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Purification, characterization of alkaline protease enzyme from native isolate *Aspergillus niger* and its compatibility with commercial detergents

M. Kalpana Devi, A. Rasheedha Banu, G.R. Gnanaprabhal, B.V. Pradeep and M. Palaniswamy
Department of Microbiology, Karpagam University, Coimbatore 641 021, Tamil Nadu, India.
m.palaniswamy@gmail.com

Abstract: An alkaline protease producing strain *Aspergillus niger* was isolated from local soil samples and enzyme production was optimized under submerged conditions. Maximum enzyme production of the culture occurred in mesophilic temperature 45°C and pH 8.5. Glucose and ammonium sulfate proved to be the best carbon and nitrogen sources respectively. The molecular weight of the enzyme determined by SDS-PAGE was found to be 38 kDa. The enzyme acted optimally at pH 10 and 50°C. It was thermo stable and retained full activity even at the end of 1 hour of incubation at 40°C. It was inhibited by Cu^{++}, Hg^{++}, Zn^{++}, EDTA and sodium azide. The enzyme retained more than 50% activity after 60 min incubation at 40°C in the presence of detergents such as Tide, Surf, Wheel and Henko indicating its suitability for application in detergent industry.

Keywords – Alkaline protease, *Aspergillus niger*, purification, characterization, detergent.

Introduction

Alkaline proteases of microbial origin possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures (Rao *et al.,* 1998; Agarwal *et al.,* 2004) The alkaline proteases find their largest use in house hold laundry with a worldwide annual production of detergents of approximately 13 billion tons (Nehra *et al.,* 2002). Alkaline proteases were in fact the first enzyme produced in bulk. Plant, animal and microbial sources are employed in enzyme production. Microbial proteases are preferred to plant and animal sources to various advantages. A variety of microorganisms such as bacteria, fungi, yeast and *Actinomycetes* are known to produce these enzymes (Madan *et al*., 2002). Molds of the genera *Aspergillus*, *Penicillium* and *Rhizopus* are especially useful for producing proteases, as several species of these genera are generally regarded as safe (Sandhya *et al.,* 2005). *Aspergillus clavatus* ES1 has been recently identified as a producer of an extracellular bleaching stable alkaline protease (Hajji *et al*., 2007, 2008).

The main draw back with production of bacterial protease is the requirement of cost intensive procedures for separation of enzymes from cells, on the other hand enzyme from fungal origin offer an advantage of separation of mycelium by simple filtration. Besides, the fungus can be grown on inexpensive substrates. The use of alkaline protease as active ingredient in laundry detergent is the single largest application of this enzyme (Nehra *et al.,* 2002). For the production of enzymes for industrial use, isolation and characterization of new promising strain is a continuous process (Kumar *et al.,* 2002). They are generally produced by using submerged fermentation due to its apparent advantages in down stream in spite of the cost intensiveness for medium components (Prakasam *et al.,* 2005).

Reports on bleach stable alkaline protease from fungal sources are scanty (Mulimani *et al.,* 2002). Therefore, a need was felt to explore native fungal isolates, capable of producing alkaline proteases and at the same relatively stable at the operating conditions.

Materials and methods

Isolation of Fungal strain

An alkaline protease producing fungi *Aspergillus niger* was isolated from local soil, Coimbatore, Tamil Nadu (Palaniswamy *et al.,* 2008).

Enzyme production

Two hundred ml of supplemented Reese medium broth in 500 ml Erlenmeyer flask was inoculated with 3% spore suspension (10^6 spores/ ml) prepared from PDA slant, and was grown at 50°C, 72 h on a rotary shaker at a speed of 150 rpm. The culture was centrifuged at 10,000 rpm for 15 min and supernatant thus obtained was used as crude enzyme extract.

Enzyme assay

Three ml of reaction mixture containing 0.5% casein in 2.95 ml of 0.1 M Tris-Hcl buffer, pH 8.5 and 0.1 ml of enzyme was incubated at 50°C. After 10 min, the reaction was stopped by adding 3ml of cold 10% TCA. After 1 hour, the culture filtrate was centrifuged at 8,000 rpm for 5 min to remove the precipitate and absorbance of the supernatant was read spectrophotometrically at 280nm. Enzyme activity was calculated by measuring mg of tyrosine equivalent released and compared with the standard. One unit (U) of enzyme activity represents the amount of enzyme required to liberate 1 µg of tyrosine under standard assay conditions.

