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"Production of Alkaline Protease by *Bacillus Circulans* Using Submerged Fermentation, Their Purification and Characterization"

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Abstract

In the present study, an extracellular alkaline protease of Bacillus circulans was cultivated on modified Reese medium at a pH of 9.5, temperature of 28°C in 168 hours of incubation. This strain was purified 16.76 folds with specific activity of 1101.0 U/mg in comparison to crude extract using ammonium sulphate precipitation (30-60%), dialysis and DEAE-Cellulose anion exchange chromatography. The molecular weight of the purified enzyme was found to be 30 kDa on Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The characterization of purified enzyme revealed 9.5 and 70°C as an optimum pH and temperature, respectively. The enzyme was activated by Ba^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+} and inhibited by Hg^{2+} and EDTA. The results thus suggested that the alkaline protease of B. circulans is a metalloprotease with serine at its active centre. The specific activity of this alkaline protease from Bacillus circulans is greater than those of other reported Bacillus sp; therefore, it was concluded that it may be suitable candidate for commercial applications.

Key words: Alkaline protease, Bacillus, Purification, Characterization, SDS-PAGE.

Introduction

Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total global enzyme market [1]. Alkaline proteases (EC 3.4.4.16) accounting for 25% of the total enzyme market are being largely used in detergent and leather industries [2-3]. Among industrial enzymes, they have a pivotal role in application areas ranging from domestic to environmental pollution abatement to neutraceutical application, healthcare product to diagnostic kit development and value-added product production to clinical applications [4-5] and also have wide range of commercial usage in detergent, leather, food, feed, recovery of silk from cocoons and that of silver from used X-ray films [6-8]. Although proteases are widespread among living organisms, microbes serve as a preferred source of these enzymes because of their rapid growth, feasibility of mass culture, limited space for cultivation, broad biochemical diversity, simplicity for generation of new recombinant enzymes with desired properties (genetic manipulation) and the ease of separation [9-10].

Among microorganisms, the genus "*Bacillus*" is an important source of industrial alkaline proteases and is probably the only genus being commercialized for alkaline protease production [11-12]. The reason for this is their wide temperature and pH tolerance and thermal stability. Although strains like *B. licheniformis* and *B. lentus* have been commercialized [13], efforts are being carried out in different laboratories to isolate and characterize more *Bacillus* species and strains with high alkaline protease activities [14-15]. In this context, a potential alkaline protease producing bacterial strain was isolated and identified as *Bacillus circulans* (procured from department of microbiology, PAU, Ludhiana) [16]. The latter has been characterized for optimum fermentation parameters for enzyme production with respect to development of low cost and easy available medium ingredients to fit for commercial use [17-18] and also molecularly characterized [19].

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, enzyme concentration and activators/inhibitors to explore the factors affecting their activity. In this paper we aimed to purify alkaline protease from *Bacillus circulans* and study the factors affecting the activity to present potential and possible application for industrial purposes.

Materials and Methods

Inoculum preparation and production of alkaline protease

The bacterial strain used in this work, *Bacillus circulans* was procured from Department of Microbiology, Punjab Agricultural University, Ludhiana. The inoculum of *B. circulans* was prepared by transferring a loopful culture of *B. circulans* into 250 ml Erlenmeyer flasks containing 50 ml of sterile inoculum Na_2CO_3-10.0 with a pH of 9.5 [20]. The inoculated medium was incubated on an orbital shaker at 150 rpm, 28°C for 48 h. Flasks (500 ml capacity) in triplicate containing 200 ml of production medium were inoculated with 2% inoculum (10^6 cells/ml) of 48 hours old inoculum culture. The flasks were incubated on an orbital shaker incubator (150 rpm) at 28°C. The samples were drawn (aseptically) periodically after every 24 hours, spun at 10,000 rpm for 10 min at 4°C and supernatant so obtained was used for estimation of enzyme activity and total soluble proteins [21].

Assay of Proteolytic Activity

Alkaline Protease activity was determined in a reaction mixture (3 ml) containing 0.1 ml of enzyme, 2 ml of 0.5% casein (in carbonate-bicarbonate buffer, 0.1 M, pH 9.5) and 0.9 ml of distilled water and was incubated at 60°C for 15 minutes. The reaction was stopped by adding 3 ml of 5% trichloroacetic acid (TCA) and free amino acids released by crude protease from casein hydrolysis were estimated by Lowry method [21]. An enzyme blank was also run along with the sample. The standard curve was prepared using 0.01-0.1 mM/ml of L-tyrosine. The amount of amino acids released in the reaction mixtures was analysed and calculated by comparing OD_{520} values with the standard curve.

