Global Journal of Molecular Sciences 4 (2): 180-186, 2009
ISSN 1990-9241

Production and Characterization of Protease Enzyme from *Bacillus laterosporus*

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Abstract: Production and partial purification of protease enzyme by *Bacillus laterosporus* was the aim of this study. *Bacillus laterosporus* was allowed to grow in shake flask broth culture for purpose of inducing protease enzyme. The protease enzyme was purified by ammonium sulfate precipitation followed by dialysis and further concentrated by Amicon tubes. After concentration, the protein was subjected to 12 % Zymogram gel with gelatin and the molecular weight of the protease enzyme was 15 kDa. The protease activity increased as the increase in enzyme concentration; optimum substrate concentration (starch) was 1.0% (w/v); an optimum incubation temperature was 40°C. Purified protease enzyme had a maximum activity at pH 7.0 of phosphate buffer and the optimum incubation time was 24 h. The protease isolated from *Bacillus latrosporus* is a mesophilic protease. It is stable at pH 7, at 40°C temperatures and this enzyme can be exploited commercially.

Key words: *B. laterosporus* · PMSF · β-mercaptoethanol · Protease · Fermentation

INTRODUCTION

Proteases are essential constituents of all forms of life on earth including prokaryotes, fungi, plants and animals. Proteases are highly exploited enzymes in food, leather, detergent, pharmaceutical, diagnostics, waste management and silver recovery [1]. Proteases [serine protease (EC. 3.4.21), cysteine (thiol) protease (EC 3.4.22), aspartic proteases (EC 3.4.23) and metalloprotease (EC 3.4.24)] constitute one of the most important groups of industrial enzymes, accounting for about 60% of the total enzyme market [2-4]. Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases [5] and among bacteria, *Bacillus* sp are specific producers of extra-cellular proteases [6]. These enzymes have wide industrial application, including pharmaceutical industry, leather industry, manufacture of protein hydrolizates, food industry and waste processing industry [7].

Thermostable proteases are advantageous in some applications because higher processing temperatures can be employed, resulting in faster reaction rates, increase in the solubility of nongaseous reactants and products and reduced incidence of microbial contamination by mesophilic organisms. Proteases secreted from thermophilic bacteria are thus of particular interest and have become increasingly useful in a range of commercial applications [8, 9].

In this study, we describe the selection of medium components for the optimal production of extra cellular protease by thermophilic *Bacillus* sp strain SMIA-2 and characterization of the enzyme.

MATERIALS AND METHODS

Micro Organism and Maintenance of Culture: The bacterial strain used in this study was the thermophilic *Bacillus laterosporus*, previously isolated from the palletized feed of prawn. The bacterial strain was cultured in 1% LB (Himedia) broth at 40°C for 24 hours and then autoclaved at 121°C for 15 minutes. A loopful of autoclaved broth was plated on nutrient agar plates and incubated at 37°C for 24-48 hours. The culture was routinely maintained on Nutrient agar slants. The organism was subculture for every month. The inoculum was prepared by dispersing the loopful of bacterial culture from a week-old nutrient agar slant culture in 1 % LB broth solution with a sterile inoculation loop.

Biochemical Characterization of Bacterial Isolate: Sub culturing was done for single colony isolation. A loopful of seed culture from overnight sample was streaked on to nutrient agar plate and was incubated for 24 hrs at 37°C.Various biochemical study was done as per the as per Bergey's manual of determinative bacteriology [10] to identify the bacterial isolate.

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Qualitative Estimation of Protease Enzyme: A loopful of strain was dispersed in 9 ml of 1% LB broth medium and the diluted sample of *B.laterosporus* was plated onto pre sterilized skim milk agar plates (Hi media – M763). The plates were incubated at 37°C for 24 hours to check the proteolytic activity.For maximum production of the protease enzyme; we checked the activities of extra cellular protease enzyme production in the culture supernatant at different stages of *B.laterosporus* growth.

