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Salt-Tolerant and Thermostable Alkaline Protease from *Bacillus subtilis* NCIM No. 64

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ABSTRACT

The proteolytic activity produced by a *Bacillus subtilis* isolated from a hot spring was investigated. Maximum protease production was obtained after 38 h of fermentation. Effects of various carbon and nitrogen sources indicate the requirement of starch and bacteriological peptone to be the best inducers for maximum protease production. Requirement for phosphorus was very evident, and the protease was secreted over a wide range of pH 5–11.

The partially purified enzyme was stable at 60°C for 30 min. Calcium ions were effective in stabilizing the enzyme, especially at higher temperature. The enzyme was extremely salt tolerant and retained 100% activity in 5*M* NaCl over 96 h. The molecular weight of the purified enzymes as determined by SDS-PAGE was 28,000. The enzyme was completely inactivated by PMSF, but little affected by urea, sodium dodecyl sulfate, and sodium tripoly phosphate.

Index Entries: Alkaline protease; salt and thermostable protease; *Bacillus subtilis*.

INTRODUCTION

Extracellularly released alkaline proteases have been known to have applications in leather processing, detergent industry, recovery of silver from X-ray films, and in producing protein hydrolysates. These proteases

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have been isolated from *Bacillus subtilis* (*1–4*), *Bacillus licheniformis* (*5*), *Bacillus amyloliquefaciens*, and *Bacillus stereothemophillus* (*6*).

Different strains of *Bacillus subtilis* produce alkaline serine protease and neutral metalloprotease in different ratios (*7*). Strains that produce only alkaline serine protease have also been reported (*8*).

The present investigation reports the production and characteristics of an alkaline serine protease by a strain of *Bacillus subtilis* NCIM no. 64 isolated by us from a tropical hot spring. The organism also produces an extracellular salt-tolerant amylase (*9*).

MATERIALS AND METHODS

Bacterial Strain

Soil and water samples were collected aseptically from a hot spring. Thermotolerant microorganisms were isolated on nutrient agar plates at 50°C. These strains were then screened for proteolytic activity on skimmed milk agar plates incubated at the same temperature. One isolate belonging to the genus *Bacillus* showed maximum zone of clearance and was selected for further work.

Identification was carried out following methods published in *Bergey's Manual* (*10*), and the microorganism was identified as *Bacillus subtilis* strain NCIM no 64.

Enzyme Production

The strain was cultivated in liquid medium containing (g/L of dist. water): casein 10 g; bactopeptone 10 g; NaCl 3 g; $MgSO_4$ 1 g; K_2HPO_4 1 g; $MnCl_2$ 8.0 mg; $ZnSO_4$ 2.0 mg; and $CaCl_2$ $2H_2O$ 300 mg. All fermentations were carried out with 100 mL of culture media in 500-mL Erylenmyer flasks at 30°C. The cultures were shaken on a rotary shaker at 200 rpm. A 10% inoculum was used to inoculate the production medium. The production medium had the same composition as the inoculum. Cultures were harvested after 38 h, and cell-free extracts of the protease were obtained after centrifugation at 3000*g* for 30 min.

Enzyme Assay

Protease activity was measured by the modified method of Kunitz (*11*) using casein as substrate. One percent of casein substrate was prepared in either 0.1*M* sodium carbonate buffer at pH 9.7 or 0.1*M* Tris-HCl buffer at pH 8.1. The reaction mixture consisting of 1 mL of casein and 1 mL of diluted enzyme was incubated for 20 min at 60°C and then terminated with 3 mL of 5% TCA. The absorbance of the filtrate was measured at 280 nm. Parallel blanks were run with each sample. One enzyme unit was arbitrarily defined as equal to change in the absorbance of 0.001. In

order to compare the protease production with other reports, 1 u of protease activity was also defined as that amount of enzyme required to liberate 1 μg of tyrosine mL^{-1} min^{-1} under the experimental conditions.

Partial Purification of the Enzyme

The total protein of the cell-free extract was precipitated with 2 vol of chilled ethanol at 4°C. The precipitate was dissolved in 10 m*M* Tris-HCl buffer at pH 8.1 (buffer A) and dialyzed against 5 L of the same buffer at 4°C to remove traces of ethanol. The dialyzed sample was centrifuged at 4000*g* for 30 min, and the supernatent was concentrated in an Amicon diaflow apparatus fitted with a YM-10 membrane.