The protease activity was defined as micro moles (µM) of tyrosine released per minute per ml of crude enzyme.

Extraction and Purification of alkaline protease of B. circulans

Extraction of the enzyme

The alkaline protease production by *B. circulans* was carried out in a 500 ml four flasks each containing 250 ml of Reese medium which was inoculated with 2.0% (v/v) of the culture and incubated at 28°C with an agitation of 100 rpm. The crude alkaline protease was prepared by spinning the culture broth at 10,000 rpm for 10 min at 4°C. The supernatant was used as crude enzyme preparation and used for determination of extracellular alkaline protease activities and proteins as described above.

Purification of alkaline protease

Ammonium Sulphate Precipitation

The cell free supernatant (obtained by centrifugation at 10,000 rpm for 20 min at 4°C) was concentrated by adding solid ammonium sulphate to get 0-30%, 30-60% and 60-90% saturations sequentially as per protocol [22], stirred for 60 min and left overnight at 4°C. The precipitates were harvested by centrifugation at 10,000 rpm for 20 min, dissolved in minimum volume of 0.1 M Tris-HCl buffer (pH 9.5) and dialyzed against the same buffer for 48 h at 4°C. The dialyzed samples were assayed for protease activity, protein content and purified further by DEAE-cellulose column chromatography.

DEAE cellulose column chromatography

Dialyzed enzyme was loaded on activated DEAE-cellulose column (3.0 cm × 45 cm) equilibrated with 0.1 M Tris-HCl buffer (pH 9.5). The same buffer containing sodium chloride gradient (0.1-1.2 M) was used for elution of enzyme with a flow rate of 1 ml per min. 25 ml buffer of each molarity was used for elution. Fractions of 5 ml each were collected and analyzed for alkaline protease activity and protein content as described earlier. The fractions which

contained the high activities of alkaline protease were separately pooled for further characterization.

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out [23] using a 11% crosslinked polyacrylamide gel. Commassie blue (0.25%) staining was used to detect the protein bands. The pattern of protein bands was recorded on white light transilluminator of a gel documentation system and observed for proteins of desired size in comparison with mobility of individual proteins in the co-run molecular weight marker.

Characterization of purified alkaline protease of B. circulans

Determination of optimum pH

The experiment was carried out to investigate the effect of different pH values on the purified alkaline protease enzyme. The purified enzyme extract was incubated at different pH values ranging from 7 to 11 using casein as a substrate and preparing buffers of different pH values (7-8 in phosphate buffer and 9-11 in carbonate-bicarbonate buffer). The enzyme activities for each case were determined under standard assay conditions as described earlier.

Determination of optimum temperature

Alkaline protease activity was determined at different temperature (30-100°C) levels by incubating the reaction mixture at appropriate temperatures for 15 minutes and analyzed for amino acids released under standard assay conditions.

Effect of enzyme concentration on partially purified alkaline protease

The effect of using different partially purified alkaline protease volumes (ml) on enzyme activity was studied by taking 0.025, 0.05, 0.075, 0.1, 0.2, 0.3, 0.4 and 0.5 ml of purified enzyme per reaction mixture under optimized conditions of pH and temperature.

Effect of various activators and inhibitors

Impact of various compounds $BaCl_2$, $CuCl_2$, $CaCl_2$, $MnCl_2$, $HgCl_2$, $CoCl_2$, $ZnCl_2$, EDTA and ammonium hydroxide as activators and inhibitors on purified alkaline protease was studied with 10 mM of concentration [24-25] by incubating them in reaction mixtures under conditions optimized for alkaline protease.

Results and Discussion

Production of alkaline Protease

Bacillus circulans was cultured in Reese medium under optimum course of action (Fig. 1). The enzyme production was gradually increased with the passage of time and highest enzyme activity (1025.6 U/ml) was obtained on 7th day (168 h) of incubation by using 2% inoculum. It was also observed that prolonged incubation period has been documented to lead auto digestion of the proteases and proteolytic attack by other proteases resulting in decreased the enzyme activity (Table 1). These results are in accordance with Muthulakshmi *et al* [26] worked on *Aspergillus flavus* obtained maximum activity (49.3 U/ml) on 7th day of incubation and *B. cereus* strain S8 has optimum protease production (174.30 U/ml) after 72h of incubation by Lakshmi *et al* [27].

