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AN EXTRACELLULAR ALKALINE PROTEASE FROM A LOCALLY ISOLATED MOULD *Aspergillus niger* JBPSI-1: PURIFICATION AND CHARACTERIZATION

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ABSTRACT

In the present investigation, an alkaline protease has been purified using two chromatographies i.e. gel filtration on dextran and ion-exchange chromatography on CM-Cellulose. The purified enzyme was completely inhibited by serine protease inhibitor, PMSF. The enzyme was optimally active at 55°C and pH-10. The Km and Vmax for casinolytic activity of the purified enzyme were found to be 1.43 mg/ml and 416.6 U/ml/min, respectively.

Keywords: Alkaline protease, *A. niger*, Km, Vmax, Inhibitor

INTRODUCTION

Proteases constitute one of the most important groups of industrial enzymes that are now used in a wide range of industrial processes, e.g. in the detergent, food, pharmaceutical, leather and silk industries **[1, 2]**.

With the exception of pharmaceutical uses, the detergent industry has emerged as one of the major consumers of hydrolytic enzymes working at alkaline pH and now accounts for more than 25% of the global enzyme production **[3]**. Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. Although there are many microbial sources available for producing protease, only a few are recognized as commercial producers **[4]**. Of these, mold of the genera *Aspergillus, Penicillium* and *Rhizopus* are especially useful for producing protease as many species of these genera are regarded as safe **[5]**.

The isolation and screening of microorganisms from naturally occurring alkaline habitats are expected to provide

new strains producing enzymes active and stable in alkaline conditions. This paper deals with purification and characterization of an alkaline protease produced by *A. niger* JBPSI-1 strain isolated from soil samples of Jabalpur, M. P. India.

MATERIALS AND METHODS

Microorganisms and Growth Conditions

The strain used throughout this study was isolated from soil samples at Jabalpur, India. The Isolate was identified as *A. niger* JBPSI-1 by MACS-Agharkar Research Institute, Pune, on the basis of their morphological and microscopic characteristics. The strain was maintained on potato-dextrose-agar plates at 28 ± 2°C. The medium used for protease production by *A.niger* JBPSI-1 was Yeast Extract 0.5%, KCl 2%, Peptone 2%, Sucrose 2%, Casein 1%, pH 7.5. *A.niger* JBPSI-1 was cultured for extracellular protease production in 250 ml Erlenmeyer flasks containing 100 ml of Yeast Extract broth for 4d at 28 ± 2°C. At the end of the incubation period, the culture was taken and centrifuged at 10,000 rpm for 10 minutes at 4°C to remove unwanted mycelia.

The crude enzyme was recovered in the supernatant.

Assay for Protease Activity

Protease activity was measured by the method of Anson **[6]** using casein as a substrate. One milliliter of the enzyme was mixed with five milliliter of 0.65% casein solution (pH 7.5), and incubated for 10 min at 37°C. The reaction was stopped by addition of 5 ml of tricholoroacetic acid (110 mM). The mixture was allowed to stand for 30 minutes and then centrifuged at 10,000 rpm for 15 min and the precipitate was removed. Free amino acids released by protease from casein hydrolysis were estimated.

One unit of protease hydrolyzed casein to produce colour equivalent to 1.0 µmol (18lµg) of tyrosine per unit at pH 7.5 and 37°C. The enzyme activity was expressed as U/ml. A control lacking the enzyme was included in each assay.

Assay of Protein Content

Quantitative estimation of proteins was determined by the method of Lowry **[7]** using Bovine serum Albumin as a standard.

Purification of Alkaline Protease

In order to purify the protease enzyme to be used for further characterization, the crude enzyme was saturated upto 60% with solid ammonium sulphate and then centrifuged. The pellets obtained were dissolved in 0.1 M Tris-HCl buffer,and loaded onto prepacked desalting columns of cross linked dextran with epichlorohydrin, equilibrated with Tris-HCl buffers, pH 7.8. The protease was eluted at 1.0M NaCl concentration. The desalted sample was further analyzed. All

the purification steps were carried out at 4°C.

CM– Cellulose Column Chromatography

The desalted sample resulting from ammonium sulphate fractionation was loaded on CM Cellulose column (8 x 1.5 cm.) previously equilibrated with Tris-HCl buffer, pH 7.5. After washing the column, the enzyme was eluted with the Tris-HCl buffer, pH 7.5. Fractions showing protease activity were pooled and used for studying the enzymatic properties.

Effect of pH and Temperature on the Activity of Enzyme

The effect of pH on protease activity was performed at 37°C in different buffers. The following buffer systems were used; phosphate buffer for 7.0 pH , Tris-HCI buffer for pH8.0, glycine-NaOH buffer for pH 9-11. To study the effect of temperature, the activity was tested at different temperatures at pH 7.5. Protease activities were assayed under standard assay conditions.

