

# Production and partial characterization of neutral protease by an indigenously isolated strain of *Aspergillus tubingensis* NIICC-08155

V Morya, D Yadav

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## Abstract

An indigenously isolated strain of *Aspergillus tubingensis* NIICC-08155 from soil of teak dominated vegetation of kusmi forest at Gorakhpur was subjected to neutral protease production and its partial characterization. The maximum production of neutral protease i.e. 68.50 U/ml was attained after 96h of incubation. The crude preparation of protease showed reasonable activity at temperature range of 40 to 60 °C with maximum activity at 40 °C and had a maximum enzyme activity at pH 6.4. The Km value of the enzyme was found to be 45.0 mg/ml and the molecular weight as determined by SDS-PAGE was approximately 45 kDa. The enzyme was completely inhibited by DTT and EDTA.

## INTRODUCTION

The microbial proteases represent 60% of the worldwide sales value of the total industrial enzymes (Godfrey, 1996; Gupta et al., 2002). Extracellular proteases (generally alkaline and acid proteases) from *Aspergillus* have high commercial value and find applications in industries like the detergent, diagnostics, food, leather, pharmaceutical, waste management and silver recovery industries (Chiplonkar et al., 1985; Godfrey & West 1996; Rao et al., 1998; Agarwal et al., 2004). Neutral proteases also have potential applications in baking, food processing, protein modification, leather processing industry, animal feeds and pharmaceutical industries. (Monod et al., 1993; Basu et al., 2007; Merheb-Dini et al., 2009; Paranthaman et al., 2009).

The members of *Aspergillus* are highly explored for the production many industrial enzymes (Adrio et al., 2003). There are few reports of neutral proteases from *Aspergillus* (Rao et al., 1998; Paranthaman et al., 2009), though substantial amount of work has been reported for alkaline and acid protease. This paper reports production and partial characterization of neutral protease secreted from an indigenous strain of *Aspergillus tubingensis* isolated from soil of teak dominated vegetation of kusmi forest at Gorakhpur.

## MATERIAL AND METHODS

### MICROORGANISM

The indigenously isolated *Aspergillus tubingensis* was isolated from kusmi forest (Ramgarh forest range, 26°45'N and 83°24'20"E INDIA) Gorakhpur, a teak dominating forest by plating method. The strain was deposited at National Institute of Interdisciplinary Science and Technology (NIIST), Thiruvananthapuram, India with accession number NIICC-08155.

### CHEMICALS

All analytical reagents and media components were purchased from Hi-Media (Mumbai, India), Merk BDH (Germany) and SISCO Research Laboratories Pvt. Ltd, (Mumbai, India).

### GROWTH MEDIA

For isolation of *Aspergilli*, Potato Dextrose Agar and Czapek's Agar was used (Raper and Fennell, 1965).

Media for fungal growth and spore production

Preliminary screening for protease production from *Aspergillus awamori* isolates was carried out by skimmed milk agar plate assay on standard media with slight modification (Ventosa et al., 1982; Vermelho 1996). For preparation of 2 liter of media 2.0 g K<sub>2</sub>HPO<sub>4</sub>, 6.0 g NaNO<sub>3</sub>,

1.0 g  $\text{MgSO}_4$ , 1.00 g KCl and 40.0 g Agar was dissolved in 1 liter of double distilled water, sterilized by autoclaving after adjusting pH to 7.0. Then 1 liter of autoclaved skimmed milk solution (13.3 g milk powder in 1 liter double distilled water) was mixed and subsequently poured approximately 20-25 ml in autoclaved Petri plates under laminar hood. After incubation for 96 hours at 30°C, the inoculated plates were observed for the clear zone formed around mycelia margins, for indicating the production of protease. The plates showing clear zone were subsequently photographed using Olympus digital micrographic camera (Model C-5050, Japan).

The proteases from different isolates of *Aspergillus* were produced under submerged culture condition using standard media comprising of Czapek solution with final composition of 0.1 g Yeast extract, 0.3 g Peptone, 2.0 g glucose, 3.0 g  $\text{NaNO}_3$ , 1.0 g  $\text{KH}_2\text{PO}_4$ , 0.5 g  $\text{MgSO}_4$ , 0.01 g  $\text{FeSO}_4$ , 10.0 g soluble casein, and 0.001 g Thymine HCl dissolved in 1.0 lit. warm double distilled water (Vermelho et al., 1996). The pH of media was adjusted to 7.0. The prepared media was then aliquoted as 50 ml dispensed in 150 ml conical flasks prior to sterilization by autoclaving. The above media was then inoculated with *Aspergillus* spore suspension with final concentration of  $10^6$  spores/ml along with penicillin (final concentration of 0.5 mg/ml (Devi et al., 2008). The flasks were incubated at 30°C under stagnant condition for 96 hrs. The culture was filtered and centrifuged at 8,000 rpm for 15 min and supernatant thus obtained was used as crude enzyme extract.