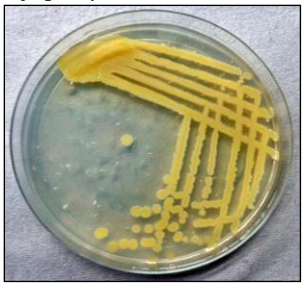

Fig: 1

Fig. 1 Growth of B. circulans on Reese medium

Table 1 Effect of incubation period on the alkaline protease activity.

S. No.	Incubation period (hr)	Enzyme Activity (U/ml)
1	24	354.3
2	48	466.2
3	72	503.4
4	96	596.7
5	120	727.2
6	144	913.7
7	168	1025.6
8	192	839.1

Enzyme purification

The supernatant with alkaline protease activity of 63200 U and specific activity of 65.69 U/mg was used as crude enzyme and subjected to partial purification by ammonium sulphate precipitation in three sequential fractions of 0-30%, 30-60% and 60-90%. A purification of 4.31 folds was achieved with a specific activity of 283.30 U/mg of protein in 0-30% fraction. The process when repeated by increasing ammonium sulphate concentration to 30-60% and 60-90% sequentially, revealed fold purification level of 8.79 and 1.69 folds and specific activity of 577.80 and 111.49 U/mg with a yield of 44.88% and 18.29%, respectively (Table 2). This showed that maximum purification could be achieved in 30-60% ammonium sulphate concentration. Hence, this fraction was subjected to DEAE cellulose anion exchange chromatography for further purification.

Table 2 Purification Profile of Alkaline protease produced by *B. circulans* MTCC 7906

Purification Step	Volume (ml)	Total Enzyme Units (U)	Total Protein (mg)	Specific Activity (U/mg)	Fold Purification	% Yield
Crude enzyme extract	1000	63200	962	65.69	1	100
Ammonium Sulphate Saturation (%)						
0-30	12.0	6431	22.7	283.30	4.31	10.17
30-60	15.0	28370	49.1	577.80	8.79	44.88
60-90	20.0	11562	103.7	111.49	1.69	18.29
DEAE-Cellulose anion exchange column chromatography	25.0	4184	3.8	1101.0	16.76	6.62

Wide-ranging results of protease purification (4.25-200 folds) with various specific activities (13.33-159381 U/mg of protein) and % recovery (2-21%) have been described for different microbial species [28-29]. The yield and fold enzyme purification was 62% and 1.5 times having specific activity of 45.8 U/mg of protein in *B. pseudofirmus* [30]. Rao *et al* [4] observed 2.2 fold increase of specific activity (1679 U/mg) with 62.2% recovery in *B. circulans*. Ahmed *et al* [31] precipitated the crude enzyme of *B. subtilis* with 1.11 fold purification and specific activity of 55.71 U/mg with 13.54% yield.

DEAE-cellulose anion exchange chromatography

The dialyzed protein fraction (30-60%) was eluted in the form of 78 fractions of 5 ml each (collected at a flow rate of 1 ml min^{-1}) and analyzed for alkaline protease activity and protein concentration. Alkaline protease enzyme was eluted at a NaCl concentration gradient of 0.6-0.7 M. Hence, the protein fractions corresponding to the concentration gradient of 0.6-0.7M were pooled that had a combined specific activity of 1101.0 U/mg with 6.62 fold purification (Table 2). These findings are in accordance with several earlier reports showing 11.9% fold purification using Sephadex G-100 with 9000 U/mg specific activity in *B. circulans* [4]. Among other reports, fold purifications of 40.38, 50 and 1.49 with specific activities of 34171.46 U/mg, 143550 APU/mg and 74.66 U/mg have been reported in *B. polymyxa* B-17, *Bacillus* sp. 2-5 and *B. subtilis*, respectively [31-33].

