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Partial Purification and Characterization of a Thermostable Alkaline Protease from *Lactobacillus brevis*

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ABSTRACT

Aims: The research was done to study the partial purification and characterization of thermostable alkaline protease from *Lactobacillus brevis.*
Methodology and Results: The enzyme was purified in a two-step procedure involving ammonium sulphate precipitation and Sephadex G-150 gel permeation chromatography. The protease was purified 8.04 fold with a yield of approximately 30% after purification with Sephadex G-150 column. It has a relative molecular weight of 33.2 kDa and optimally active at a temperature of 60 ^{o}C and pH 9.0. The maximum velocity V_{max} and Michaelis constant K_m of the protease produced during the hydrolysis of casein were 66.66 U/mg protein and 3.33 mg/ml. It was strongly activated by Ca^{2+} and ethylene diamine tetra acetic acid (EDTA), mildly inhibited by Na^{+}, K^{+}, Mg^{2+} and Fe^{2+} and strongly inhibited by Cu^{2+} and Hg^{2+}. The ability of the enzyme to improve the cleansing power of various detergents was also studied.
Conclusion, significance and impact of study: The findings in this study suggest that the protease is a suitable candidate for detergent formulation and biotechnological applications.

Keywords: protease, *Lactobacillus,* alkaline, inhibitors and detergent

INTRODUCTION

Proteases are one of the most important classes of enzymes and are expressed throughout the animal kingdom, plant and as well as microbes (Dubey *et al.*, 2007). Among the various proteases, bacterial proteases are the most significant compared with those of animals and plants (Gupta *et al.*, 2002). This enzyme accounts for nearly 60% of the total worldwide enzyme sales (Adinarayana *et al.*, 2003; Beg *et al.,* 2003). There are acidic, alkaline and neutral proteases, but of all these, alkaline proteases are employed primarily as cleansing additives (Ward, 1995; Nehra *et al.*, 2004). Alkaline protease 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; Devi *et al.*, 2008). One of the major drawbacks affecting the stability at alkaline pH of enzymes recovered from thermophiles is that enzymes from alkalophile confer stability in a wide pH range but are usually thermolabile (Griffin *et al*., 1992).

In this present study, the protease produced by *Lactobacillus brevis* was characterized. The results obtained showed that the protease may be applied as an effective detergent additive which is recommended for laundry/detergent producing industries.

MATERIALS AND METHODS

Organisms and culture conditions

The test isolate, *Lactobacillus brevis* used for this research was isolated from the hindgut of kola nut weevil *Balanogastris kolae* Desbr. It was cultured on nutrient agar to obtain the pure culture of this organism. The organism was grown in a basal medium containing (g/L): K_2HPO_4, 1.5; KH_2PO_4, 0.5; $MgSO_4$, 0.05; NaCl, 1.5; $(NH_4)_2SO_4$, 1.0; $CaCl_2{\cdot}2H_2O$, 0.02; $FeSO_4{\cdot}7H_2O$ 0.02; yeast extract, 0.5; sucrose, 0.5 and casein, 0.1. The inocula for the experiments were prepared by growing the organism in nutrient broth (NB, Oxoid) at 35 °C for 18 h on a rotary shaker (Gallenkamp). Sterilized medium (500 mL) in 1000 mL conical flasks was inoculated with 10mL of inocula (1.2×10^5 cells/mL). The flask was incubated at 35 °C on a rotary shaker (120 rpm) for 48 h and then centrifuged at 5000 rpm for 20 min at 4 °C to remove bacterial cells. The supernatant obtained was used as the crude extract for further studies.

Protease assay

The protease activity was determined a reaction mixture consisting of 1 mL of substrate solution (1% casein in Tris-HCl buffer, pH 8.0) and 1 mL of the enzyme solution. The reaction mixture was incubated for 60 min at 40 °C. The proteins were precipitated by adding 3 mL of 0.5% TCA and free amino acids released by protease from casein

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hydrolysis were estimated by Lowry *et al.* (1951) method. The protease activity was defined as mol of tyrosine released per min per mL of the enzyme.