## ENZYME ASSAY

The production of protease was quantified by tyrosine methods (Kunitz, 1947). The reaction mixture comprising of 1 ml of 2% casein solution, 1.0 ml of 50 mM buffer (pH 7.0) and 0.1 ml of crude enzyme was incubated for 15 min. at 30°C. After incubation, the reaction was stopped by adding 2ml of cold 10% TCA. After one hour, the mixture was centrifuged at 10,000 g for 15 min. to remove the precipitate. The acid soluble material was estimated spectrophotometrically at 280 nm. Enzyme activity was calculated by measuring mg of tyrosine equivalent released and compared with the standard. One enzyme unit activity was defined as the amount of enzyme required to liberate 1  $\mu\text{g}$  of tyrosine per minute under experimental conditions.

## ENZYME KINETICS

The protease activity subjected to temperature ranging from

20 -100°C was monitored by standard enzyme assay while thermostability of the enzyme was determined by incubating the crude enzyme preparation at temperatures ranging from 40-100°C for 30 minute in a constant-temperature water bath. The residual enzyme activities were then assayed after each treatment. Optimum pH for enzyme activity was determined by subjecting the crude enzyme preparation to three different types of buffers namely citrate buffer (pH range 2-5), Phosphate buffer (pH range 6-9) and Glycine NaOH (pH range 10-12). The assay conditions were similar except variability of pH from 2 to 11 as mentioned above. The  $K_m$  value was calculated by Michaelis- Menten and double reciprocal graph (Lineweaver & Burk, 1934). The effect of different metal ions namely  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Cu}^{2+}$ , chlorides and inhibitors like pepstatin, EDTA, DTT and PMSF was studied by taking metal ions in assay buffer at a final concentration of 5 mM.

## PARTIAL PURIFICATION AND CHARACTERIZATION

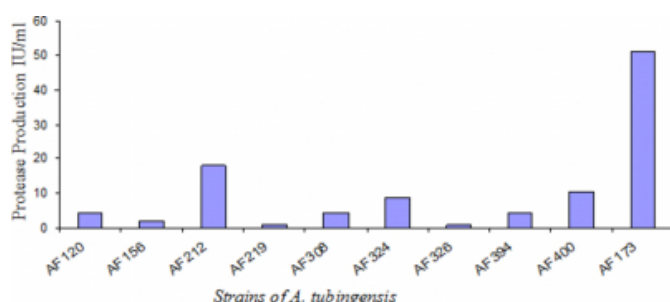
Partial purification of crude enzyme preparation was achieved by standard method using ammonium sulphate fractionation (Ogundero and Osunlaja, 1986). The crude enzyme preparation were fractionated in different ranges of ammonium sulfate namely 0-30%, 30-60% and 60-90% saturation. During ammonium sulphate precipitation, the salt was added in small quantity under constant stirring to prevent increase of high local concentrations. The precipitate is then dialyzed against 1.0 mM Phosphate buffer and protein concentration was estimated by standard Lowery method. The purified fractions were then assayed individually for total soluble proteins and protease activity. The approximate molecular weight was determined by subjecting the partially purified enzyme to 12 % SDS PAGE (Laemmli, 1970).

## RESULTS AND DISCUSSION

A total of ten indigenously isolated strains of *Aspergillus tubingensis* were used for neutral protease production under submerged culture conditions. The production of neutral protease varied among the ten strains with maximum production of 60.55 IU/ml (Figure 1). The strain showing maximum production was further investigated for preliminary enzymatic characterization like optimum pH, temperature,  $K_m$  and effect of metal ions and inhibitors.

**Figure 1**

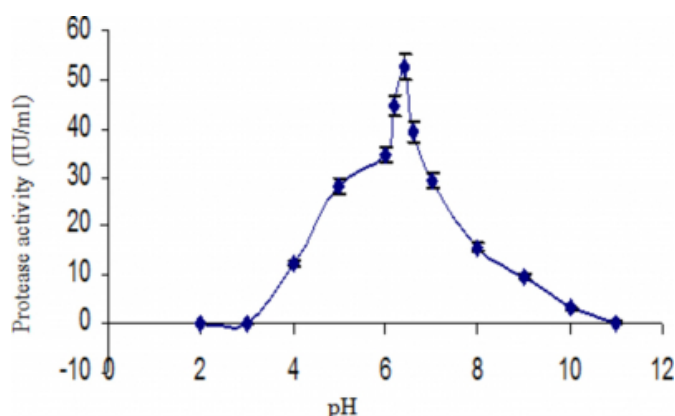
Figure. 1 Production of neutral proteases by 10 strains of under submerged culture condition