Production of Protease in Shake Flask Fermentation: The bacterium strain was cultured in 2.5 L conical flask containing the following ingredients: Yeast extract (0.5g) Casein (0.1g) KH_2PO_4(0.2g) Na_2CO_3 (1.0g) Distilled water (100ml) pH, 7.0. Inoculation was performed with 10% (v/v) seed culture of 1 day grown in 1% LB broth medium. The fermentation conditions were maintained at 37°C, 50 rpm agitation for 2-4 days. The culture broth was harvested and centrifuged at 8,000 rpm for 20 min at room temperature. Cell free supernatants were used for measuring protease activity.

Determination of Protease Activity: The activity of protease was assessed in triplicate by measuring the release of trichloroacetic-acid soluble peptides from 0.2% (w/v) azocasein in 50 mM HEPES/NaOH buffer (pH 7.5) at 50°C for 10 min. The 1-mL reaction was terminated by the addition of 0.5 mL of 15% trichloroacetic acid and then centrifuged at 10.000g for 10 min, after cooling. One unit (U) enzyme activity was defined as the amount of enzyme required to produce an increase in absorbance at 420nm equal to 1.0 in 60 min under the assay conditions [11]. Protein was measured by the method of Lowry, as modified by Petterson [12].

Enzyme Purification: The bacterial strain was grown for 48 hours at 37°C and the culture broth was harvested and centrifuged at 8,000 rpm for 20 min at room temperature. Cell free supernatant was fractionated by ammonium sulfate precipitation. All subsequent steps were carried out at 4°C. The protein pellet obtained after precipitation with ammonium sulphate was resuspended in 0.1M Tris-HCl buffer, pH 7.8 and dialyzed against the same buffer. After dialysis, the contents were collected in the Amicon tubes Ultra-5 kDa (Millipore) and centrifuge at 10000 rpm for 20 minutes at 27°C [13]. The purified enzyme was subjected to 12 % Zymogram gel with gelatin [14].

Effect of Culture Conditions on Enzyme Production: The effects of carbon sources 1% (w/v) on enzyme secretion were investigated replacing trisodium citrate by glycerol, D(+) galactose, lactose, sucrose, maltose, starch, D(+) glucose, D(+) manose, L (+) arabinose, casein, D(+) xylose and citric acid. Different nitrogen sources including Soya oil, Casein, Wheat bran, Tryptone, Skim milk NH_4NO_3, peptone, yeast extract, meat extract, casein, $(NH_4)2SO_4$, $(NH_4)2HPO_4$, NH_4Cl, KNO_3, urea and ammonium citrate were employed in preliminary studies to determine growth and production of extra cellular protease.

Effect of Ph on Activity and Stability of Protease: The optimum pH was determined with azocasein 1% (w/v) as substrate dissolved in different buffers (citrate phosphate, pH 5-6, sodium phosphate, pH 7.0, Tris-HCl, pH 8.0 and glycine NaOH, pH 9-13). The effect of pH on enzyme stability was determined by pre-incubating the enzyme without substrate at different pH values (5.5-9.0) for 24h at room temperature and measuring the residual activity at 40°C. Reaction mixtures were incubated at 40°C for 2 hours and the relative activity of the enzyme was measured at standard assay conditions

Effect of Temperature on Activity and Stability: The effect of temperature on the enzyme activity was determined by performing the standard assay procedure at pH 7.5 within a temperature range from 30 to 100°C. Thermostability was determined by incubation of crude enzyme at temperatures ranging from 30-100°C for 2h in a constant-temperature water bath and the relative protease activities were assayed at standard assay conditions.

Effect of Inhibitors and Chelators on Protease Activity: The effect of various protease inhibitors (5mM) such as serine inhibitors (phenylmethylsulphonyl fluoride [PMSF]] and β-mercaptoethanol [β-ME] and a chelator of divalent cations (ethylene diamine tetra ace-tic acid [EDTA]) were determined by the addition of the corresponding Inhibitors and Chelators at a final concentration of 1.0 mM to the reaction mixture and assayed under standard condition. The relative protease activity was measured [15-17].

