

# PRODUCTION OF A THERMOSTABLE EXTRACELLULAR PROTEASE FROM THERMOPHILIC *BACILLUS* SPECIES

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## ABSTRACT

Isolation and identification of thermophilic *Bacillus* sp was carried out from soil samples of a tannery area. The isolate KCPSS-3, when cultivated in liquid cultures containing trisodium citrate, produced protease. The production reached a maximum in 9 h, with levels of 2.15 U/mg protein. The microorganism utilized several carbon sources for the production of protease. Starch was found to be the best substrate, followed by trisodium citrate, citric acid and sucrose. Among the various organic and inorganic nitrogen sources used, ammonium nitrate was found to be the best. Studies on the effects of temperature and pH on protease production revealed that the *Bacillus* sp could grow up to 65 °C within a broad pH range of 5-10 with an optimal growth temperature and pH at 60 °C and 8.0, respectively. Thermostability studies revealed that the enzyme was stable for 2 h at 30 °C, while at 40 °C and 80 °C, 14% and 84% of the original activities were lost. Hence, it's an indication that the enzyme produced is thermostable.

**Key words:** Protease, thermophilic bacterium, *Bacillus* sp.

## INTRODUCTION

Proteases are one of the three largest groups of industrial enzymes. They account for nearly 60 % of the total enzyme sales covering about 20 % of the world market, and they are used mainly in detergents (3,8,10). Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases (9) 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 hydrolyzates, food industry and waste processing industry (4).

Global requirements of thermostable biocatalysts are far greater than those of the mesophiles of which proteases contribute two thirds (8). Thermostable proteases are advantageous in some applications because higher processing temperatures can be employed, resulting in

faster reaction rates, increase in solubility of nongaseous reactants and products, and reduced incidence of microbial contamination by mesophilic organisms (2).

In this study, bacteria isolated from soil samples collected from different places from a tannery area, was screened for proteolytic activity. The selection of medium components for the optimal production of extracellular protease by thermophilic *Bacillus* sp strain KCPSS-3, along with some biochemical properties of the enzyme is also described. The effects of pH on protease production and temperature on the activity and stability of the enzyme are also carried out.

## MATERIALS AND METHODS

### Organism

The bacterial strain used in this study was the thermophilic *Bacillus* sp strain KCPSS-3, previously isolated from soil samples collected from different places

from a tannery area. Growth in 5% nutrient broth at pH 5-8 and temperature 37-70 °C were examined. Different biochemical tests were employed for its identification.

### Screening for proteolytic activity

The bacterial isolates were plated onto nutrient broth-casein-agar medium and were incubated at 37 °C for 24 h. The strain was screened on the basis of formation of a clear zone of casein hydrolysis on the test plate and was transferred to nutrient agar slant for growth and maintenance.

### Protease production

The culture medium used in this investigation for protease production contained: 0.5% w/v D-glucose, 0.75% w/v peptone, 0.5% w/v MgSO<sub>4</sub>.7H<sub>2</sub>O, 0.5% w/v KH<sub>2</sub>PO<sub>4</sub>, and 0.01% w/v FeSO<sub>4</sub>.7H<sub>2</sub>O maintained at 37 °C for 24-72 h in a shaker incubator (120 rpm). At the end of each fermentation period, the whole fermentation broth was centrifuged at 10,000 rpm at 4 °C for 15-20 min and the clear supernatant was used as crude enzyme preparation.

### Effect of culture conditions on enzyme production

The effects of carbon sources 1% w/v on enzyme production were investigated replacing trisodium citrate by glycerol, lactose, D(+)-galactose, sucrose, maltose, starch, D(+)-glucose, casein and citric acid. Different nitrogen sources including NH<sub>4</sub>NO<sub>3</sub>, peptone, yeast extract, casein, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, NH<sub>4</sub>Cl, KNO<sub>3</sub> and ammonium citrate were employed for preliminary studies to determine growth and production of extracellular protease.

### Determination of protease activity

The activity of protease was assessed in triplicate by measuring the release of trichloroacetic acid soluble peptides from 0.5% w/v azocasein in Tris-HCl buffer (pH 7.2) when 500 µl of the same was incubated with 100 µl of the enzyme for 60 min at 37 °C. The reaction was stopped by adding 500 µl of 15% TCA with shaking. This was left for 15 min and then centrifuged at 5000 rpm at 4 °C for 15-20 min. 1 ml of the supernatant was added to 1 ml of 1M NaOH and the absorbance was read at 440 nm. One unit (U) of protease activity was defined as the number of micromole of substrate converted to product per minute under standard assay conditions.

### Effect of pH on protease production

The effect of pH on protease production from *Bacillus* sp was determined by growing each species in fermentation medium of different pH using appropriate buffers: phosphate buffer (pH 5-6.0), Tris-HCl buffer (pH 7.0-8.0) and glycine-NaOH buffer (pH 9-10). Protease production was measured and monitored every 6 h over a period of 72 h of fermentation through assay of protease activity.