The concentrate (2 mL) was loaded on a Bio-gel P100 (Bio-Rad, USA) column (1.5× 100 cm). Buffer A was run at a flow rate of 10 mL/h, and 2-mL fractions were collected. Fractions showing protease activity were pooled and concentrated. This fraction was used as a partially purified preparation to study the enzyme characteristics. For the determination of molecular weight, the partially purified enzyme was then loaded onto a preparative gel electrophoresis, and after activity staining the casein agarose, the appropriate band was cut and the eluted protein was run on 10% SDS PAGE with appropriate standards. By this method, the molecular weight was determined to be 28,000± 1000.

Effect of Calcium Ions and Protease Inhibitors

The effect of calcium ions and inhibitors on enzyme activity was tested at the pH optimum of the protease. All solutions were prepared at a concentration of 2 m*M*. Enzyme solution was mixed with calcium ion solution or inhibitor solutions in the ratio of 1:1 and preincubated for 15 min at 25°C. Results were recorded as percent residual activity of controls without added calcium ions and inhibitors.

Reactivation of EDTA-Inactivated Protease

The protease was inactivated with 1 m*M* EDTA by incubation at 25°C for 15 min. To this inactivated enzyme solution, Ca ions were added to yield a final concentration of 2 m*M*. Each of these mixtures was further incubated at 25°C for 15 min, and the restored enzyme activity was measured.

RESULTS

Growth and Enzyme Production

The growth curve of *Bacillus subtilis* NCIM No. 64 in a media containing bacteriological peptone as the sole nitrogen source is shown in Fig. 1.

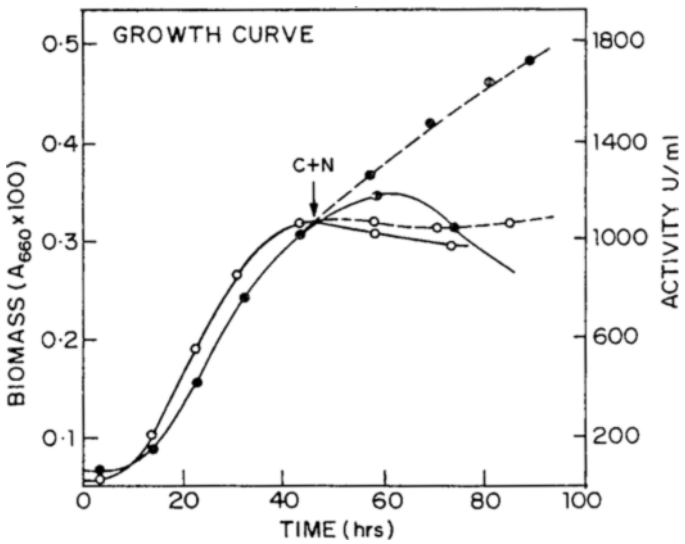

Fig. 1. Time-course of cell growth (●—●) and protease activity (○—○) by *Bacillus subtilus* no. 64. --- shows biomass yields and proteolytic activity after the addition of carbon and nitrogen source at the end of log phase.

Data for total enzymatic activity in the supernatant indicate that proteolytic activity appears after 12 h and peaks at 38 h just before the organism reaches stationary phase. The activity remains fairly constant during the stationary phase of growth. However, addition of more carbon and nitrogen source at this stage, increases the biomass steadily without increasing the enzyme production.

Effect of Nitrogen and Carbon Sources

Various carbon and nitrogen sources were tested as inducers for proteolytic activity (Fig. 2 gives the proteolytic activity with the biomass yields). Among nitrogen sources (supplemented with maltose), defatted soyaflour was the best inducer, whereas the combination of bactopeptone with starch gave maximum yield of the protease. Inorganic nitrogen sources repressed protease activity.

Effect of pH of the Growth Medium

When grown at different pH, the organism showed a broad pH activity profile. Further, the *Bacillus* released a similar amount of protease over a wide range of pH 6–11 (Fig. 3), yielding similar amounts of biomass.