The optimum temperature of the neutral protease was found to be 40 °C (Figure 2) while thermal stability was up to 60 °C for 30 minutes, showing similarity with earlier reports (Monod et al., 1993; Basu et al., 2007; Sumantha et al., 2006). The optimum pH found was 6.4 though the enzyme showed reasonable activity in the pH range of 3.0 to 9.0 (Figure 3). The pH optima of most of the neutral protease reported in literature also supports this finding (Monod et al., 1993; Basu et al., 2007; Merheb-Dini et al., 2009). Optimum substrate concentration for maximum enzyme activity was determined in terms of  $K_m$  using casein as substrate. The  $K_m$  values were interpreted from Line Weaver Plots. The  $K_m$  values (Figure 4 and 5) for neutral protease from *Aspergillus tubingensis* was found to be 45.0 mg/ml, which is in the range, as reported in literature (Monod et al., 1993; Basu et al., 2007; Merheb-Dini et al., 2009).

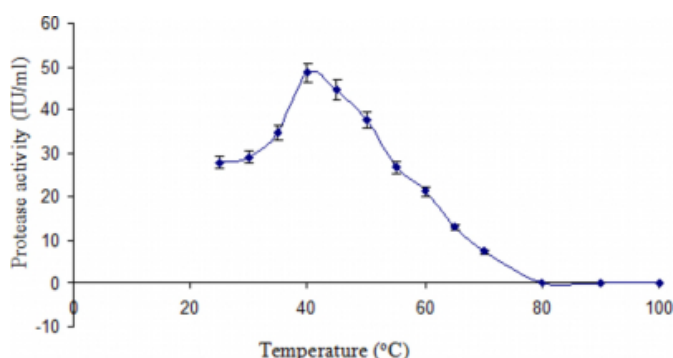
**Figure 2**

Figure 2 Effect of pH on activity of neutral protease secreted by NIICC-08155.



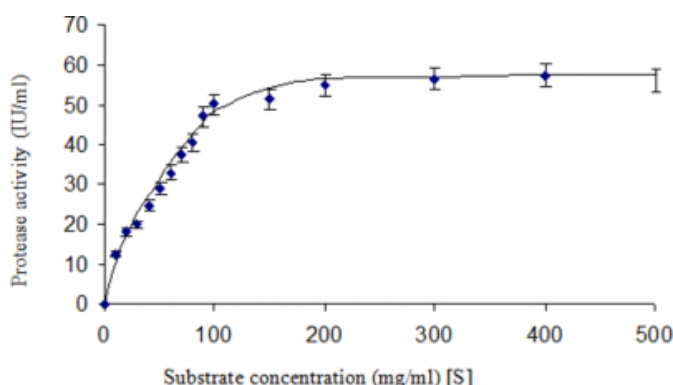
**Figure 3**

Figure 3 Effect of temperature on activity of neutral protease secreted by NIICC-08155



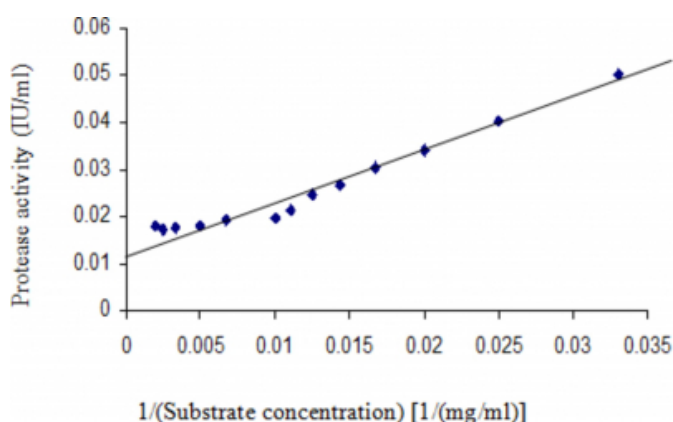
**Figure 4**

Figure 4. Graph showing effect of substrate concentration on enzyme activity of neutral protease secreted by NIICC-08155.



