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Partial Purification and Characterization of Alkaline Protease from *Bacillus sp.* Isolated from Soil

R. Agrawal, R. Singh, A. Verma, P. Panwar and A.K. Verma

Department of Biochemistry, GBPUA &T, Pantnagar, Uttarakhand 263145, India

Abstract: *Bacillus* isolates were analyzed for alkaline protease production on casein containing agar plates and identified by clear zones of casein hydrolysis around colonies. Two gram positive *Bacillus* isolates S5 and C3 which showed the best enzyme production were studied for purification by precipitation and dialysis. The intracellular alkaline protease enzyme was purified about 4 fold with a yield of 9.8% for isolate S5 and about 2 fold with a yield of 18% for isolate C3. The pH and temperature optima of partially purified alkaline protease were determined to be 9 and 40°C, respectively. Such alkaliphilic proteases from these isolates have a great economical and environmental impact to alleviate the pollution problem created by leather and other industries.

Key words: Alkaline protease · *Bacillus* sp. · Organic solvent precipitation · Dialysis

INTRODUCTION

Soil and water pollution from hazardous lime, sodium sulphide, solvents, etc. the end-products of the pre-tanning stages in the leather processing industries is increasing at a high pace. Proteases can be used for the replacement of chemicals involved during tanning procedures to solve this problem. Of particular importance are the proteases with activity at alkaline pH and high temperatures [1, 2].

Protease is responsible for cell growth and differentiation and is found in all living organisms. Out of many commercial protease producers only few bacterial strains are recognized. *Bacillus sp*. dominate the industrial sector being active producers of extracellular proteases. Alkaline proteases alone account for 20% of the world enzyme market with their predominant use in leather processing and detergent industries [3]. It has been proved that alkaline protease from *Bacillus* can be used as a dehairing agent, indicating elastolytic, keratinolytic activities and also a low hydrolytic collagen activity [4-7].

In the present study, it could be attemped to isolate bacteria from soil and optimize it for protease production by a promising strain. Different bacterial strains were isolated from soil and screened for their ability to produce protease and two potential producers were obtained. Here, we report the purification and characterization of the extracellular alkaline protease from *B. strains*.

MATERIALS AND METHODS

Materials: All the bacteriological media components were product of Hi-Media, India. All other chemicals were of analytical grade. *Bacillus* isolates were procured from the gene pool of the Dept. of Biochemistry, G.B.P.U.A.T. Pantnagar.

Methods:

Cultivation and Screening of Positive Strains: *Bacillus* isolates (procured from the gene pool of the Dept. of Biochemistry, G.B.P.U.A.T. Pantnagar) plated onto 'Skim Milk Agar' plates [8] containing peptone (0.5% w/v), NaCl (0.5% w/v), agar (1.5% w/v) and skim milk (10% w/v). Plates were incubated at 37°C for 24 hours. A clear zone of skim milk hydrolysis gave an indication of protease producing organisms.

Enzyme Production: Production of protease from *Bacillus* strains was carried out in a 'Protease Specific Medium' [6] containing glucose (0.5% w/v), peptone (0.75% w/v) and salt solution (5% v/v) i.e. $MgSO_4.7H_2O$ (0.5% w/v), KH_2PO_4 (0.5% w/v); and $FeSO_4.7H_2O$ (0.01% w/v) maintained at 37°C for 48 hours in a shaker incubator (120 rpm). The pH of the medium was adjusted. The contents were then centrifuged at 7, 000 rpm for 20 min. at 4°C and the cell-free supernatant was used for determining extracellular protease activity.

Corresponding Author: Ruchi Agrawal, Department of Biochemistry, GBPUA&T, Pantnagar, Uttarakhand 263145, India. Tel: +91- 9045601431.

Optimization of Cultural Parameters: Using Luria Bertani broth, protease production was studied at different pH (4, 7 and 9) and temperatures (40, 50, 60°C). Effect of salt (NaCl) concentration was also studied. Absorbance was taken at 660nm.

Enzyme Assay: Alkaline protease activity was measured by Yang & Huang Method [9]. Absorbance was read spectrophotometrically at 280 nm. 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. A blank was run in the same manner, except the enzyme was added after the addition of TCA. All experiments were done in duplicate.

Protein Determination: Total protein content was measured by Folin-Lowry Method [10] using bovine serum albumin (BSA) as a standard protein.

Partial Purification of Crude Enzyme

Organic Solvent Precipitation: Acetone was added to the cell free culture supernatant up to 70% saturation, precipitates were centrifuged at 10,000 rpm for 10 min at 4°C and resuspended in 0.1 M phosphate buffer (pH 9).