### Effect of temperature on protease production

The effect of temperature on protease production was studied by growing each *Bacillus* sp in fermentation media set at different temperatures (37, 45, 50, 55, 60, 65 and 70 °C). Protease production was monitored every 6 h over a period of 72 h of fermentation through protease activity assay.

### Effect of temperature on activity and stability of enzyme

The effect of temperature on the enzyme activity was determined by performing the standard assay procedure at pH 7.5 at different temperatures (37, 45, 50, 55, 60, 65 and 70 °C). Thermostability was determined by incubating the crude enzyme at above mentioned temperatures for 2 h in a thermostatic water bath. After treatment the residual enzyme activities were assayed.

## RESULTS AND DISCUSSION

### Effect of culture conditions on enzyme production

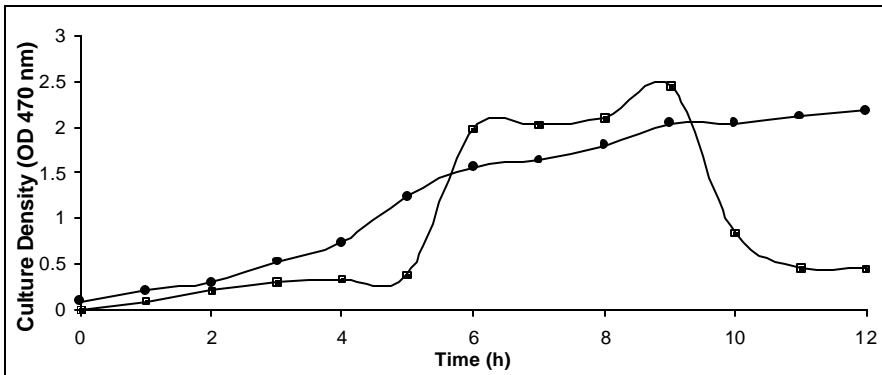
The growth pattern of *Bacillus* sp. KCPSS-3 and protease production was observed for 12 h in liquid medium with 1% trisodium citrate as a carbon source in a 250 ml conical flask (Fig. 1). *Bacillus* sp grew very fast and the formation of protease started from 5<sup>th</sup> hour of the growth and reached a maximum in 9 h (2.15 U/mg protein) then began to fall. This implies that the production of protease was directly linked to the metabolically active state of the culture. Ward (9) reported that *Bacillus* sp usually produce more protease during the late exponential phase.

*Bacillus* sp. KCPSS-3 was capable of utilizing a wide range of carbon sources. However, the best carbon sources in the present study, for protease production were starch and trisodium citrate, followed by citric acid and sucrose (Table 1).

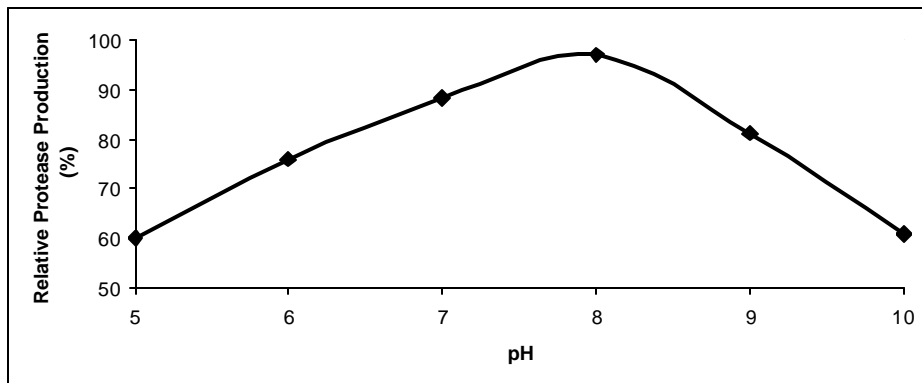
The type of nitrogen sources also affected the production of protease. Among the various organic and inorganic nitrogen sources, the maximum enzyme activity (1.1 U/mg Protein) was obtained when NH<sub>4</sub>NO<sub>3</sub> was used in the medium (Table 2). Moderate to good levels of enzyme activities were obtained when NH<sub>4</sub>Cl, KNO<sub>3</sub> and ammonium citrate were used as nitrogen sources. When various organic nitrogen sources were tested for protease production, it was found that protease formation by *Bacillus* sp. KCPSS-3 was repressed, although growth in some cases was stimulated. Similar results were obtained by Ponsare *et al.* (5) to *Aeromonas hydrophila* and by Banerjee *et al.* (1) to *Bacillus brevis*.