Effect of Metal Ions on Protease Activity: The effect of different metal ions on protease activity was determined by the addition of the corresponding ion at a final concentration of 1.0 mM to the reaction mixture and assayed under standard conditions. The enzyme assay was carried out in the presence of KCl, CaCl2, $MgSO_4$, $FeSO_4$, $CoCl_2$, $ZnCl_2$, MnSO4, $HgCl_2$, $CuSO_4$ $BaCl_2$and NaCl. The activity is expressed as a percentage of the activity level in the absence of metal ion. The enzyme was pre-incubated with metal ion (60°C, 5 min.) Separate blanks with individual metal ions were prepared.

RESULTS AND DISCUSSION

The isolated bacterial strain was identified as *Bacillus laterosporus* using various biochemical tests (Table 1) as per the Bergey's manual of determinative bacteriology (Holt et al, 1994). The strain *B.laterosporus* hydrolysis the skim milk and the zone of hydrolysis on skim milk agar is shown in Fig. 1. The highest activity of extra cellular protease enzyme was at 3 days as shown in Fig. 2. The crude enzyme was concentrated using ultra 5kDa Amicon tube (Millipore product) and it was subjected to 12 % Zymogram gel. The molecular weight was determined by interpolation from a linear semi logarithmic plot of relative molecular mass versus the Rf value (relative mobility). Depending on the relative mobility, the molecular weight of the protein band was calculated to be 15 kDa, which coincided with the band of á- lactalbumin marker protein.

B.laterosporus was capable of utilizing a wide range of carbon sources. However, the best carbon sources in the present study, for protease secretion were soluble starch trisodium citrate, citric acid and glycerol (Table 2). In a similar study Johnvesly and Nailk [18] showed that citric acid, soluble starch and trisodium citrate were the best carbon sources for protease production by *Bacillus* sp JB-99. According to these authors, culturing this organism in 1% glucose (w/v) repressed completely the synthesis of alkaline protease. However, in the present study glucose was found to be a relatively good carbon source for enzyme production since moderate amount of protease activity was detected. The type of nitrogen sources also affected enzyme production. Among the various organic and inorganic nitrogen sources, the maximum enzyme activity (100%) was obtained when ammonium nitrate, wheat bran and ammonium chloride was used in the medium (Table 3). Moderate to good levels of enzyme activities were obtained when citric acid, ammonium citrate and potassium nitrate were used as nitrogen sources. When various organic nitrogen sources were tested for protease production, it was found to be increased.

The effect of different metal ions on protease is shown in Table 3. A stronger inhibitory effect was observed in the presence of KCl, BaCl2, CuS04, $ZnCl_2$ and $HgCl_2$ inhibited completely the enzyme at 1mM concentrations. The protease secreted by *Brevibacillus* (*Bacillus*) *brevis* was also inhibited by CuS04, $ZnCl_2$ and $HgCl_2$ [19]. The inhibitory effect of heavy metal ions is well documented in the literature. It is known that the ions mercury, cadmium and lead react with the protein thiol groups (converting them to mercaptides), as well as with

Table 1: Biochemical characterization of *Bacillus laterosporus*

Gram staining	+	Growth at	
Rod shaped	+	37 C	+
Endospores produced	+	40 C	+
Mobility	-	45 C	+
Indole production	-	Growth at	
Methyl red	+	05 % Nacl	+
Voges Prokauer	-	Starch hydrolysis	-
Beta –galactosidase	-	Gelatin hydrolysis	+
Nitrate reduction	+	Catalase	+
TSI	+	Oxidase	+
H2S production (TSI)	-	Urease	+
Gas from TSI	-	Utilization of Esculin	-
Lysine decarboxylase	+		
Ornithine decarboxylase	+	Citrate	-
Arginine decarboxylase	-	Malonate	-
Lecithinase activity	+	Mannital	-
Acid formation from		Tyrosine	+
Arabinose	-	Lecithinase activity	+
Xylose	-	Resistance to Lysozyme	+
Adonitol	-		
Raminose	-		
Cellobiose	-		
Mellibiose	-		
Saccharose	-		
Raffinose	-		
Trehalose	-		
D-glucose	-		
Lactose	-		