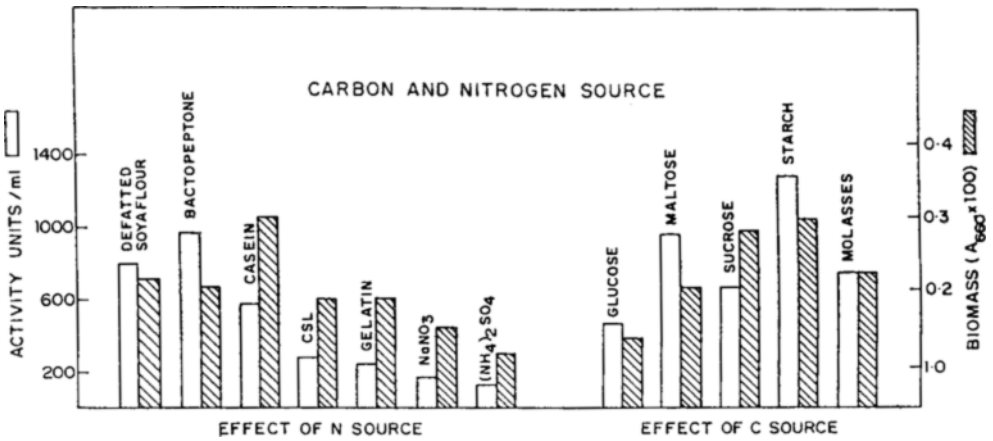

Fig. 2. Effect of carbon and nitrogen source added to the basal media on protease production. All carbon sources were at a concentration of 0.2%, and the nitrogen sources were at a concentration of 1%.

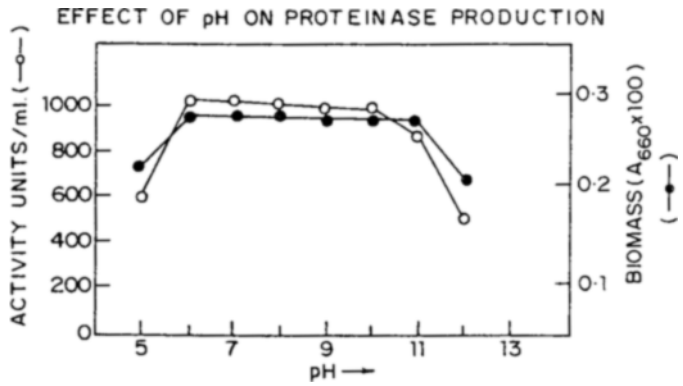

Fig. 3. Effect of the pH of the basal media on enzyme production (○—○) and biomass (●—●).

Effect of Phosphorus

Potassium dihydrogen phosphate was used as phosphorus source in this study. This is frequently used as a phosphorus source, since it also serves as a buffer. Results with varying initial concentrations of phosphate in the medium are presented in Fig. 4. There was a twofold increase in the protease production when phosphate concentration was increased from 0.25 to 1.5% and remained fairly constant thereafter (Fig. 4).

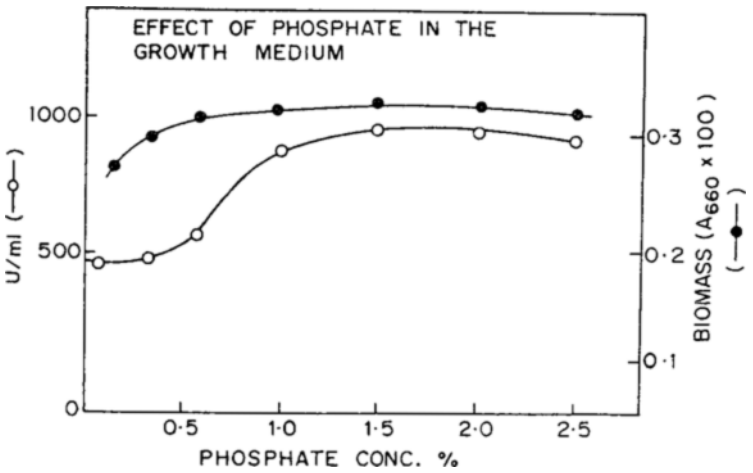

Fig. 4. Effect of phosphorous added to the basal media on protease production (○—○) and biomass (●—●).

