium Sulfate fractionation	1000	150	7	80	1.3
DEAE Cellulose Ion -exchange chromatography	950	61	16	76	3.1

Fig-IV: Effect of pH on activity of protease from *Penicillium janthinellum*(♦) and *Neurospora crassa*( )



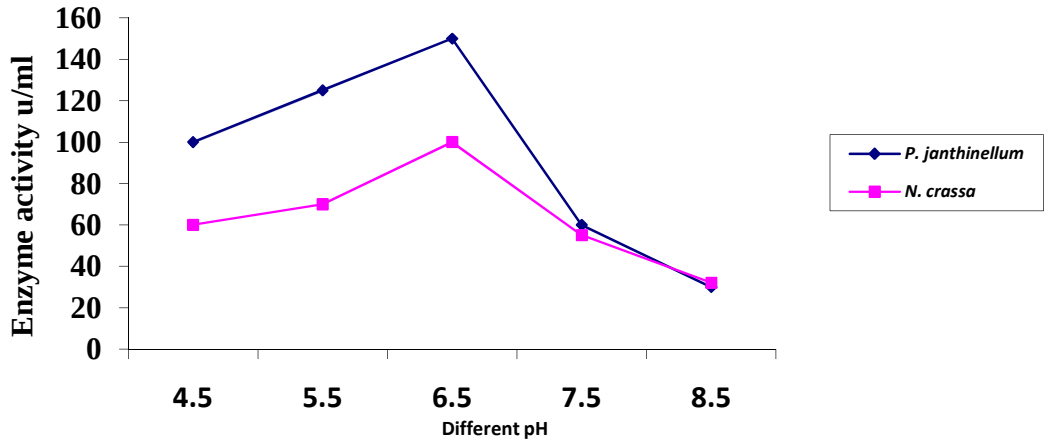


FIG-V: Effect of pH on stability of protease from *Penicillium janthinellum*(♦) and *Neurospora crassa*( )

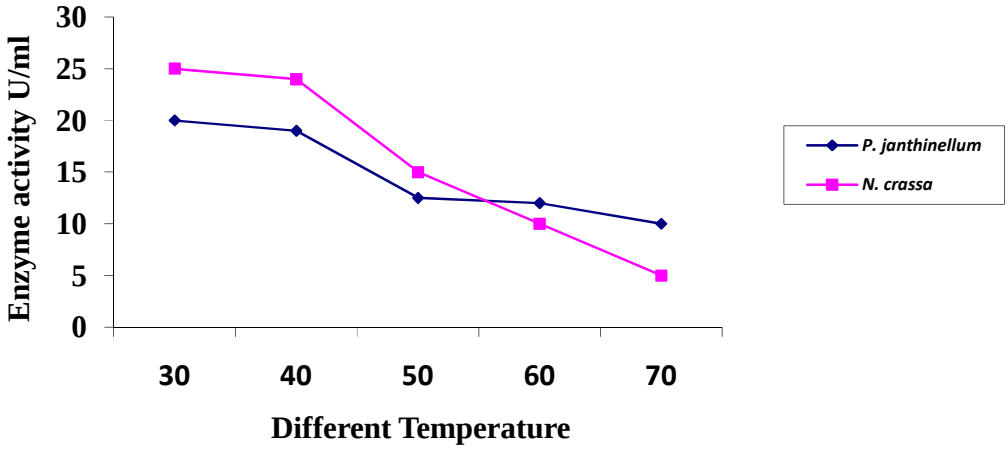


Fig-VI: Effect of Temperature on activity of protease from *Penicillium janthinellum*(♦) and *Neurospora crassa*( )

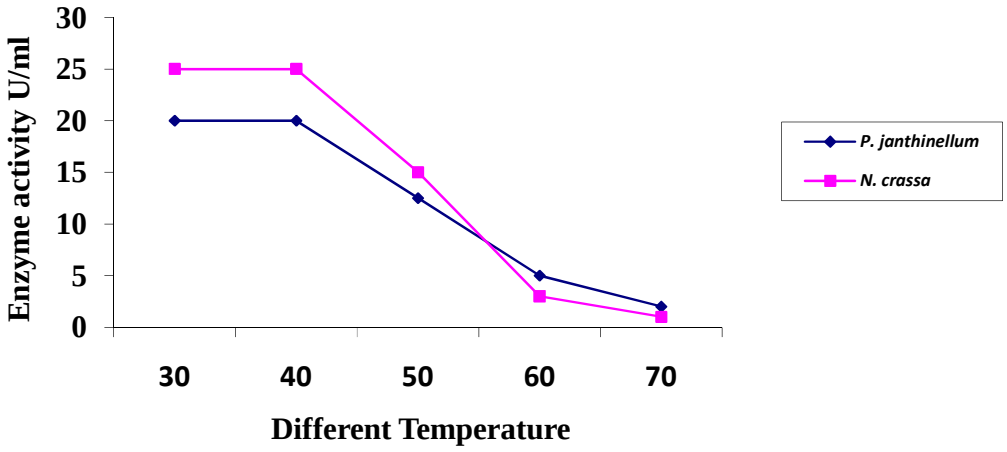


Fig-VII: Thermostability of protease from *Penicillium janthinellum*(♦) and *Neurospora crassa*( )