### Effect of pH on protease production

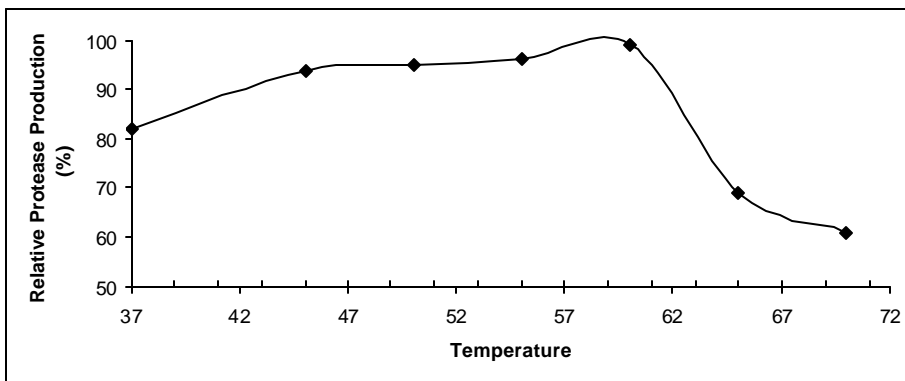
The *Bacillus* sp. KCPSS-3 produced protease over the entire range of pH investigated (pH 5-10). However, the



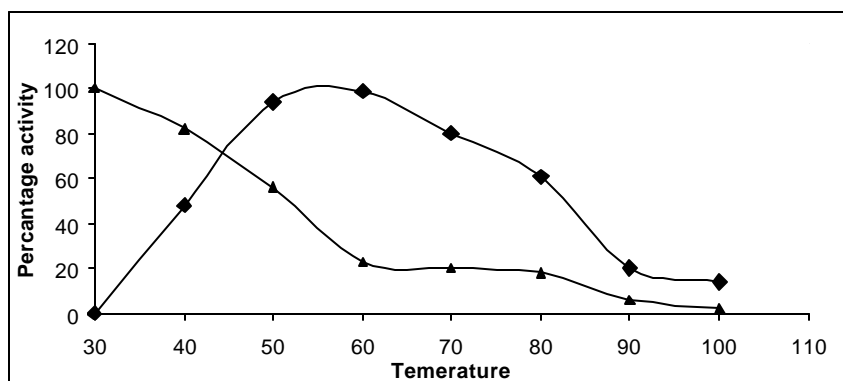
**Figure 1.** Growth (OD) and protease production (%) as a function of cultivation time by *Bacillus* sp KCPSS-3 grown on 1.0% trisodium citrate in shake flasks at initial pH 7.0 and at 50°C.



**Figure 2.** Protease production from *Bacillus* sp KCPSS-3 at different pH



**Figure 3.** Protease production from *Bacillus* sp KCPSS-3 at different temperatures



**Figure 4.** Optimum temperature (%) and stability (%) of protease produced by *Bacillus* sp KCPSS-3 grown at 50 °C for 9 h. Relative activity is expressed as percentage of the maximum.

**Table 1.** Growth and protease activity by *Bacillus* sp KCPSS-3 using different carbon sources. The culture density and extracellular protease activity were determined after 9h incubation at 50°C and initial pH 7.0.

Carbon source	Culture density (OD 470 nm)	Protease activity (U/mg Protein)
Glycerol	0.91	0.34
Galactose	0.36	0.27
Lactose	0.37	0.27
Starch	1.26	1.16
Sucrose	0.69	0.78
Maltose	0.21	0.43
Glucose	0.48	0.50
Casein	0.41	0.34
Trisodium citrate	1.04	1.13
Citric acid	0.46	0.79

**Table 2.** Growth and production of protease by *Bacillus* sp KCPSS-3 using different nitrogen sources. The culture density and extracellular protease activity were determined after 9h incubation at 50°C and initial pH 7.0.

Nitrogen source	Culture density (OD 470 nm)	Protease activity (U/mg Protein)
Peptone (0.5%)	1.5	0.24
Yeast extract (1.0%)	1.40	0.14
Casein (1.0%)	0.62	0.12
Ammonium citrate (1.0%)	0.57	0.72
Ammonium nitrate (1.0%)	0.65	1.10
Ammonium sulphate (1.0%)	0.24	0.17
Potassium nitrate (1.0%)	0.54	0.57

maximum protease production was observed at pH 8.0 while at pH 10, the protease production was about 60% (Fig. 2).

#### Effect of temperature on protease production

Based on the exhibited proteolytic activity and growth temperature range of 37-70 °C, the effect of temperature on protease production was determined. The *Bacillus* sp. KCPSS-3 showed maximum protease production at 60 °C. Protease production was about 70% even at 65 °C (Fig. 3). Results reveal that the *Bacillus* sp under study is a thermophile, and hence, this may be the source of thermostable proteases.

#### Effect of temperature on activity and stability of enzyme

The protease activities were assayed at different temperatures ranging from 37-70 °C at a constant pH of 7.5 (Fig. 4). Enzyme activity increased with temperature within the range of 30 °C to 60 °C. A reduction in enzyme activity was observed at values above 60°C. The thermostability of the enzyme was examined by measuring the remaining activities at 60 °C, after incubation of the enzyme without substrate at various temperatures between 30 & 90 °C for 2 h. The thermostability profile indicated that the enzyme was stable 30 °C for 2 h while at 40 °C & 80 °C, 14% and 84% of the original activities were lost, respectively.

#### CONCLUSIONS

The results obtained in this study show that there is appreciable high production, activity and stability of the enzyme at high temperatures. This suggests that KCPSS-3 can be a potential producer of extracellular thermostable protease which could find applications in industry and biotechnology. The enzyme thus produced is presently under characterization.

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