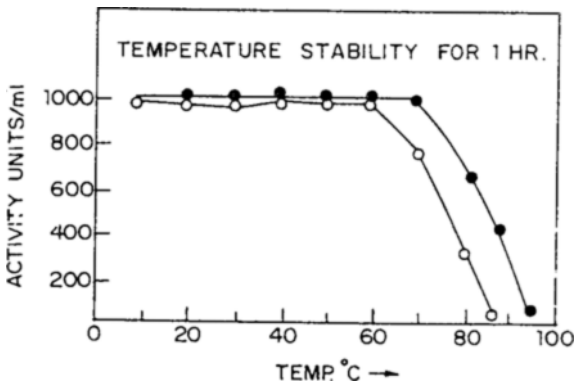

Fig. 5. Effect of temperature on enzyme stability. The enzyme was added to the carbonate-bicarbonate buffer (pH 9.7) incubated for 30 min, and the residual activities were measured. Closed circles show inclusion of calcium ions in the reaction mixtures.

Enzyme Stability

The enzyme was extremely thermostable, and the native enzyme is stable at 60°C when incubated for 30 min. Inclusion of 2 m*M* $CaCl_2$ further increased the thermostability from 60 to 70°C as seen in Fig. 5.

The enzyme was stable when incubated in the presence of NaCl (concentrations varying from 0.25 to 5*M*) (Fig. 6). Inclusion of NaCl in the reaction mixture also increased the thermostability, and as seen in Fig. 7, the optimum temperature was 72°C when incubated in the presence of 4*M* NaCl. The enzyme was active in the alkaline range with a pH optimum of 9.7 and had a pH stability between pH 5–12 over a period of 90 h (Fig. 8).

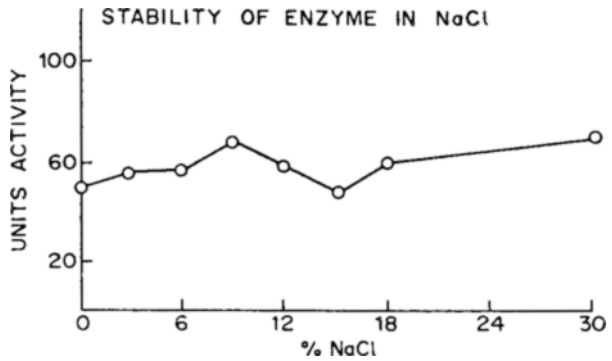

Fig. 6. Effect of NaCl on enzyme activity. The enzyme was incubated in varying amounts of NaCl at 4°C for 90 h, and the residual activities were measured at 60°C at pH 9.7.

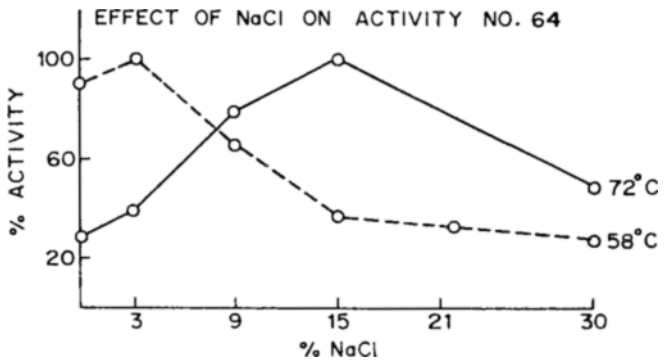

Fig. 7. Effect of NaCl on thermostability. The enzyme reaction was carried in varying concentrations of NaCl and the residual activities measured at 50°C and 72°C.

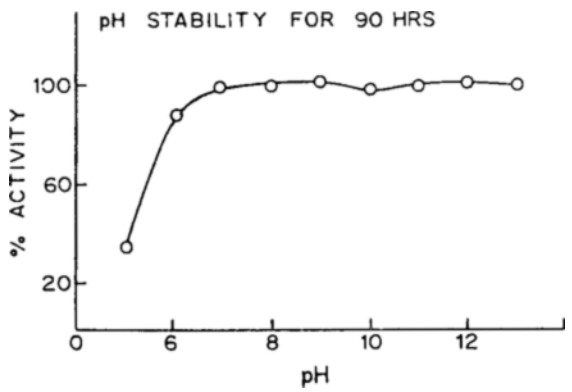

Fig. 8. Effect of pH on enzyme stability. The reaction mixtures were incubated at 60°C for 10 min, and the residual activity was measured at pH 9.7.