Protein assay

Protein was quantified by the method of Lowry *et al.,* (1951), with bovine serum albumin as standard.

Optimization of cultural parameters

Using Reese medium, protease production was studied at different pH (7-9) and temperature range (30-50°C). Effect of different carbon and nitrogen sources were also studied.

Partial purification for enzyme characterization

Ammonium sulfate fractionation: Solid ammonium sulfate was added to the crude extract to 40-80% saturation. The precipitate was collected by centrifugation, dissolved in minimal volume of 0.1%Tris-Hcl buffer (pH 9) and dialyzed against same buffer at 4˚C.

DEAE-cellulose chromatography: The enzyme solution obtained in the above step was applied to DEAE-cellulose column (2.0 X 25cm) pre equilibrated with Tris-Hcl buffer. The enzyme was eluted with the same buffer at a flow rate of 15 ml/h.

Characterization of purified alkaline protease

SDS-PAGE: SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12.5% (w/v) acrylamide slab gel with 25 mM Tris / 192 mM glycerin buffer (pH 8.3) that contained 0.1% (w/v) SDS as the running buffer, as described by Laemmli (1970).

Determination of kinetic parameters

Enzyme was incubated with various concentrations of casein (2-20 mg/ml) in Tris-Hcl buffer (pH 9) at 50°C. Kinetic parameters K_m and V_{max} were calculated by linear regression from Lineweaver- Burk plots (Lineweaver & Burk, 1934).

Determination of temperature and pH optima and stability

Optimum temperature for activity of the alkaline protease was determined by carrying out at selected temperatures from 30 to 50°C. In each case, the substrate was preincubated at the required temperature before the addition of enzyme. The optimum pH was determined by monitoring protease activity (50°C) at pH values between pH 7 and 11, using Tris-Hcl buffer (pH 9).

Effect of various metal ions and inhibitors on protease activity

The effects of various metal ions and inhibitors on the activity of the purified protease were assessed (including the appropriate metal ion salts) by following standard assay at 5 mM final concentration.

Compatibility with commercial detergents

Detergents solutions at a concentration of 7 µg/ml were prepared in double distilled water. The solution were boiled for 10min to destroy any protease already present and cooled. Fixed enzyme concentration was added to each detergent solution and the mixture was incubated at 35°C for different time intervals. The activity was then assayed by Anson's method (1938).

Results and discussion

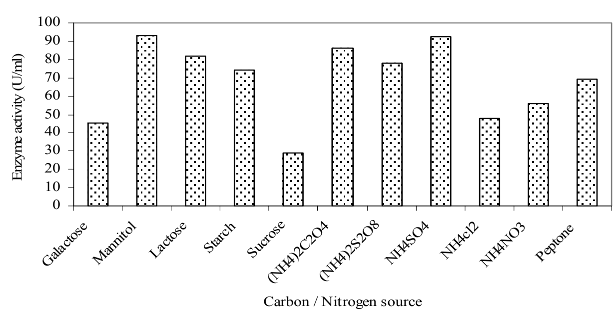
Optimization of cultural parameters

Influence of temperature and pH: Protease production at different temperature was examined for 72 h keeping the other fermentation conditions constant. Protease production increased with increase in temperature from 35 to 45°C. Maximum production of protease (89.1 U/ml) was obtained at 45°C. Growth and protease production ceased at higher temperature (50°C) similar observation were shown by Morimura *et al.,* (1994) for *Aspergillus usami*. It was revealed that environmental temperature not only affects growth rates of organism but also exhibit marked influence on the levels of protease production. Another important factor significantly affecting the production of protease is the initial pH of the medium. Protease production by *Aspergillus niger* was observed in the range 7-9 pH. Growth and protease production ceased at 9 pH. Maximum protease production 80.6 U/ml was found at 8.5 pH. The results clearly indicated alkalinophilic nature of the fungus. Optimum pH 8.4 has been reported for alkaline protease of *Conidiobolus coranalis*. Likewise pH 7 has been reported to be optimum for *Aspergillus flavus* (Sutar *et al.,* 1992).