SDS-PAGE of alkaline protease

The purified alkaline protease resolved on a SDS-PAGE was found to be a homogenous monomeric protein as evident by a single band (Fig. 2a). Molecular weight of the protease was determined by interpolation from a linear logarithmic plot of relative molecular mass versus the Rf value. The molecular weight of the protease band was accordingly calculated and found to be as nearly around 30 kDa (Fig. 2 b). These results are in accordance with literature reports where most of the molecular mass of protease from *B. circulans* was 39.5 kDa [4], *Bacillus firmus* Tap 5 was 34 kDa [34] and from *Bacillus licheniformis* MP1 was 30 kDa [8]. In literature, the molecular masses of alkaline proteases from various *Bacillus* species range between 15-45 kDa [35-36]. Gimenez *et al* [37] and Studdert *et al* [38] also reported halophilic alkaline proteases with molecular weight in range from 40 to 130 kDa. Hence, a wide variation in the molecular weight of alkaline protease from different *Bacillus* sp. is observed. In some *Bacillus* sp., multiple electrophoretic forms of alkaline proteases were observed. The multiple forms of these enzymes may be due to the non-enzymatic, irreversible deamination of glutamine or asparagine residues in the protein molecules or of auto proteolysis [35].

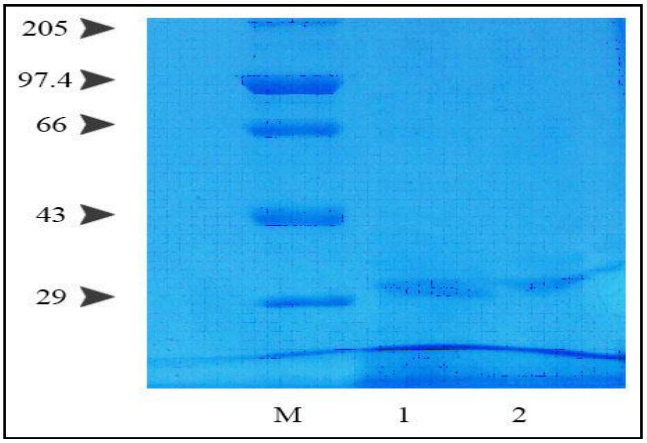

Fig. 2a SDS-PAGE of the purified alkaline protease.

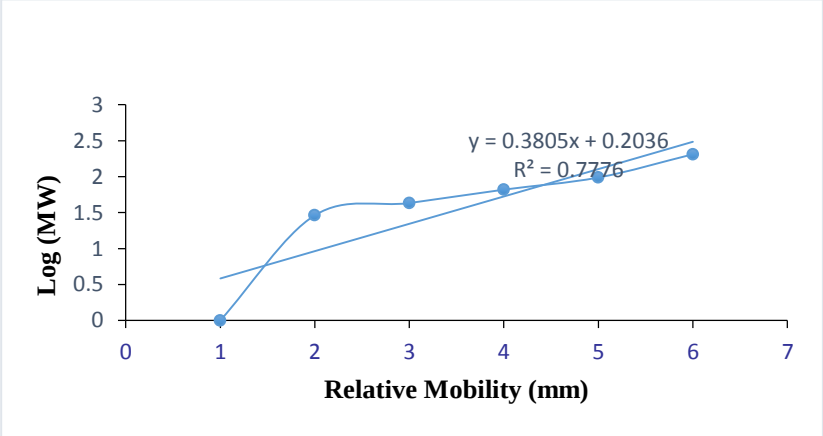

Fig. 2b Molecular weight determination

Characterization of purified alkaline protease

Effect of pH on the activity of alkaline protease

Productivity of the enzyme by culture is greatly dependant on pH of the fermentation medium. Therefore, the effect of pH (7.0 - 10.5) was studied for the production of alkaline protease by *Bacillus circulans*. The enzyme produced was found to be active in alkaline pH range with maximum activity of 1230.7 U/ml at pH 9.5 (Table 3). However, the enzyme activity declined sharply near neutral pH and thereafter decreased rather marginally at alkaline pH levels. Any further variation of the pH of the reaction mixture caused reduction in catalytic activity. This finding was in accordance with previous studies that reported pH optimum for alkaline protease is species specific and found to vary between the range of 7-11 of *Bacillus* sp [39] with a few exceptions of higher pH optima of 12-13 [40].

Table 3 Effect of pH on the activity of alkaline protease.