Protein assay

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (BSA) as the standard. The concentration of protein during purification studies was measured at an absorbance of 280 nm.

Purification and characterization of protease

Ammonium sulphate precipitation

Fifty milliliters (50 mL) of crude enzyme was precipitated (fractional) with $(NH_4)_2SO_4$ (Analytical grade, B.D.H) at 70% (w/v) saturation for 24 h at 4 °C. The precipitate was centrifuged at 10,000 rpm for 10 min. The precipitate was redissolved in Tris-HCl buffer (pH 7.8) and dialyzed against several volumes of the same buffer for 24 h at 4 °C using acetylated cellophane tubing prepared from Visking dialysis tube (Gallenkamp) as described by Whitaker *et al.* (1963).

Sephadex G-150 Gel filtration chromatography

Sephadex G-150 (Sigma, Aldrich) was packed into a column (1.5 × 75 cm) and equilibrated with Tris-HCl buffer (pH 7.8).The column was eluted with the same buffer at a flow rate of 20 mL/h. A fraction of 2.0 mL were collected at interval of 30 min and the absorbance at 280 nm was read using spectrophotometer (Jenway, 6305). For determination of molecular weight by gel filtration the standards used were: gamma globulin, 15 kDa; alpha chymotrypsinogen, 25.7 kDa; ovalbumin, 45 kDa; bovine serum albumin, 66 kDa and creatine phosphokinase, 81 kDa (Sigma, UK).

Effect of pH on protease activity

Substrates (1% casein) having pH ranging from 5.0 to 8.0 were prepared using 0.05 M of different buffer system (Glycine-HCl, pH 3.0; acetate buffer, pH 4.0 and 5.0; phosphate buffer pH 6.0 and 7.0; Tris- HCl, pH 8.0). Enzyme activity was determined at 40 °C.

Effect of temperature on protease activity and stability

Protease activity was assayed by incubating the enzyme reaction mixture at different temperatures, ranged from 20 °C to 80 °C for 1 h. The thermal stability at temperature from 50 °C to 80 °C was also determined. Samples were taken at 5 min intervals and analyzed for protease activity.

Effect of substrate concentration on protease activity

The effect of substrate concentration [S] on the rate of enzyme action was studied using [S] values from 2.0 mg/mL to 10.0 mg/mL. The Lineweaver-Burke plot was made. Both the V_{max} and K_m of the enzyme were calculated.

Effect of heavy metals on enzyme activity

A stock solution of 0.01 M of $HgCl_2$ and EDTA were prepared. Two milliliters of each salt solution was mixed with 2 mL of substrate solution. The substrate/chemical mixture was incubated at room temperature for 5 min before it was used in enzyme assay.

Effect of cations

A stock solution of 0.01 M of each salt was prepared. The effects of some salts/cations (NaCl, KCl, $CaCl_2$, $CuSO_4$, MgSO4, and $FeCl_2$) on enzyme activity was also determined. The substrate/salts mixture was also incubated at room temperature for 5 min before it was used for enzyme assay.

De-staining ability of the enzyme

The application of protease as a detergent additive was studied on white cloth pieces (4 × 4 cm) stained with blood. The de-staining property was studied by dipping pieces of cloth artificially stained with blood either in detergent solution supplemented with crude enzyme followed by incubation for 15 min at 60 °C. The stained cloth pieces were taken in separate flasks. The following sets were prepared and studied:

Flask with distilled water (100 mL) + stained cloth
Flask with distilled H_2O (100 mL) + stained cloth + 1 mL Omo detergent (7 mg/mL)
Flask with distilled H_2O (100 mL) + stained cloth + 1 mL Omo detergent (7 mg/mL) + 2 mL enzyme solution.

After incubation cloth pieces were taken out, rinsed with water and drained. The untreated cloth pieces stained with blood were taken as control.