Influence of carbon and nitrogen sources: There are general reports showing that different carbon sources have different influences on extracellular enzyme production by different strains (Wang & Lee, 1996; Nehra, 2002). Among the various substrates tested mannitol and ammonium sulfate were found to be the most effective for protease production (Fig.1). The mechanism that shows the formation of extracellular enzymes is influenced by the availability of precursors for protein synthesis.

The effect of different nitrogen sources like gelatin, peptone, aspartic acid, casein and acetamide has reported that nitrogen sources

Fig. 1. Effect of carbon and nitrogen sources on production of alkaline protease by A. niger

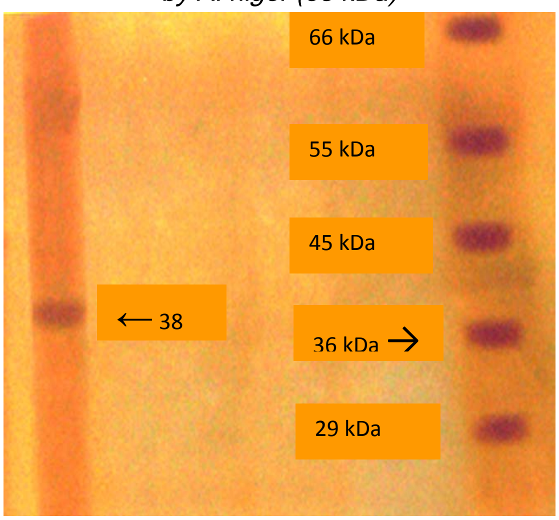


stimulate equal accumulation of protease in the culture medium of *Aspergillus terreus* (Ashour, *et al.,* 1996).

Table 1. Purification of alkaline protease from Aspergillus niger

Fraction	Activity (U/ml)	Protein (mg/ml)	Specific Activity (U/mg)	Purification (fold)	Recovery (%)
Culture filtrate	28.0	5.8	4.82	1.0	100
Ammonium Sulfate Precipitation	69.3	2.56	27.07	5.62	74.25
Dialysis	81.1	1.50	54.06	11.21	57.92
DEAE - Cellulose	89.6	0.54	165.92	34.42	32

Fig. 2. SDS - PAGE showing the molecular weight of alkaline protease enzyme produced by A. niger (38 kDa)



Partial purification and enzyme characterization

A summary of purification steps for alkaline protease from *Aspergillus niger* is given in Table 1. The purification of alkaline protease resulted in 6 fold purification with 74% recovery by ammonium sulfate precipitation. The purification of crude enzyme through DEAE cellulose column chromatography gave 34 folds increase in purity with 32% recovery of alkaline protease from *Aspergillus niger.* The similar observation was reported by Ogundero and Osunlaja (1986) for *Aspergillus clavatus*. The molecular weight of purified enzyme as determined by SDS-PAGE was found to be 38 kDa. The appearance of a single band on SDS-PAGE further suggests the enzyme to be monomeric (Fig.2). The molecular weight in the range of 32- 33 kDa has also been reported for the enzyme from *Malbranchea inlchella* (Voordouw *et al.,* 1974).

Enzyme characterization

Optimum pH: The effect of pH on the activity of alkaline protease was studied with various pH from 8-11 (Fig.3). The optimum pH for alkaline protease enzyme from *Aspergillus niger* was determined as 10.0. These findings are in accordance with earlier reports showing pH optima of 10.0-10.5 for protease from *Bacillus* species, *Thermus aquaticus, Xanthomonas maltophila* and *Vibrio metscnikovii* (Durham *et al.,* 1987). In an early study, the protease from *Thermus* sp strain RT 41A exhibited stability for at least 4 hours over a pH range of 5-10 (Adinarayana *et al.,* 2003).