Table 1
Effect of Inhibitors

Effectors	% Residual activity
PMSF	0.08
EDTA (5 m*M*) at 50°C	97.00
EDTA (5 m*M*) at 65°C	3.00
EDTA (5 m*M*)+10 m*M* Ca at 65°C	95.00
Pepstatin	100.00
PCMB	100.00
IAA	100.00
STPP 0.5%	99.00
SDS 10%	100.00
Urea 8*M*	85.00
Isopropanol	92.00

Effect of Surface-Active Reagents and Inhibitors

PMSF completely inhibited the enzyme (Table 1). STPP, SDS, and urea did not inhibit the activity of the enzyme.

DISCUSSION

In several respects, the enzyme produced by the present strain of *Bacillus subtilis* NCIM No. 64 is quite different from reported data on *Bacillus subtilis* (*3,4,12*) and *Bacillus licheniformis* (*5,13*). It was therefore considered to be of interest to report these data. The use of soya-meal in the production medium for protease has been reported by many workers (*5,13,14*) who reported that their strains grew on soyaflour alone. We have found that 1% of soya-meal alone was not an effective nitrogen source, but the addition of 0.2% carbon source, like starch, significantly increased the enzyme production. Among the different media, starch with bactopeptone supported better production of protease, which was followed by maltose in combination with bactopeptone.

Boyer and Carlton (*4*) and Sinha and Satyanarayana (*5*) have shown in *Bacillus subtilis* and *Bacillus licheniformis*, respectively, that protease production is maximum after the culture has reached the stationary phase with units of activity varying between 20–40 (μg of tyrosine mL^{-1} min^{-1}). In the present strain, maximal activity of 100 U (μg of tyrosine) is reached before the stationary phase, in fact, in the late logarithmic phase of growth. One explanation could be that the protease is secreted in the logarithmic phase to scavenge nitrogen for the organism. As it reaches the stationary

phase, the available protein source becomes depleted, and accumulation of oligopeptides and amino acids represses further secretion of the enzyme (*15*). It was confirmed that on addition of carbon and nitrogen source at the end of log phase, there was an increase in the biomass without further increasing the proteinase production. The organism was able to grow in a wide pH range of 5–11. Between pH 6 and 11, the curve flattened. Similar amounts of biomass yields indicated that the organism was tolerant over this pH range. Similar results were reported by Nehete et al. (*13*) and Sinha and Satyanarayana (*5*) for *Bacillus licheniformis*.

Phosphate plays a vital role in enhanced production of proteolytic enzymes, especially serine proteases (*16*). In our case, requirement of phosphate was very evident by the fact that there was a twofold increase in protease production when phosphate level was increased from 0.25 to 1.5%. The exact role of phosphate in increasing the yield of protease is not known, but as suggested by the other authors, it is likely to mediate a large number of enzymatic reactions of primary metabolism.

The enzyme is an alkaline serine protease, by the fact that it was completely inhibited by PMSF. The organism produces a thermostable protease with a temperature optima of 60°C.

Divalent ions, neutral salts, and sugar-alcohols have been used for stabilizing various enzymes (*8*). Calcium chloride increased the thermostability of the enzyme from 60 to 70°C, the requirement of calcium was confirmed by titration against EDTA, and full activity was restored on readdition of calcium. Sodium chloride also stabilized the enzyme, and the thermostability was raised from 60 to 72°C in the presence of 15% NaCl. We do not have any concrete explanation for this observation, but one explanation could be the formation of salt bridges around the protein, which stabilize the enzyme at higher temperature. However, the low activity with high salt at lower temperature could be owing to substrate accessibility. Sugar-alcohols of varying molecular weights did not affect the thermostability.

Bacillus subtilis NCIM No. 64 is significantly different from the strains of *Bacillus subtilis* reported by Boyer and Carlton (*4*), Hunt and Ottensen (*3*), and Millet (*12*). Hence, we conclude that the protease from *Bacillus subtilis* NCIM No. 64, being both temperature and pH tolerant, has a potential in the detergent and leather industries.

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