RESULTS AND DISCUSSION

Purification of *Lactobacillus brevis* protease

The partial purification of this enzyme using ammonium sulphate precipitation and gel filtration chromatography are shown on Table 1 and Figure 1 respectively. Multiple protein peaks produced by the isolate are shown on the elution profile and a single activity peak was observed. The two step purification process yielded a partially purified protease with a specific activity of 29.15 U/mg protein and a purification of approximately eight fold with 29.9% yield of proteins. The molecular weight of the protease produced was estimated to be 33.2 kDa.

Characteristics of the purified enzyme (pH optimum)

The highest protease activity was found to be at pH 9.0

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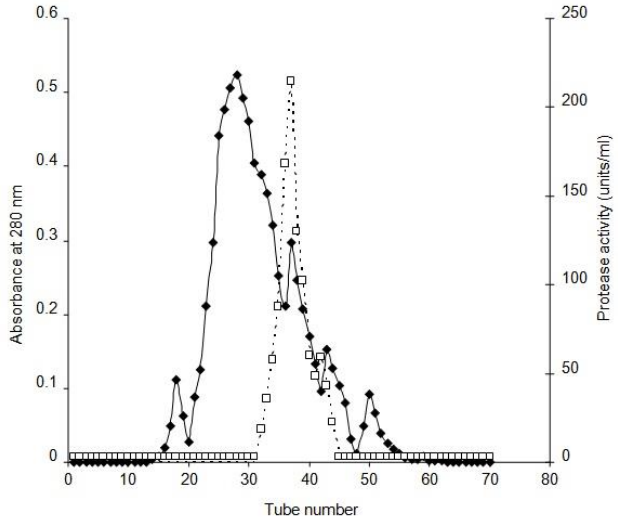
Table 1: Purification of extracellular protease of *L. brevis*

Fraction	Vol. (mL)	Protein content (mg/mL)	Protease activity (U)	Specific activity (U/mg of protein)	Yield (%)	Purification fold
Crude enzyme $(NH_4)_2SO_4$	50	1835.5	6650	3.62	100	1.00
Precipitation	20	591.2	5926	10.02	32.4	2.76
Gel filtration	45	550.3	516047	29.15	29.9	8.04



Abs 280 nm (♦), Protease activity (µmol/min/mL) (□)

Figure 1: Elution profile of protease produced by *Lactobacillus brevis* on Sephadex G – 150 (1.5 x 75) column equilibrated with 0.1M Tris – HCl buffer, pH 7.8. Flow rate at 20 mL/h.

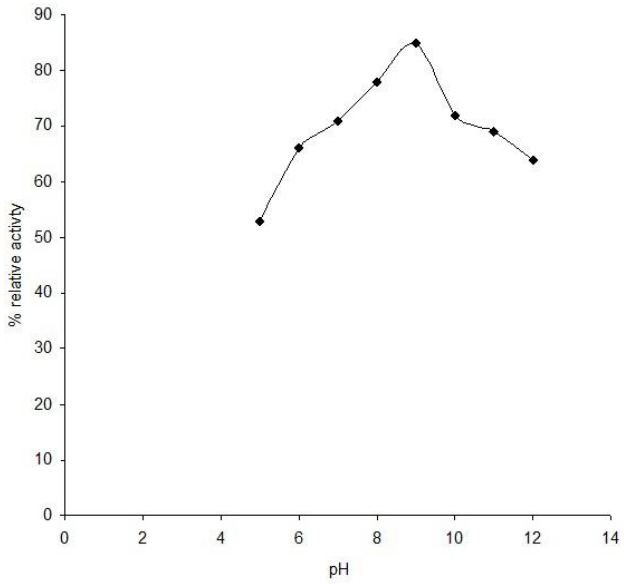


Figure 2: Effect of pH on the activity of *L. brevis* protease.