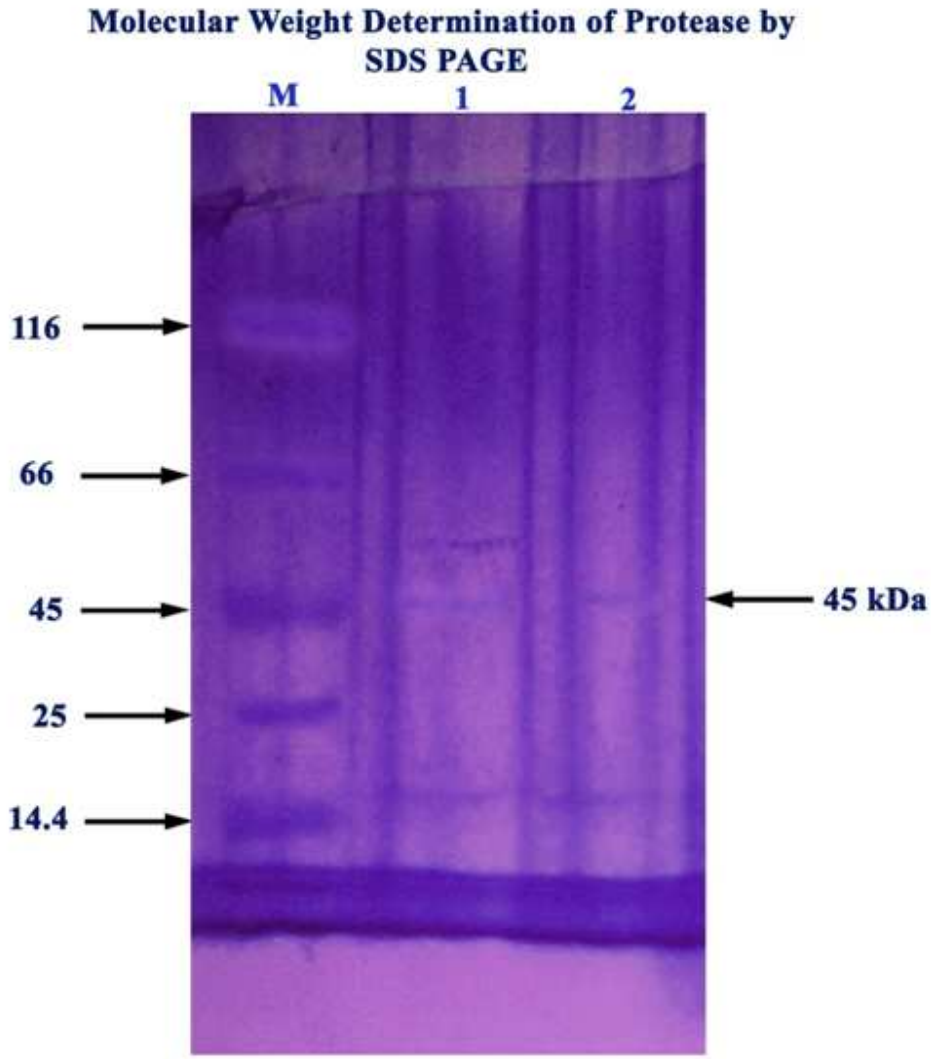
Effect of pH on enzyme activity and Stability

The optimum pH of protease from *Penicillium janthinellum* protease was estimated to be 6.5 and from *Neurospora crassa* protease 6.5, as shown in Fig IV. So the enzyme appeared to be slightly acid, which is expected for proteases produced by fungi (Reed & Nagodawithana, 1993) proteases from *Mucor*, described by Maheshwari *et al*., (2000) also exhibited low optimum pH.

The pH stability of protease enzyme *Penicillium janthinellum* and *Neurospora crassa* 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, as shown in Fig 4 .Proteases from *Penicillium*

duponti K 1014 (Hikotaka hashimoto *et al.*, 1973) also exhibited a broad range of stability from pH 2.5 – 6.0.

FIG-III: Determination of molecular mass of purified protease by SDS-PAGE.



M - Molecular Weight Markers :

14.4 kDa - Lysosyme, 25 kDa - Endonuclease Bsp 981
45 kDa - Ovalbumin, 66 kDa Bovine serum albumin,
116 kDa - β - Galactosidase

Lane1 - Purified fraction of protease from *Penicillium janthinellum*
Lane2 - Purified fraction of protease from *Neurospora crassa*

Effect of Temperature on Enzyme Activity & Stability

The optimum temperature of activity ranged between 30 to 40^{o}C (Fig-6). Yet, proteases from the genera *Aspergillus* (coral *et al*., 2003, Tunga *et al*., 2003) and from *Penicillium* sp.(Germano *et al.,2003)*showed optimum activities at lower temperatures 40^{o}C and 45^{o}C respectively.

The thermal stability of protease from *Penicillium janthinellum* and *Neurospora crassa* was determined to be 30 - 40 ^{o} C and 30-40^{o}C respectively (Fig -7) *Aspergillus parasiticus,*which maintained 100% of activity at only 40^{o}C for 1 hour (Tunga *et al* .,2003)

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 45 kDa and 45 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 approximately48 kDa for protease from *Aspergillus* species.

CONCLUSION

In the present study, an extracellular protease were partially purified and characterized from *Penicillium janthinellum* and *Neurospora crassa.*The isolated strain of *Penicillium janthinellum* was found to be a potential producer of extracellular protease than *Neurospora crassa*.The enzyme yield were more in *Penicillium janthinellum* than that of *Neurospora crassa*.

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