Effect of Protease Inhibitors on Protease Activity

Protease type was determined by employing the following inhibitors; ρ-chloromercuribenzoic acid (ρ-CMB), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonylfluoride (PMSF), β-mercaptoethanol and iodoacetate. The purified enzyme was preincubated with each inhibitor (5mM) in 100 mM Tris-HCl buffer for 30 minutes at 37°C and then the remaining enzyme activity was measured. The activity of the enzyme without any inhibitor was taken as control.

Effect of Metal Ions on Protease Activity

The effect of various metal ions at 5mM conc. was investigated by adding them to enzyme solution for 30 min at 37°C. Protease activity was measured by adding the substrate and carrying out the enzyme assay under the optimum conditions.

Kinetic Studies

The Michaelis constant Km and the maximum reaction velocity (Vmax) of the protease for casein were determined at different substrate concentrations. They were evaluated by plotting the data on a Lineweaver Burk double reciprocal graph **[8]**.

RESULTS AND DISCUSSION

Purification of Protease from *Aspergillus niger* JBPSI-1

Table 1 shows the different steps of purification for *Aspergillus niger* with 55% yield and 27 fold purification.

Optimum pH

The effect of pH on the activity of alkaline protease was studied with various pH from 7-11. **(Figure 1)**. The optimum pH for alkaline protease from *A.niger* JBPSI-1 was determined as 10.0. These findings are in

accordance with earlier reports showing pH optima of 10-10.5 for protease from Bacillus Sp., *Thermus aquaticus, Xanthomonas maltophila, Vibrio metscnikovii* **[9]** and *A.niger* **[10]**. The important detergent enzymes, subtilisin carlberg and subtilisin Novo or BPN also showed maximum activity at pH 10.5.

Optimum Temperature

Optimum temperature is a critical factor for maximum enzyme activity and is a prerequisite for industrial enzymes to be active and stable at higher temperature **[11]**. The activity of the purified enzyme was determined at 20°C to 90°C. The optimum temperature recorded was at 55°C for protease activity. The enzyme activity gradually declined at temperature beyond 55°C **(Figure 2)**. Li *et al*., **[12]** also reported that alkaline protease isolated from *Thermomyces lanuginose* P_{134} had a broad temperature optimum of 50°C.

Effect of Metal Ions and Inhibitors

The influence of various metal ions and inhibitors on enzyme activity was studied. Protease activity may be enhanced by various metal ions that also help to know their biochemical nature. Among the metal ions tested, addition of Zn^{+2} and Cu^{+2} enhanced the activity of alkaline protease enzyme produced by *Aspergillus niger* JBPSI-1. Hg^{+2} and Ca^{+2} inhibited alkaline protease enzyme activity to the level of 21 and 20% **(Table 2)**. The inhibitory effect of heavy metal ions is well documented in the literature. It is known that the mercury ion react with the protein thiol groups (converting them mercaptides), as well as with histidine and tryptophan residues **[13]**.

Inhibition studies primarily give an insight into the nature of the active center **[14]**. The effect of different inhibitors on the enzyme activity of the purified protease was studied **(Table 3)**. Of the inhibitors tested, PMSF was able to inhibit the protease completely, while ρ-CMB exhibited 88% inhibition. This indicated that it is a serine alkaline protease. Gold and Fahrney **[15]** also explained that alkaline protease was completely inhibited by PMSF in which PMSF sulfonated the essential serine in the active site and resulted in complete loss of activity.

Enzyme Kinetics

Enzyme kinetics continues to be the most fundamental aspect of enzymology. For commercial use of any enzyme it is desirable to choose an enzyme, which will have the fastest reaction rate per unit amount of enzyme as this indicates the maximum effect for minimum amount of added catalyst **[16]**.

On examining the effect of substrate concentration on proteolytic activity of protease from *A.niger* JBPSI-1, it was found to obey the Michaelis-Menten kinetics.

There was an increase in substrate hydrolysis upto 4mg/ml and thereafter there was saturation of the enzyme. From Line weaver Burk plot, the Km and Vmax of the reaction was found to be 1.43 mg/ml and 416.6 U/ml/min, respectively **(Figure 3)**.

Table 1: Purification of Protease from *Aspergillus niger* JBPSI-1

Purification Step	Total Protease Activity	Total Protein (mg)	Specific Activity	Fold Purification	%
Culture Supernatant	7857	20	394	1	100
Salting out (Gel filtration)	6993	1.8	3885	9.9	89
CM-Cellulose Chromatography	4290	0.4	10725	27	55

Table 2: Effect of different metal ions on the activity of protease from *Aspergillus niger* JBPSI-1

Metal Ions (5mM)	Residual Relative Enzyme activity (%)
Control	100%
Na^{+} (NaCl)	103%
Co^{+2} (CoCl2)	101%
Ca^{+2} (CaCl2)	80%
Cu^{+2} (CuCl2)	120%
Hg^{+2} (HgCl2)	79%
Zn^{+2} (ZnCl2)	120%
Mg^{+2} (MgCl2)	97%

Table 3: Effect of different inhibitors on the activity of protease from *Aspergillus niger*-JBPSI-1