(Figure 2). This is similar to the result of the protease activity of *Bacillus subtilis* PE-11 whose maximum activity was found to be at pH 10.0 (Adinarayana *et al.*, 2003).

Temperature optimum and stability

The optimum temperature for the protease activity was 60 °C. The activity gradually declined at temperature beyond 60 °C (Figure 3). Adinarayana *et al.*, (2003) reported a similar result of temperature optimum of 60 °C for protease derived from *Bacillus* sp BZI-2. Thermo stability profile shows that the enzyme activity was stable at 50 °C and 60 °C as it retained almost all its initial activity. Its activity was reduced at 70 °C and 80 °C respectively (Figure 4).

Lactobacillus brevis protease was however confirmed as stable thermally as it retained 60% activity at 80 °C for 60 min.

Kinetics

The V_{max} and K_m values for thermo stable alkaline protease from *Lactobacillus brevis* were determined from

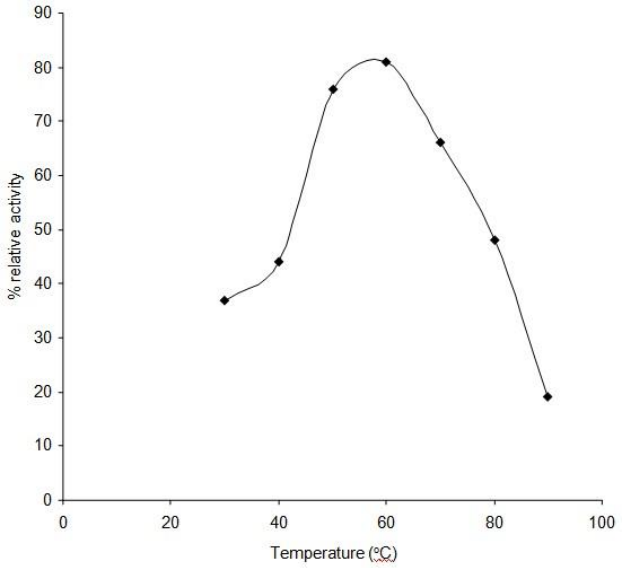


Figure 3: Effect of temperature on the activity of *L. brevis* protease.

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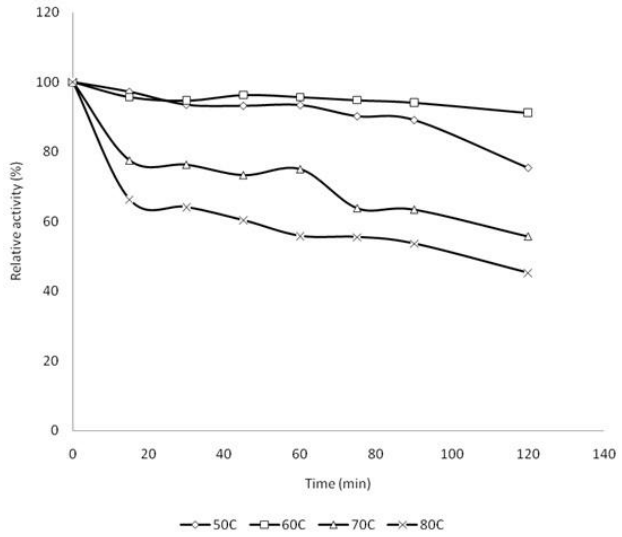

Figure 4: Thermal stability of *L. brevis* protease.