Optimum temperature:

Temperature is a critical factor for maximum enzyme activity and it is a prerequisite for industrial enzymes to be active and stable at higher temperature. Assay mixture was incubated at different temperature ranging from 30-60°C and enzyme activity was maximum at 45°C (Fig. 4). However, the enzyme was completely inactivated at 60°C. Li *et al.,* (1997) reported that alkaline protease isolated from *Thermomyces lanuginose* P_{134} had a broad temperature optimum of 50°C.

Fig. 3. Effect of pH on the activity of alkaline protease A. niger

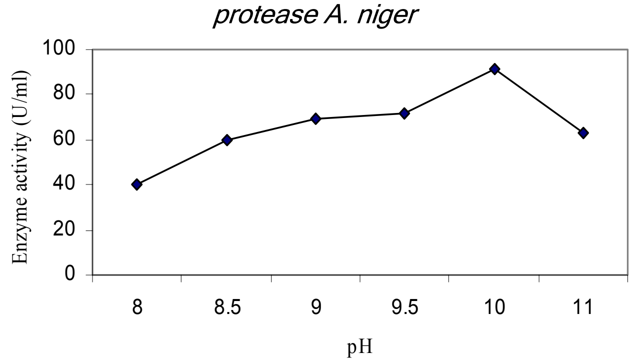


Table 2. Effect of various metal ions and inhibitors (5mM) on the activity of alkaline protease from Aspergillus niger

Metal ion	Residual activity (%)
None	100
$CaCl_2$	105.3
$MgCl_2$	99.3
$ZnCl_2$	21.3
$HgCl_2$	45.8
$CoCl_2$	2.37
$CuSo_4$	63.74
Urea	68.3
Sodium Azide	69.3
Mercaptoethanol	61.2
EDTA	11.0

Samal *et al.,* (1991) reported an alkaline protease from *Tritirachium albumlimber* to be quite thermostable even up to 50°C.

The thermostability activity for alkaline protease enzyme produced by *A. niger* was stable up to 60 minutes at 40°C. It lost activity gradually after 60 minutes (Fig. 5). The similar reports were observed

Fig. 4. Effect of temperature on the activity of alkaline protease A. niger

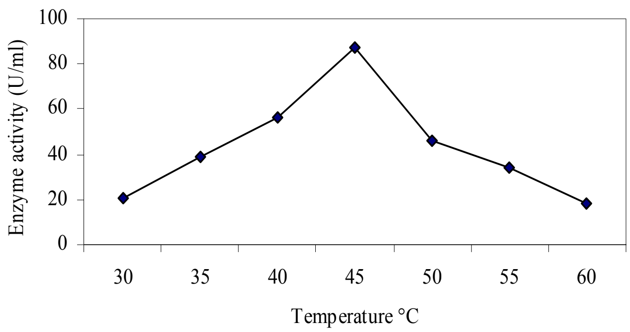


Fig. 5. Effect of incubation time on the activity of alkaline protease A. niger

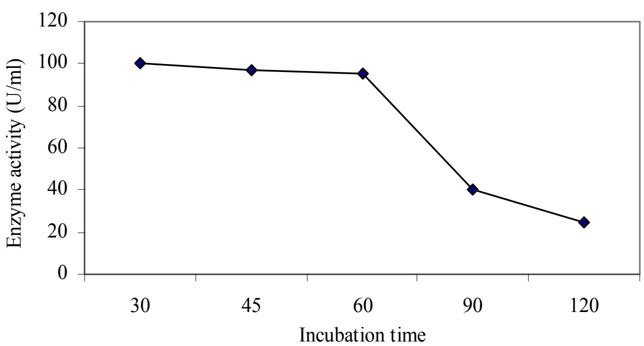
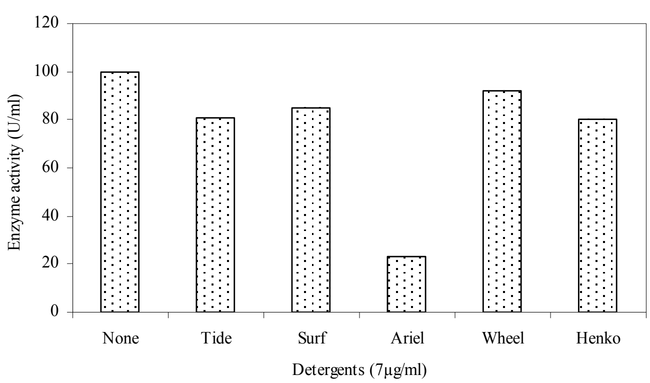