Dialysis Against Distilled Water and Buffer: The obtained organic solvent precipitate (in solution) was introduced into 'dialysis tubing' for dialysis against distilled water for overnight, followed by dialysis against phosphate buffer at pH 7.0. The obtained protease enzyme preparation was concentrated against crystals of sucrose and then by lyophilization. All steps were performed at 4°C. The fraction obtained was then assayed for enzyme activity.

Enzyme Characterization

Effect of Salt (NaCl): NaCl in various concentrations of 0, 0.2, 0.4, 0.6 M was added into the substrate and protease assay for crude and purified enzyme was carried out.

Effect of pH: The effect of pH on enzyme activity was evaluated with 0.05 M citrate buffer (pH 4) and 0.05M phosphate buffer (pH 7, 9). Enzyme assay was carried out.

Effect of Temperature: The effect of temperature on enzyme activity was assessed by carrying the enzyme assay at various temperatures (37-60 °C). Enzyme activity was measured as described earlier.

RESULTS

Growth and Protease Production: The two strains S-5 & C-3 showed maximum growth and maximum enzyme production after 48 hours showing a large clear zone of hydrolysis on milk agar (Fig. 1).

Optimization of Cultural Parameters: Using Luria Bertani broth, protease production was studied at different pH (4, 7 and 9) and temperatures (40, 50, 60°C). Effect of different salt (NaCl) conc. (0, 0.2, 0.4, 0.6 M) was also studied. Absorbance was taken at 660nm. The highest protease production was found to be at pH 9.0, 40°Cand 0.2 M salt conc. for both the strains (Fig.2-4).

Partial Purification of Protease from S-5 and C-3strains: Protease activities were measured for the cell free filtrate obtained after organic solvent precipitation and dialysis. The summary of purification profile of alkaline protease from S-5 and C-3 isolates is presented in Tables 1 and 2, respectively.

Characterization of Partially Purified Enzyme

pH Optimum: For the determination of the pH optimum, citrate (pH 4.0), phosphate (pH 7.0, 9.0) and glycine-NaOH (pH 10.0) buffers were used. The highest protease activity was found to be at pH 9.0 using phosphate buffer for both the strains (Fig. 5).

Temperature Optimum: The activity of the crude and purified enzyme was determined at different temperatures ranging from 30°C to 90°. The optimum temperature recorded was at 40°C for protease activity (Fig. 6).

Table 1: Purification Profile for the strain S-5

Sample	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude enzyme	10.854	39.3	0.76	100	-
70% Organic Solvent Precipitation	2.782	10.08	2.8	25.6	3.6
Dialysis	1.068	3.14	3.4	9.8	4.4

Table 2: Purification Profile for the strain C-3

Sample	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude enzyme	10.68	38.70	1.53	100	-
70% Organic Solvent Precipitation	3.16	11.44	2.46	29.6	1.6
Dialysis	1.926	6.97	2.76	18.03	1.8

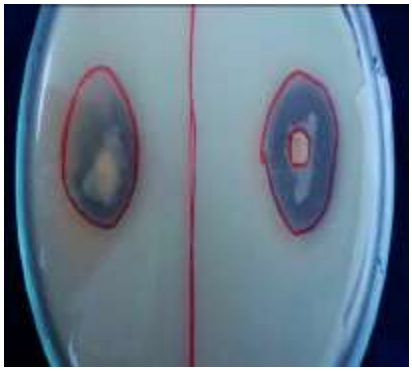

Fig. 1: Zone of hydrolysis for the two strains S-5 & C-3 on 'skim milk agar'.