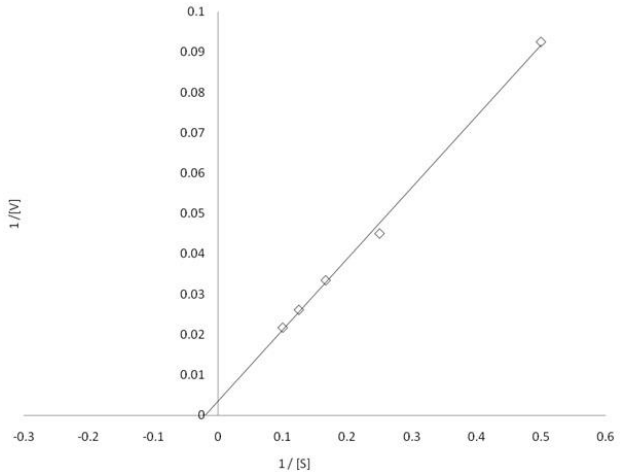

Figure 5: Lineweaver- Burke plot of protease produced by *L. brevis.*

Lineweaver- Burke plots. This revealed that the protease had a V_{max} of 250 U/mg protein and K_m value 50mg/mL (Figure 5). Several authors have reported values far lower than these (Kaur *et al.*, 1998; Devi *et al.*, 2008).

Effect of various salts

The salts that were used are categorized into activators ($CaCl_2$ and EDTA), mild inhibitor (NaCl, KCl, $MgSO_4$ and $FeCl_2$) and strong inhibitor ($CuSO_4$ and $HgCl_2$) (Table 2). Devi *et al*. (2008) reported that $CaCl_2$ enhanced the activity of protease enzyme produced by *Aspergillus niger*. Tsuchiya *et al*. (1987) reported that the proteases isolated from *Cephalosporium* sp. was inhibited by Hg^{2+}, Mn^{2+}, Cu^{2+}, Ca^{2+} and these same ions were found to inhibit the activity of the alkaline proteases secreted by *Bacillus polymyxa* (Kaur *et al*., 1998). Nehra *et al*. (2004) reported that Mg^{2+} activated the alkaline protease produced by *Aspergillus* sp. These reports are in agreement with our results.

De-staining properties with detergents

As shown in Figure 6, after 10 min of incubation at 60 °C the detergent solution supplemented with the enzyme was able to remove the blood strains completely, while the detergent solution only could not remove it. Bhosale *et al*. (1995) has reported high activity of alkaline proteases of *Conidiobolus coronatus* showing compatibility at 50 °C in the presence of 25 mM $CaCl_2$ with varieties of detergents. Adinarayana *et al*. (2003) worked with *Bacillus subtilis* PE II proteases and reported its compatibility and stability with various locally available detergents at 60 °C in the presence of $CaCl_2$ and glycine as stabilizers.

Table 2: Effect of salts on the activity of *L. brevis* protease

Salt	Relative activity (%)
Control	100
NaCl	83
KCl	92
$CaCl_2$	101
$MgSO_4$	79
$CuSO_4$	49
$FeCl_2$	76
$HgCl_2$	34
EDTA	106

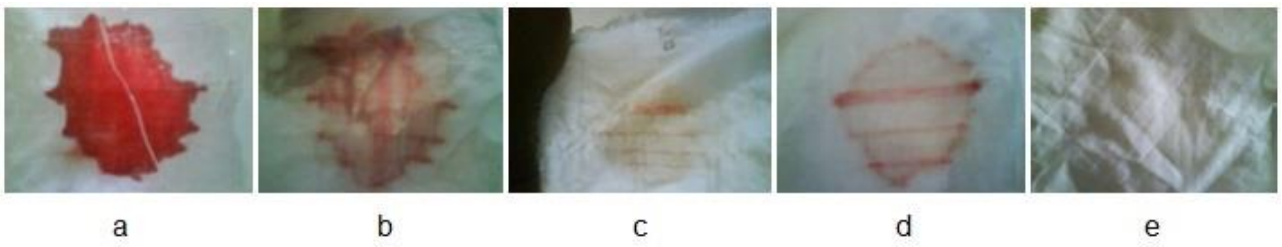

Figure 6: Blood de-staining activity of crude alkaline protease of *Lactobacillus brevis* {a, 0 min; b, 5 min (detergent only); c, 5 min (detergent + enzyme); d, 10 min (detergent only); e, 10 min (detergent + enzyme)}.

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