Compound	Residual Relative Enzyme Activity (%)
Control	100%
ρ-CMB	88%
EDTA	85%
PMSF	07%
β-Mercaptoethanol	101%
IAA	98%

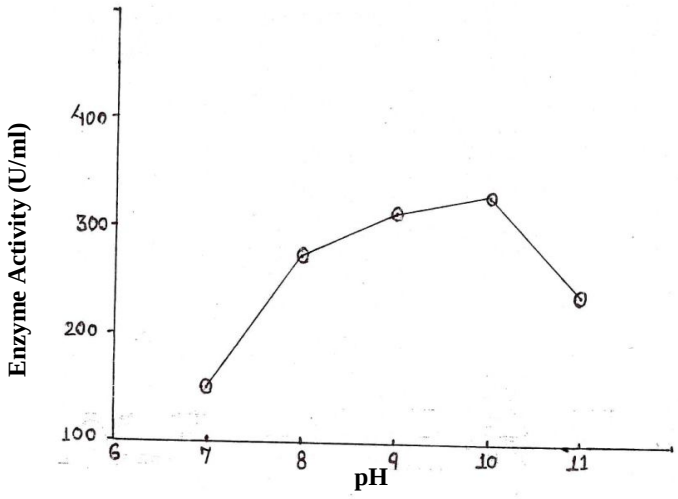

Figure 1: Effect ofpH on protease activity from *Aspergillus niger* JBPSI-1

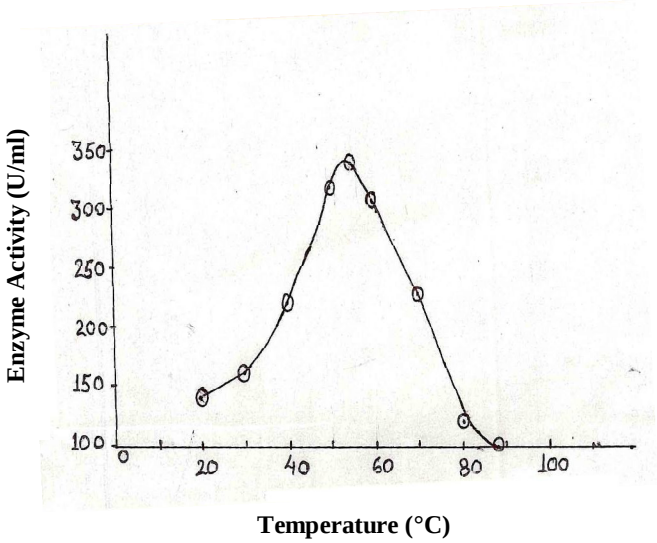

Figure 2: Effect of Temperature on Protease activity from *Aspergillus niger* – JBPSI-1

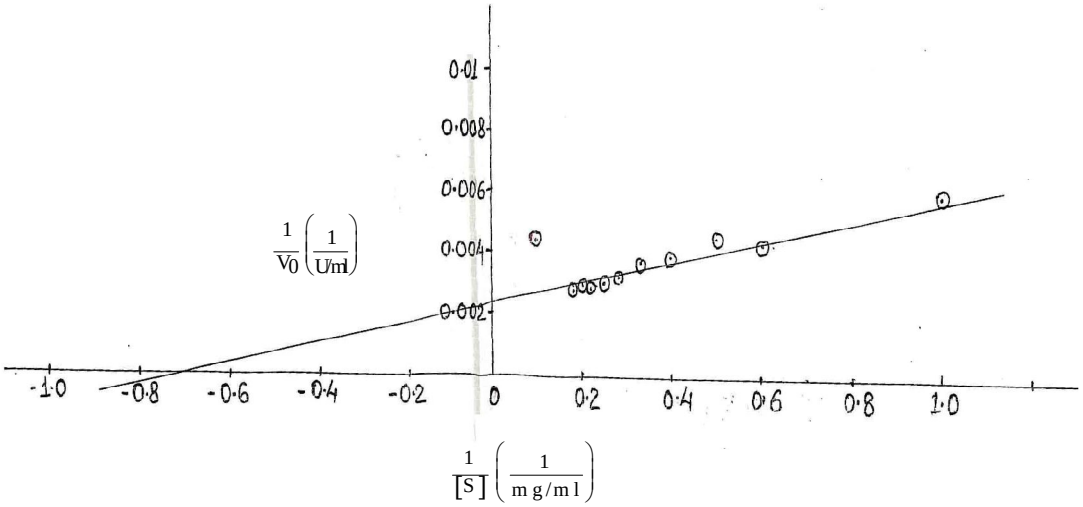

Figure 3: Lineweaver-Burk Plot of Alkaline Protease from *Aspergillus niger*-JBPSI-1

CONCLUSION

In the present study, the alkaline protease production capability of a native isolate of *Aspergillus niger* JBPSI-1 from soil was exploited by submerged fermentation of Yeast Extract broth. Purified enzyme was characterized for optimum pH, temperature and their kinetic study was also done with the purpose to obtain an alternative of available proteases in detergent industry.

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