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Purification and Kinetic Parameters Characterization of an Alkaline Protease Produced from *Bacillus subtilis* through Submerged Fermentation Technique

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Abstract: This paper reported the purification and kinetic parameters characterization of an alkaline protease produced from *Bacillus subtilis* through submerged fermentation process using rice husk as growth supporting substrate (by-product of rice industry) collected from Pearl Rice Mill Hafiz Abad, Faisalabad. *Bacillus subtilis* was cultured in fermentation medium under some pre-optimized growth conditions. Maximum alkaline protease activity of 216±4.32U/mL was obtained when fermentation medium of rice husk was inoculated with 4% (4mL/100mL) inoculum size, 7% substrate concentration at pH 11 for 48 h fermentation time period with 2% molasses as additional supplement material for fermentation medium. An alkaline protease was purified 1.49-fold with specific activity of 74.66 U/mg in comparison to crude enzyme extract using ammonium sulfate precipitation, dialysis and Sephadex-G-100 column chromatography. The enzyme was shown to have a relative low molecular weight of 27kDa by sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE). The enzyme displayed 10 and 45°C as an optimum pH and temperature respectively. Using casein as substrate, the enzyme showed maximum activity (V_{max}) of 148U/mL with its corresponding K_M value of 58µM. Among activators/inhibitors EDTA and Ca^{2+} gave enhancing effect on purified alkaline protease where as SDS, Tween-81, Na^{+} and Hg^{2+} caused enzyme inhibition and inactivation to variable extents. The specific activity and substrate affinity of this alkaline protease from *Bacillus subtilis* is greater than those of other reported *Bacillus sp*; therefore, it was concluded that it may be potentially useful for industrial purposes.

Key words: Alkaline protease · *Bacillus subtilis* · Purification · SDS-PAGE · Characterization

INTRODUCTION

Proteases (EC 3.4.21-24) are enzymes that hydrolyze proteins and form a bulky cluster of enzymes which are ubiquitous in nature and most central category of enzymes from an industrial point of view. An extensive range of microorganisms has great potential to produce alkaline proteases under suitable growth conditions [1]. Bacteria are the most dominant group of alkaline protease producers with the genus *Bacillus* being the most prominent and serve as an ideal source of these enzymes [2, 3] because of their rapid growth and limited space required for their cultivation [1]. An ease with which they can be genetically manipulated to generate new enzymes with altered properties that is desirable for their diverse applications [4].

The vast diversity of proteases, in contrast to their mode of action and specificity, has attracted worldwide attention in attempts to exploit their physiological and biotechnological application [5, 6]. Proteases are the one of the most important groups of industrial enzymes used in detergent, protein, brewing, meat, photographic, leather, dairy industries, pharmaceutical and food industry [7, 8]. They used in pharmaceutical and food industry for peptide synthesis in leather industry for dehairing and in detergent industry as an additive of detergent formulation [2, 9] to substitute currently used toxic chemicals, is a relatively new development and has conferred added biotechnological importance [6].

Alkaline proteases have traditionally detained the predominant share of the industrial enzyme market accounting for about 60-65% of total global sale of enzymes [6, 10, 11]. About 35% of total microbial enzymes used in detergent industry are derived from bacterial sources and most of them produced by *Bacillus* sp. [12, 13]. *B. subtilis* is a highly favorable bacterium for

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protease production because it is non pathogenic, well explored as a model of gram positive bacteria and have remarkable potential to synthesize various types of protease.

With respect to the factors affecting culture conditions, productivity and properties of alkaline protease, it was considered of significance to purify and characterize this enzyme through kinetic studies by studying the effect of varying pH, temperature, substrate concentration and activators/inhibitors to explore the factors affecting their activity. In this paper we aimed to purify alkaline protease from *Bacillus subtilis* and study the factors affecting the activity to present potential and possible application for industrial purposes.

MATERIALS AND METHODS

Chemicals and Substrate: All the chemicals used were of analytical grade unless otherwise stated and purchased from Merk (Germany), Scharlau (Spain) and Sigma Chemical Co., USA. Rice husk was used as growth supported substrate and collected from Pearl Rice Mill Hafiz Abad, Faisalabad, Pakistan. The substrate was crushed, sun and oven dried (60°C) and stored in moisture free polyethylene bags.