Table 2: Effect of the carbon sources on the production of the protease. Cells were grown in the basal medium containing, 1% wheat bran and supplemented with each carbon sources at 37°C for 48 hours

S.No:	Nitrogen source	Concentration	Relative activity(%)
1	Glycerol	1 %	100
2	Galactose	1 %	75
3	Lactose	1 %	86
4	Sucrose	1 %	100
5	Maltose	1 %	89
6	Starch	1 %	100
7	Glucose	1 %	89
8	Manose	1 %	95
9	Arabinose	1 %	65
10	Casein	1 %	91
11	Xylose	1 %	76
12	Fructose	1 %	67
13	Trisodium citrate	1 %	100
14	Citric acid	1 %	95

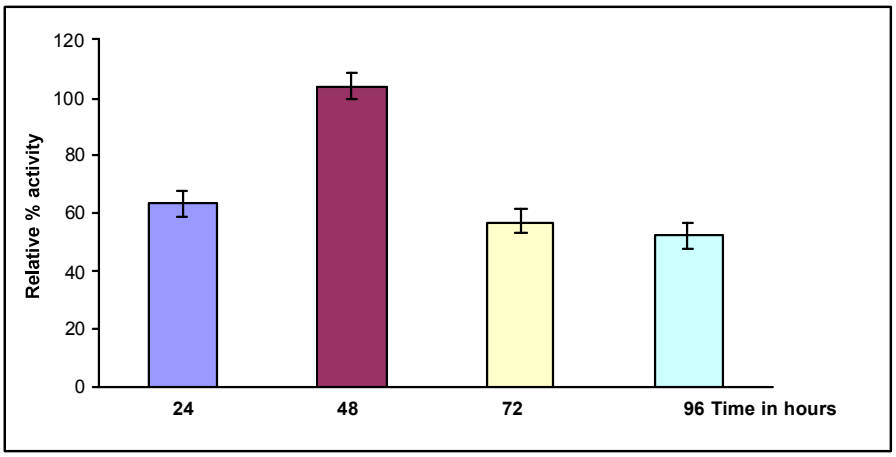

Fig. 1: Protein production in different time intervals

Fig. 2: Zone of hydrolysis of *B.laterosporus* on skim milk agar

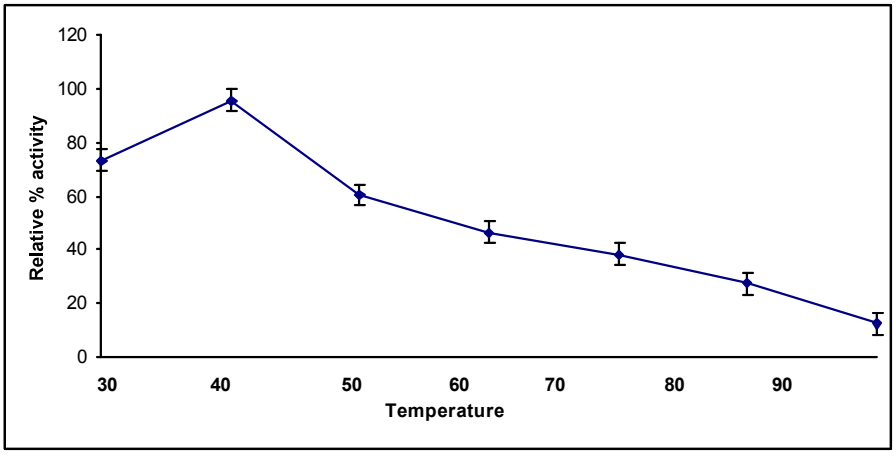

Fig. 3: Effect of temperature on protease enzyme activity

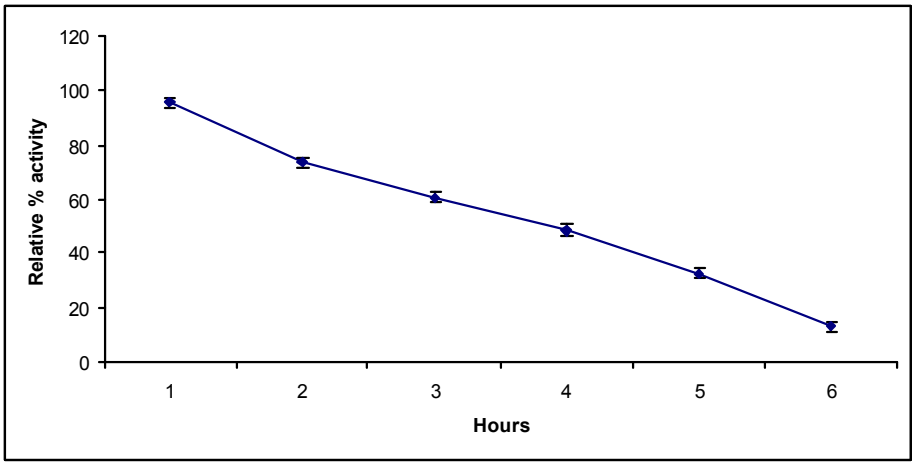

Fig. 4: Stability of enzyme activity at 40℃

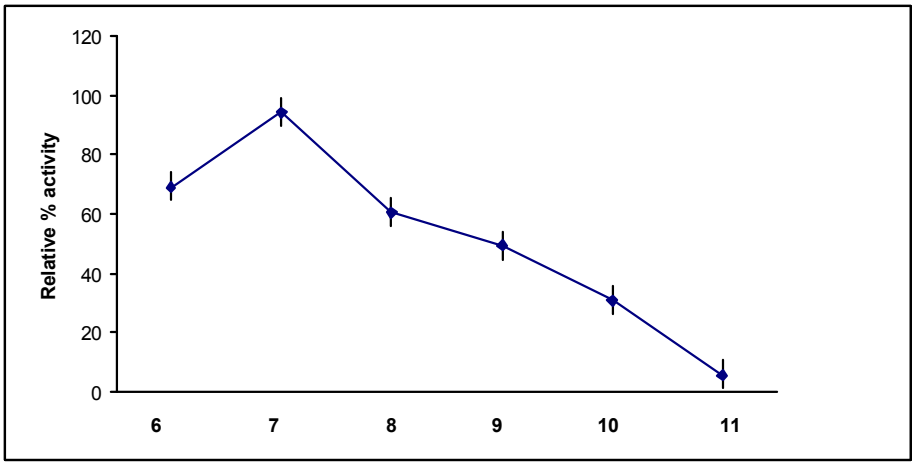

Fig. 5: Effect of pH on enzyme activity

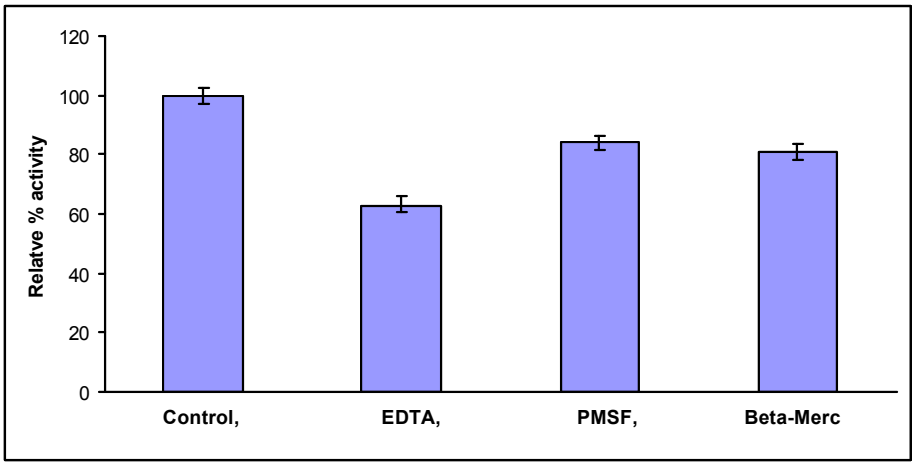

Fig. 6: Effect of inhibitors on protease enzyme activity

Table 3: Effect of the nitrogen sources on the production of the extra cellular protease. Cells were grown in the basal medium containing 1 % glucose and supplemented with each nitrogen source at 37°C for 48 hours

S.No:	Nitrogen source	Concentration	Relative activity (%)
1	Soya oil	1 %	86
2	Casein	1 %	82