S. No.	pH	Enzyme Activity (U/ml)
1	7	634.0
2	8	764.5
3	9	1062.9
4	9.5	1230.7
5	10	1100.2
6	10.5	1025.6

Effect of Temperature on the activity of alkaline protease

Temperature has profound influence on production of microbial enzymes. Analysis of the temperature dependent alkaline protease activity revealed it as a broad range enzyme with maximum activity at 70°C (Table 4). However, further increase in the incubation temperature drastically reduced the enzyme activity. As enzymes are proteins and undergo essentially irreversible denaturation (i.e. conformational

alteration entailing a loss of biological activity) at temperatures above those to which they are ordinarily exposed in their natural environment. For a variety of industrial applications relatively high thermostability is an attractive and desirable characteristic of an enzyme [10]. Elsewhere, reports from literature also suggest the alkaline proteases display maximum activity between 40°C to 85°C [41-42]. Our results are correlated with Rao *et al* [4] and Tambekar and Tambekar [43] who reported that 70°C as an optimum temperature for alkaline protease of *B. circulans* and *B. pseudofirmus*, respectively.

Table 4 Effect of Temperature on the activity of alkaline protease.

S. No.	Temperature (ºC)	Enzyme Activity (U/ml)
1	30	1137.5
2	40	1156.1
3	50	1249.4
4	60	1268.0
5	70	1305.3
6	80	1230.7
7	90	1025.6
8	100	876.4

Effect of enzyme concentration

The effect of enzyme concentration on alkaline protease activity was determined at different enzyme concentration ranging from 0.025-0.5 ml. It was observed that optimum enzyme concentration required for maximum activity of protease was 0.1 ml (1268.0 U/ml) (Table 5). The maximum activity at 0.1 ml (100 µl) enzyme concentration is due to the utilization of total amount of substrate in enzyme-substrate complex. Jaswal and Kocher [39] reported that among the different enzyme concentrations (0.05-0.5 ml) in a reaction mixture of 3 ml, 0.1 ml of enzyme that constitutes 3.33% of reaction mixture produced maximum protease activity. In earlier reports, Tambekar and Tambekar [43] reported optimum enzyme concentration for *Bacillus pseudofirmus*, *Cohnella thermotolerans* and *Bacillus odysseyi* were 2.5, 3.5 and 1.5 ml, respectively.

Table 5 Effect of Enzyme Concentration on the activity of alkaline protease.

S. No.	Enzyme Concentration (µl/3ml of reaction mixture)	Enzyme Activity(U/ml)
1	25	317.0
2	50	689.9
3	75	988.3
4	100	1268.0
5	200	1193.4
6	300	1118.8
7	400	1006.9
8	500	932.4

Effect of various activators and inhibitors on the activity of alkaline protease

The effect of metal ions ($BaCl_2$, $CuCl_2$, $CaCl_2$, $MnCl_2$, $HgCl_2$, $CoCl_2$ and $ZnCl_2$) and compounds (EDTA) on alkaline protease activity at a concentration of 10 mM was studied by incubating them in reaction mixtures under optimized protease activity conditions. Among different activators and inhibitors studied for their influence on alkaline protease activity, results revealed that the enzyme activity is increased (Table

6) by $BaCl_2$ (1156.1 U/ml), $CaCl_2$ (1118.8 U/ml), $CoCl_2$ (1006.9 U/ml), $CuCl_2$ (1081.5 U/ml), and $MnCl_2$ (1342.6 U/ml), indicating that enzyme may be a metalloprotease. This fact was further strengthened by the use of a metal chelator EDTA (708.6 U/ml) that inhibited the enzyme activity. The metal ion $HgCl_2$ also inhibited the enzyme activity (634.0 U/ml). Ramakrishna *et al* [41] reported that metal ions such as Ca^{2+}, Mg^{2+} and Mn^{2+} increased the protease activity and Hg^{2+} and Na^{+} ions resulted in utmost inhibition, respectively while EDTA slightly activated the protease activity. Whereas, according to the report of Arulmani *et al* [44] and Tekin et al [45] EDTA showed mild inhibitory effect on serine protease from thermostable alkalophilic *Bacillus laterosporus*-AK1 and *Bacillus cohnii*, respectively.

Table 6 Activity of alkaline protease in the presence of different metal ions and compounds.

S. No.	METAL IONS / COMPOUNDS	ENZYME ACTIVITY (U/ml)
1	$BaCl_2$	1156.1
2	$CaCl_2$	1118.8
3	$CoCl_2$	1006.9
4	$CuCl_2$	1081.5
5	$MnCl_2$	1342.6
6	$ZnCl_2$	1081.5
7	$HgCl_2$	634.0
8	EDTA	708.6

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