Micro-organism and Inoculum Development: Pure culture of *Bacillus subtilis* available in Enzyme Biotechnology laboratory, University of Agriculture Faisalabad, Pakistan and used as fermentation micro-organism for protease production. Pure culture was grown on nutrient agar slants at 37°C for 24h and preserved at 4°C for one month. The preserved culture was revived on fresh agar slants after every week for whole experiment. The inoculum was prepared by transferring a loopful culture of *B. subtilis* into 100mL of inoculum medium composed of g/100mL; casein hydrolysate, 0.5; peptone, 0.5; NaCl, 0.05; $CaCl_2$, 1; $MgSO_4$, 0.5 and K_2HPO_4, 0.5. The inoculated medium was incubated in a temperature controlled shaker (Eyela-Japan) at 37°C and 140 rpm agitation speed for the propagation of bacter up to 10^{8-10} cells/mL.

Production of Alkaline Protease: An alkaline protease from *Bacillus subtilis* was produced under some pre-optimizes fermentation conditions (4mL/100mL of inoculum size, 7% substrate concentration at pH 11 for 48 hrs fermentation time period and 2% molasses was also used as additional supplement for substrate to get better production of alkaline protease from *Bacillus subtilis*. After stipulated time period, the fermented cultures were harvested by centrifugation at 10000×g for 10 min. at 4°C to get clear supernatant containing enzyme solution. The clear supernatant was used as crude enzyme extract for protease assay and also for purification purposes.

Protease Enzyme Assay: The enzyme activity was determined by the method of McDonald and Chen [14]. According to this method three sets of test tubes were made one was control and the other two were experimental. In all these tubes 2mL of 1% casein in Glycine-NaOH buffer pH 10, was added in all three test tubes. In control 2mL of 1% casein solution, 1mL of enzyme and 3mL of 10% TCA was added. The rest of the two tubes contained 2mL of 1% casein solution and 1mL of enzyme. All these three test tubes were incubated at 60°C for 15 minutes. After the incubation, 3mL of 10% TCA was added in the experimental and then centrifuged for three minutes. Then took 1mL of supernatant in test tube and add 5mL of alkaline copper reagent. After 15 minutes 0.5mL Folin-ciocalteau reagent (diluted in 1:1 ratio i.e. 1mL (Folin-ciocalteau reagent: 1mL distal water) was added in each test tubes and stands for 30 minutes. After the completion of the time the absorbance was read out at 700 nm spectrophotometrically. One unit enzyme activity was defined as the amount of enzyme that releases 1 μg of tyrosine per mL per min under the above assay conditions. Specific enzyme activity was expressed as units/mg of protein.

Determination of Protein Content: Protein contents of the crude and purified enzyme extracts were determined by following the method of Bradford [15] with Bovine serum albumin as standard.

Partial Purification and Dialysis of Protease: Crude extract of protease obtained from Bacillus was centrifuged 10,000g for 15minutes at 4°C to increase clarity to maximum. After obtaining clarity to maximum level, the crude enzyme concentrate was placed in ice bath and crystals of ammonium sulfate were added to attain 40% saturation at 0°C and kept for overnight at 4°C. The resulting precipitate was collected by centrifugation at 10,000g for 20 min at 4°C. The pellet of precipitated proteins was discarded. In the supernatant, more crystals of ammonium sulfate were added to attain 70% saturation at 0°C. It was again kept for a night at 4°C and centrifuged as previously. The pellets were dissolved in minimal volume of 0.1%Tris-Hcl buffer (pH 9) and dialyzed against

distal water for several times with 4 equal changes of water after every 6 hrs to remove ammonium sulfate. Total proteins and activity of partially purified protease were determined before and after dialysis of ammonium sulfate precipitation as mentioned before. The partially purified protease was lyophilized and used for further studies related to gel filtration chromatography and SDS-PAGE for further purification and molecular weight determination, respectively.

Gel Filtration Chromatography: Further purification of partially purified protease was carried out by gel filtration chromatography using Sephadex G-100 (Sigma, USA) column. The column was packed to the height of 120cm in a glass column with an internal diameter of 2.0cm and equilibrated with 0.1 M Tris-HCl buffer (pH 9) [16]. The flow rate was maintained at 0.5 mL min-1. Up to 20 fractions were collected each of 1mL and both the enzyme activity as well as the protein content was determined for each separate fraction, as mentioned in the previous section.