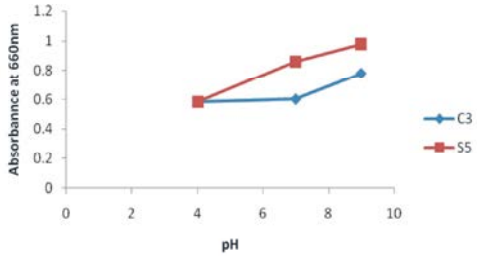

Fig. 2: Effect of pH on the growth of the isolates

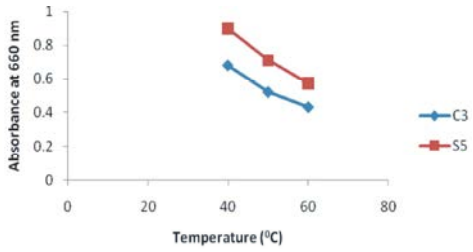

Fig. 3: Effect of temperature on the growth of the isolates at pH 9.0

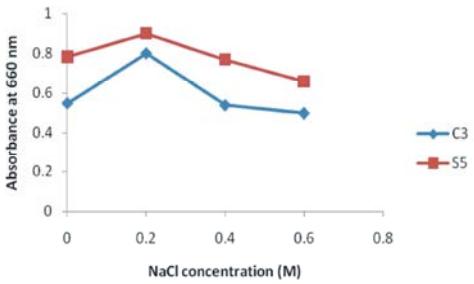

Fig. 4: Effect of NaCl concentrations on the growth of the isolates

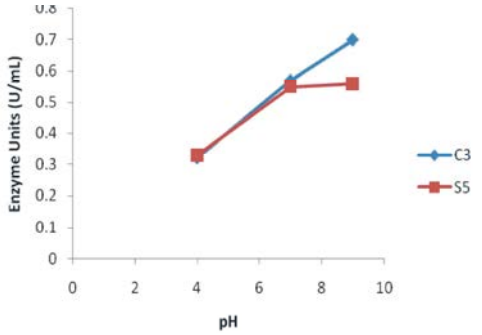

Fig. 5: Optimization of pH for partially purified proteases from the isolates

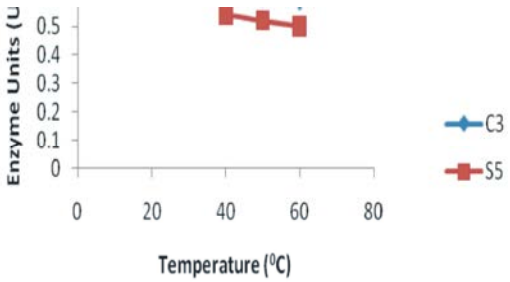

Fig. 6: Optimization of temperature for partially purified proteases from the isolates

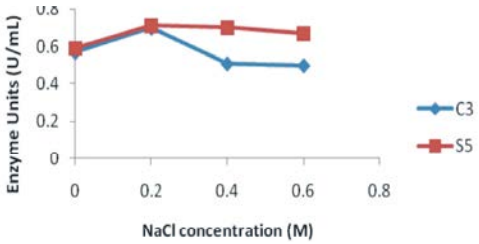

Fig. 7: Optimization of salt conc for partially purified proteases from the isolates

Optimum Salt (Nacl) *Concentration*: Various NaCl conc. (0, 0.2, 0.4, 0.6 M) were taken to determine optimum salt conc. which was found to be 0.2 M for both the strains (Fig. 7).

DISCUSSION

Studies on the effect of different parameters on the growth of the two strains S5 and C3 indicated pH 9, 37°C and 0.2 M to be the optimum pH, incubation temperature

and medium salinity for the cell growth, respectively. The optimum incubation time and agitation speed were 48 h and 120 rpm for both growth and the protease production (data not shown). However, *Bacillus proteolyticus* CFR3001 isolated from fish processing wastes (both fresh water and marine) produced an alkaline protease with optimum conditions for cell growth and protease production being 37°C, 96 h, agitation speed of 100 rpm and medium pH 9 [7].

The activity profile of the supernatant from the two strains grown under optimum conditions as compared to partially purified protease is shown in Tables 1 & 2. Upon partial purification, the specific activity increased up to 3.4 U/mg compared to 0.76 U/mg for the crude enzyme, purification fold and yield being 4.4 and 9.8% for the strain S5. Similar results were reported in *Bacillus proteolyticus* CFR3001, where the activity increased by >4-folds upon partial purification [7]. While for that of the strain C3, it was 2.76 U/mg increased from 1.53 U/mg of the crude enzyme, purification fold about 2 but with a yield of about 18%.

The optimum pH for the original protease concentrate (i.e. ammonium sulphate precipitated and dialyzed fraction) from both the strains were 9 as also reported in *Alcaligenes faecalis* by Berla and Suseela, 2002. On the other hand, the protease enzyme had an optimum pH of around 8 [11-12]. The optimum incubation temperature was 37°C at 0.2 M salt concentration. However, the partially purified enzyme from the two strains showed comparable activity over a range of 40°C to 60°C and partially purified enzyme from the strain S5 was able to tolerate a salinity level up to 0.6 M. *Bacillus proteolyticus* CFR3001 alkaline protease was active between 40°C and 50°C and lost >20% of its activity around 60°C [7]. However, *Yersinia ruckeri* exoprotease was more active in the range of 25 to 42°C and had an optimum activity at 37°C [11].

CONCLUSION

A high optimum pH is a feature of alkaline proteases *(8)*. In our study, proteases produced by two different *Bacillus* strains showed activity even at high temperatures. The alkaline proteases isolated from isolates S-5 and C-3 are thermostable and halophilic proteases. These have a relevant economical and environmental impact to alleviate the pollution problem created by leather industries as it has desirable properties such as stability at alkaline pH, high temperature. These properties indicate the possibility of using the protease in the pre-tanning processes of leather and in other biotechnological applications that would require higher working temperatures. Additional work is necessary in order to enable the full characterization (amino acid analysis, column chromatography) of the examined proteases.

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