Fig. 6. Showing Alkaline protease compatible with various commercial detergents



for some fungal origin by Li *et al.* (1997), *Thermomyces lanuginosus* and in *Aspergillus* species by Nehra *et al.* (2004). The results of Sumandeep *et al.* (1999) showed that alkaline protease appeared to be more stable at alkaline pH 9. More than 40% of the initial activity was preserved after 1 h incubation with the substrate at 80°C in *Bacillus* sp.

The influence of various metal ions and inhibitors on enzyme activity was studied. Among the metal ions tested, addition of 5mM $CaCl_2$ enhanced the activity 105.3% of alkaline protease enzyme produced by *Aspergillus niger.* $ZnCl_2$ and $CoCl_2$ inhibited alkaline protease enzyme activity to the level of 78.8 and 97.63%. Tsuchiya *et al.* (1987) reported protease isolated from *Colosporium* sp.KM388 inhibited by Hg^{++}, Mn^{++}, Cu^{++} ,Ca^{++} were found to inhibit the enzyme activity of alkaline protease secreted by *Bacillus polymyxa* (Kaur *et al.,* 1998). Nehra *et al.,* (2004) reported that Mg^{++} was found to be an activator of the alkaline protease enzyme produced by *Aspergillus sp.* The effect of inhibitors on enzyme activity of the partially purified protease from *A. niger* retained 69.3% activity when incubated in sodium azide (5mM) for 1 h, as the enzyme was 90% inhibited by metal chelator EDTA. The similar results were observed by Madan *et al.* (2002) for *Bacillus polymyxa* alkaline protease (Table 2).

Effect of substrate concentration on alkaline protease activity

Optimum substrate concentration for maximum enzyme activity was determined in terms of V_{max} and K_m using casein. V_{max} and K_m values were interpreted from Line Weaver and Eadie-Hofstee Plots (Table 3).

Optimum substrate concentration: V_{max} and K_m values for alkaline protease enzyme from *Aspergillus niger were* determined from Line Weaver and Eadie-Hofstee plots. The results revealed that alkaline protease from *Aspergillus niger* had a V_{max} of 85.0 U/mg of protein and K_m value of 0.8mg/ml. Matta *et al.,* (1994) has reported proteases with lower K_m values with casein substrate from *Bacillus alkalophilus* and *Pseudomonas* species, which showed K_m values of 0.4 and 2.5 mg/ml respectively. A slightly higher K_m value of 3.7 mg/ml has been reported for the enzyme from *Bacillus polymyxa* strain indicating higher affinity of the enzyme towards casein (Kaur *et al.,* 1998).

Application study

Compatibility with various commercial detergents: 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 80-92% of its original activity in various detergents (Fig. 5&6). However, Arial detergents retained only 23% of enzyme activity. Phadatare *et al.* (1993) reported high activity alkaline protease

Table 3. Properties of alkaline protease enzyme

Properties	*Aspergillus niger*
Optimum pH	8.5
Optimum temperature(°C)	45
Thermo stability (minutes)	60
V max (U/mg protein)	85
Km (mg/ml)	0.8
Molecular weight(kDa)	38

from *Conidiobolus coranatus* showed compatibility at 50°C in the presence of 25 mM Calcium chloride with a variety of commercial detergents. They observed the enzyme protease to retain more than 80% of its activity in the presence of various detergents. Similarly, among the three proteases isolated from *Tritirachium album* Limber, proteinase R and T were reported to retain 90 and 89% activity respectively up to one hour in the presence of detergents like ERA plus, Dyanamo, while BPN was highly unstable in all the detergents and retained just 4% activity even after 10 minutes. Madan *et al.,* 2002 studied the compatibility of alkaline protease from *Bacillus polymyxa* retained 20-84.5% of its activity in various detergents. They also reported 16, 11.4 and 6.6% activity in Revel, Ariel and wheel respectively (Adinarayana, 2003).

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