SDS-PAGE for Determination of Molecular Weight: Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) was performed on a 5% stacking and a 12% running gel according to the method of Laemmli [17] to determine the molecular weight of purified protease. To 50µL of protein sample, 50 µL of sample buffer was added and boiled in boiling water bath for 5 min, cooled at room temperature and loaded into the gel. The protein bands were visualized by staining with Coomassie Brilliant Blue G (Sigma) and destaining was done with methanol:glacial acetic acid:water (30:10:60). Then gel was taken to the gel documentation system for pictures of molecular weight of protease. The molecular weight of the purified protease was determined in comparison with standard molecular weight markers (Sigma, USA).

Characterization of Purified Alkaline Protease: The partially purified protease was subjected to characterization through kinetic studies by studying the effect of different pH values (6-13) using 0.2M phosphate buffer, effect of different incubation temperatures (25-60°C), effect of varying concentrations of substrate and effect of various compounds as activators and inhibitors on purified alkaline protease produced from *B. subtilis*. The enzyme activities for each case were determined under standard assay conditions using casein as substrate as described earlier.

RESULTS AND DISCUSSION

Production of Alkaline Protease: Bacillus subtilis was cultured in fermentation medium containing rice husk as growth supported substrate under optimum course of action and maximum alkaline protease activity of 216±4.32U/mL obtained when rice husk as a substrate was inoculated with 4% (4mL/100mL) inoculum size, 7% substrate concentration at pH 11 for 48 h fermentation time period with 2% molasses as additional supplement material for fermentation medium. Among the several growth factors particularly substrate particle size is one of the most critical parameter affecting the productivity and growth of microorganisms [18]. It has been reported in literature that a low cost substrates like wheat flour, wheat bran, rice straws (husk) and molasses are suitably effective for growth and enzyme production [19, 20].

Extraction and Purification of Alkaline Protease: After stipulated fermentation time period, the fermented broth was centrifuged at 9000×g for 10 minutes at 4°C to get clear supernatant extract containing crude enzyme. The supernatant with alkaline protease activity of 43200 U/200mL and specific activity of 50 U/mg was used as crude enzyme solution and subjected to partial purification by ammonium sulphate precipitation in two fractions of 0-40% and 40-70%. The crude enzyme was precipitated at 70% saturation with specific activity of 55.71 U/mg and 1.11 fold purification. The pellets (precipitate) were dissolved in minimal volume of 0.1 M Tris-Hcl buffer (pH 9) and dialyzed against distal water with 4 equal changes of water after every 6 hrs to remove the extra salt. This dialyzed fraction was made up to a known volume and referred to as partially purified alkaline protease and loaded on Sephadex-G-100 gel filtration column. By gel filtration the enzyme was purified to 1.49 fold with a yield of 3.11 % and specific activity of 74.66 U/mg (Table 1).

SDS-PAGE: The purified alkaline protease was resolved on a SDS-PAGE (5% stacking and 12% running gel) found to be a homogenous monomeric protein as evident by a single band corresponding to 27kDa on SDS-PAGE relative to the standard molecular weight markers (Fig. 1). In literature, the alkaline protease from *Bacillus* sp. is reported as a single band with molecular weight ranging from 16-32kDa [21, 22]. Patel *et al.* [23] also reported a single band protease with the molecular weight of 30kDa where as, other halophilic and alkaline proteases have molecular weight in range from 40 to130kDa [24, 25].

Table 1: Purification summary of an alkaline protease produced from *Bacillus subtilis* under optimum fermentation conditions

Sr. No.	Purification Steps	Total Volume (mL)	Total Enzyme Activity (IU)	Total Protein Content (mg)	Specific Activity (U/mg)	Purification fold	% Yield
1	Crude Enzyme	200	43200	864	50	1	100
2	$(NH_4)_2SO_4$ Precipitation	30	5850	105	55.71	1.11	13.54
3	Dialysis	25	4000	67.5	59.25	1.18	9.25
4	Sephadex-G-100	12	1344	18	74.66	1.49	3.11

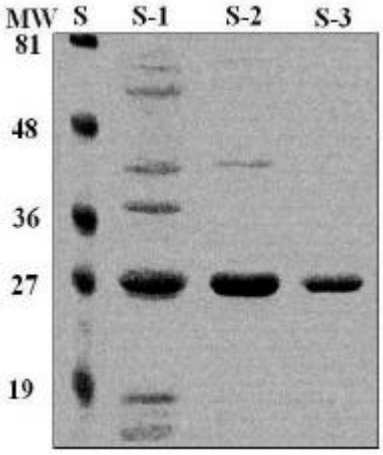

Fig. 1: Molecular mass determination of purified alkaline protease by SDS-PAGE
[Lane MW, Molecular weights in kDa of standard marker; lane S, standard protein markers; lane S-1, Crude alkaline protease; lane S-2, Partially purified Alkaline protease; lane S-3, Purified Alkaline protease]