**Figure 5**

Figure 5. Double reciprocal graph between substrate concentration and enzyme activity of neutral protease secreted by NIICC-08155

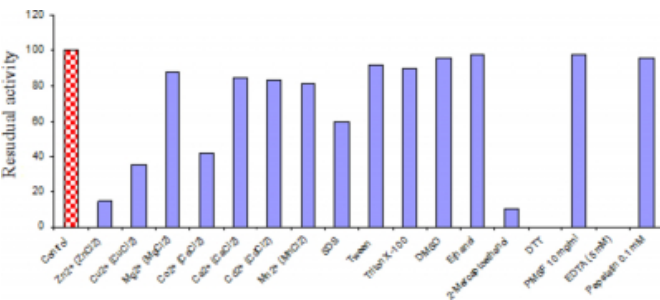


The influence of various metal ions and inhibitors on enzyme activity was studied (Figure 6). Among the metal ions tested,  $Mn^{2+}$ ,  $Cd^{2+}$ ,  $Co^{2+}$  and  $Mg^{2+}$  were insignificant as

they do not influence the enzyme activity (Basu et al., 2007; Merheb-Dini et al., 2009 ) while  $Zn^{2+}$ ,  $Cu^{2+}$  and  $Co^{2+}$  showed significant inhibitory effect (Figure 6). The effect of inhibitors and detergents on enzyme activity of the partially purified protease was also studied (Figure 6). The enzyme retained its activity in the presence of PMSF and pepstatin while it was completely inhibited by 0.1 mM EDTA (Figure 6) as reported in literatures (Monod et al., 1993; Basu et al., 2007; Merheb-Dini et al., 2009). Enzyme activity and stability in presence of some available commercial detergents was studied with a view to exploit the enzyme in detergent industry. The enzyme retained its activity in the presence of detergents like Tween, Triton X-100 and DMSO (Figure 6). However, the enzyme activity was drastically reduced when subjected to SDS detergent. The enzyme activity was completely inhibited in the presence of 0.5 % 2-Mercaptoethanol and DTT (Figure 6).

**Figure 6**

Figure 6 Effect of metal ions, detergents and inhibitors on activity of neutral protease secreted by NIICC-08155



A summary of purification steps for neutral protease from *A. tubingensis* (NIICC-08155) is given in Table 1. The purification of Neutral protease resulted in 7.38 fold purification with recovery by 30-60 % ammonium sulfate precipitation. The molecular weight of partially purified enzyme as determined by SDS-PAGE (Figure 7) was found to be approximately 30 kDa similar to what has been reported earlier (Basu et al., 2007; Merheb-Dini et al., 2009).

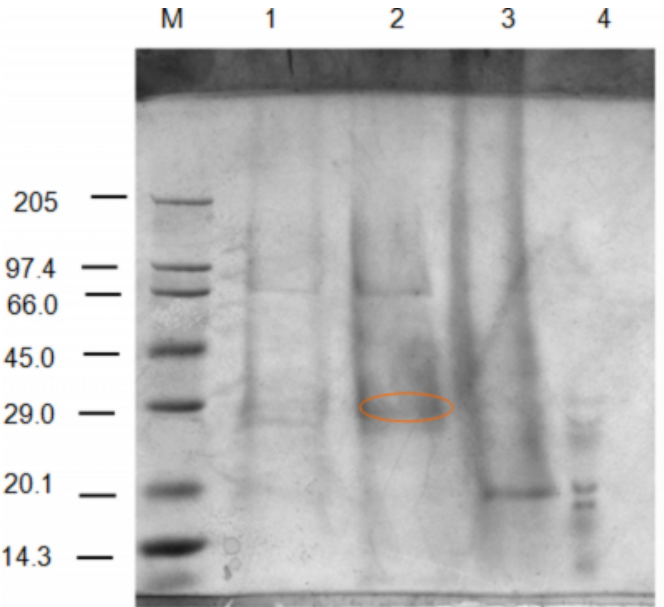
**Figure 7**

Table 1 Partial Purification of Protease produces by (NIICC-08155)

Protease Producing strains	Sample	Volume (ml)	Protein (mg)	Activity (IU)	Sp. activity (IU/mg protein)	fold
<i>A. tubingensis</i> (NIICC-08155)	Crude	200	64.0	12000	1875	-
	0-30% fraction	10	19.4	300	15.4	.082
	30-60% fraction	10	7.23	10000	1383.2	7.38
	60-90% fraction	10	31.1	400	12.8	0.068

**Figure 8**

Figure 7 12% SDS Page showing partially purified neutral Protease secreted by NIICC-08155; Lane M (Protein Marker), 1, 2, 3, and 4 are 0-30%, 30-60 %, 60-90% and crude enzyme respectively



The partially characterized neutral protease from *Aspergillus tubingensis* NIICC- 08155 needs to study further in view of its possible industrial applications after complete purification and characterization.

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**Author Information**

**Vivek Kumar Morya, (M.Sc.)**

Department of Biotechnology, Jawahar Lal Nehru Center For Biotechnology, DDU Gorakhpur University

**Dinesh Yadav, (Ph.D.)**

Department of Biotechnology, Jawahar Lal Nehru Center For Biotechnology, DDU Gorakhpur University