3	Wheat bran	1 %	100
4	Tryptone	1 %	73
5	Skim milk	1 %	78
6	Yeast extract	1 %	65
7	Meat extract	1 %	61
8	Ammonium citrate	1 %	98
9	Peptone	1%	45
10	NH_4NO_3	1 %	67
11	$(NH_4)2HPO_4$	1 %	72
12	NH_4Cl	1 %	100
13	KNO_3	1 %	86
14	$(NH_4)2SO_4$	1 %	49

Table 4: Effect of various metal ions on protease activity

S.No:	Nitrogen source	Relative activity (%)
1	KCl	7
2	$CaCl_2$	153
3	$MgSO_4$	69
4	$FeSO_4$	57
5	$CoCl_2$	109
6	$ZnCl_2$	12
7	MnSO4	138
8	$HgCl_2$	0
9	$CuSO_4$	11
10	$BaCl_2$	9
11	NaCl.	29
12	Control	100

histidine and tryptophan residues. Moreover, by action of silver and mercury, the disulphide bonds were found to be hydrolytically degraded [20]. Protease activity was stimulated by $MnS0_4$ $CoC1_2$and $CaC1_2$. These results suggest that these metal ions apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active conformation of the enzyme at higher temperatures [21]. Similar effects of $MnSo_4$ on the activity of proteases were also observed by Rahman *et al.* and by Manachini *et al.* [22, 23].

The protease activities were assayed at different temperatures ranging from 30°C-90°C at a constant pH of 7.0 (Fig. 3, 4). Enzyme activity increased with temperature within the range of 30°C to 40°C. A reduction in enzyme activity was observed at values above 40°C. The optimum temperature of this protease was 40°C. The thermo stability of the protease was examined by measuring the remaining activities at 40°C, after incubation of the enzyme without substrate at various temperatures between 30 and 90°C for 2h. Thermo stability profile indicated that the enzyme was stable at 40°C for 1h and gradually decreased after 1h when incubation increased. A pH range between 6 and 11 was used to study the effect of pH on protease activity (Fig. 5). Optimum pH was found to be 7.0. At pH 6.0 only 63% of the maximum enzyme activity was obtained, increasing to 87% at pH 7.0 and the enzyme activity was declined gradually after pH 7.0. Inhibition studies primarily give an insight into the nature of an enzyme, its cofactor requirements and the nature of the active centre. The effect of different inhibitors on the enzyme activity of the crude protease was studied (Fig. 6). Of the inhibitors tested (at 5mM concentration), EDTA was able to inhibit the protease considerably, while ß mercaptoethanol, PMSF exhibited 18%, 13% inhibition respectively. The protease isolated from *Bacillus latrosporus* is a mesophilic protease. It is stable at pH 7, at 40°C temperatures and this enzyme can be exploited commercially.

ACKNOWLEDGEMENTS

The authors are grateful to the authorities of Biocorporals, Chennai, Tamil Nadu, India for their timely supply of materials all through the study.

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