Characterization of Purified Alkaline Protease

Effect of pH on Protease Activity: The experiment was carried out to investigate the effect of different pH values on the purified protease enzyme. The purified enzyme extract was incubated at different pH values ranging from 7-13 using 0.2M phosphate buffer. Normal enzyme assay as described earlier was performed after ten to fifteen minutes of incubation using casein as substrate on spectrophotometer at the wavelength of 700 nm. Results of enzyme assay showed that the alkaline protease enzymes was completely stable in a large alkaline pH range (9-11) and presented an optimum activity for 126U/mL at a pH value of 10 (Fig. 2) whereas any further increase in pH up to 12 showed decreasing trend in activity. The pH optima for alkaline protease of *Bacillus* sp has been reported to be vary from 8-11 and species specific [26, 27]. Our results are correlated with Adinarayana *et al*. [13]; Patel *et al*. [23] and Deng *et al*. [28] who reported that enzyme was active in the range of pH 8.5-12 with an optimum between pH 10 and 11.

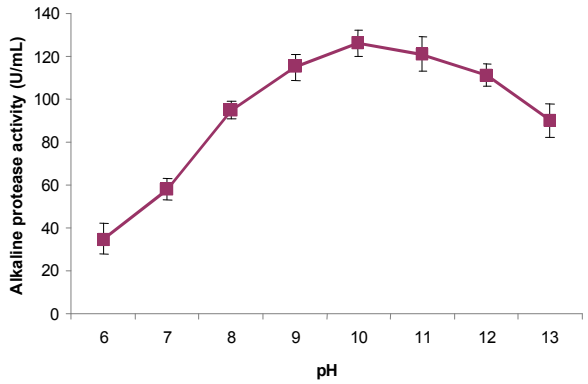

Fig. 2: Effect of varying pH values on purified alkaline protease activity

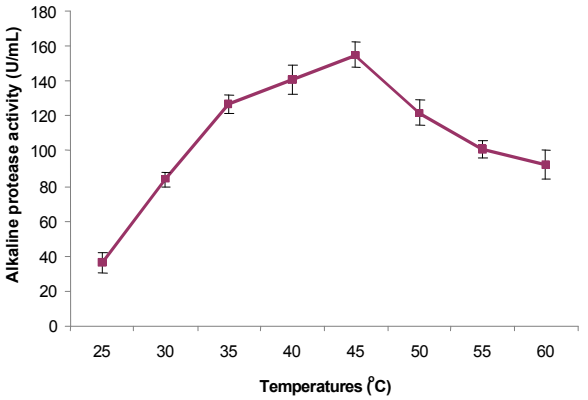

Fig. 3: Effect of different temperatures on purified alkaline protease activity

Effect of Temperature on Protease Activity: The experiment was conducted to determine the effect of different incubation temperatures (25-60°C) on the purified protease enzyme. The purified alkaline protease was incubated under temperature controlled conditions. After ten to fifteen minutes of incubation protease was assayed to determine the effect of temperature on enzyme activity with the same procedure as mentioned previously. Temperature optimum for purified alkaline protease was observed at 45°C. Results of Fig. 3 showed that at temperatures higher than 45°C enzyme starts to losses its activity rapidly. For a variety of industrial applications relatively high thermostability is an attractive and desirable characteristic of an enzyme [4, 29, 30].

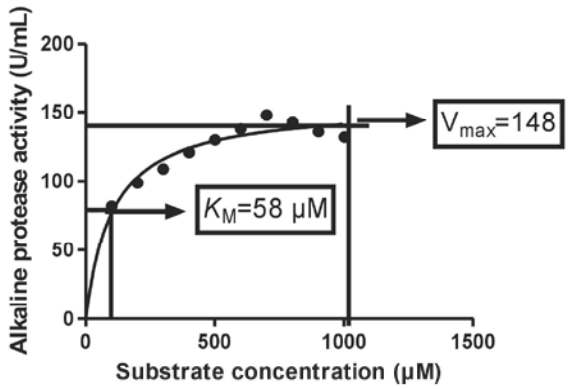

Fig. 4: Determination of K_M and V_{max} for purified alkaline protease through Michaelis-Menten equation

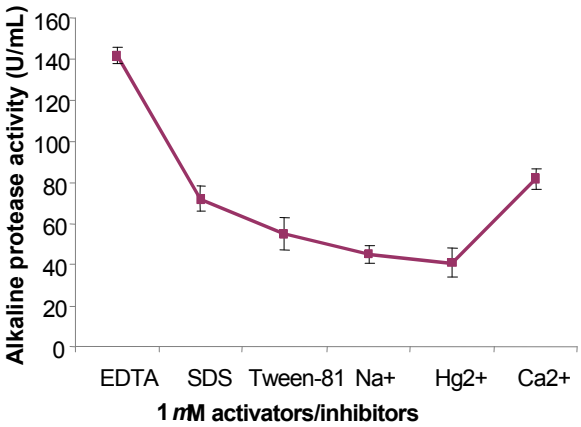

Fig. 5: Effect of various activators and inhibitors on purified alkaline protease activity

Hussain *et al*. [26] reported maximum proteolytic activity at 40°C while, further increase decrease the activity and showing 80% loss in activity at 70°C. Kalpana *et al*. [31] reported maximum enzyme activity of alkaline protease from *Aspergillus niger* at 45°C.

Effect of Substrate Concentration: Determination of K_M and V_{max}: Enzymes are natural catalysts that speed up the chemical reactions. However, the speed of any fastidious reaction being catalysed by a particular enzyme can only reach a certain maximum value. This rate is known as V_{max} while, K_M can define as the concentration of substrate at which half of the maximal velocity obtained. The Michalis-Menten kinetic constants K_M and V_{max} for purified alkaline protease were determined by using varying concentration of casein ranging from 100-1000µM. Enzyme activities were measured under standard assay conditions as described earlier and results obtained were plotted as a graph of Enzyme activity (U/mL) against concentration of substrate [µM], which yielded a hyperbolic curve, as shown in the Fig. 4 with K_M and V_{max} values. The relationship between rate of reaction and concentration of substrate depends on the affinity of the enzyme for its substrate. This is usually expressed as the K_M (Michaelis constant) of the enzyme. From the catalytic properties, K_M and V_{max} values of purified alkaline protease from *Bacillus subtilis* were 58µM and 148U/mL respectively. An enzyme with low K_M has a greater affinity for its substrate. An alkaline protease is highly substrate specific and exhibit maximum activity towards casein as substrate [1, 32]. Adinarayana *et al*. [13] reported that protease have a high level of hydrolytic activity against casein as substrate and poor to moderate hydrolysis of BSA and egg albumin respectively. Patel *et al*. [23] reported the K_M and V_{max} of Ve1 protease were 0.153 g/100mL and 454 U/mL respectively.

Effect of Various Activators and Inhibitors: An effect of various compounds (EDTA, SDS, Tween-81, Na^+, $Hg2^+$ and Ca^{2+}) as activators and inhibitors on purified protease was studied with 1 *m*M of concentration. The purified alkaline protease extract was incubated along with activators and inhibitors for ten to fifteen minutes at 45°C followed by the normal assay protocol using casein as substrate. As shown in Fig. 5 that among various compounds and metal ions SDS, Tween-81, Na^+ and Hg^{2+} showed inhibitory effect on purified alkaline protease whereas, the enzyme activated by EDTA and Ca^{2+} at a concentration of 1*m*M. Joo and Chang [8] reported that SDS used with H_2O_2, slightly inhibit the proteolytic activity. Gupta *et al.* [33] reported that as the concentration of SDS increased, the activity of protease decreased. From metal ions such as Ca^{2+}, Mg^{2+} and Mn^{2+} increased the protease activity and Hg^{2+} and Na^+ ions (1*m*M) resulted in utmost inhibition respectively [3,34, 35]. An inhibtory effect of Tween 20, Tween 80 and Tween 81 were also reported by Gupta *et al.* [33] and Nadeem *et al.* [36]. Ramakrishna *et al*. [3] reported that EDTA slightly activated the protease activity whereas, according to the report of Arulmani *et al*. [1] EDTA showed mild inhibitory effect on serine protease from thermostable alkalophilic *Bacillus laterosporus*-AK1.

In conclusion, the purified alkaline protease has molecular weight of 27kDa with an optimum activity at pH 10 and 45°C temperature. The purified enzyme showed maximum activity (V_{max}) of 148U/mL with its corresponding K_M value of 58µM. The specific activity and substrate affinity of this alkaline protease from *Bacillus subtilis* is greater than those of other reported *Bacillus sp*; therefore, it is concluded that it may be potentially useful for industrial purposes.

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