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*Full Length Research Paper*

# Alkaline Protease from *Bacillus firmus* 7728

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**Extracellular alkaline protease producing *Bacillus firmus* MTCC 7728 was isolated from the soil samples taken from the leather factories in Nacharam industrial area, Hyderabad. Maximum activity was found after 48 h of fermentation. Optimum pH and temperature for maximum enzyme activity were 9 and 40°C, respectively. The potential of mesophilic alkaline protease produced by *Bacillus firmus* MTCC 7728 in various industries is yet to be exploited.**

**Key words:** Isolation, Extracellular alkaline protease, *Bacillus firmus* MTCC 7728.

## INTRODUCTION

Proteases play a crucial role in many physiological and pathophysiological processes. Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Godfrey and West, 1996). Microbial proteases are preferred to the enzymes from plant and animal sources, since they possess almost all the characteristics desired for biotechnological applications. Proteolytic enzymes can be classified as acidic, neutral and alkaline proteases, with regard to their pH working range. Acidic proteases have application in meat tenderization, in the production of fermented foods and also in acidic cleaning compositions (Rao et al., 2007).

Neutral and alkaline proteases hold great potential for application in the detergent and leather tanning industries due to the increasing trend in developing environment friendly technologies (Rao et al., 1998). Alkaline proteases have numerous applications in food industries (Kalisz, 1988; Outtrup and Boyce, 1990), silver recovery from X–ray films (Fujiwara et al., 1991) and several bio-remediation processes. In a recent study (Puri, 2001), the silk degumming efficiency of an alkaline protease from *Bacillus sp*. RGR – 14 was reported. Proteases of the *subtilisin* group are used in the pharmaceutical industry for the treatment of burns and wounds.

The involvement of proteases in the life cycle of disease causing organisms has led them to become a potential target for developing therapeutic agents against fatal diseases, such as cancer and AIDS (Rao et al., 1998). Oral administration of proteases produces an anti-inflammatory response in burn patients and speeds up the healing process (Bogner and Snyder, 1962; Shaw, 1969; Tsomides and Goldberg, 1969).

Alkaline proteases are classified under serine proteases. They are produced by a wide variety of microbial species like *Bacillus subtilis, Aspergillus oryzae, Streptomyces cellulasae,* and *Aeromonas hydrophila* species. Most commercial proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus *Bacillus. Bacillus* sp. are attractive industrial tools for a variety of reasons, including their high growth rates leading to short fermentation cycle times, their capacity to secrete proteins into the extracellular media and the GRAS (generally regarded as safe) status with the food and drug administration for species such as *B. subtilis* and *Bacillus licheniformis* (Schallmey et al., 2004). One of the most important and noteworthy features of many alkalophiles is their ability to modulate their environment. They can convert any neutral or high alkaline medium in their favour to optimize external pH for their growth (Krulwich et al., 1998).

In this paper, we report the isolation of alkaline protease producer, *Bacillus firmus* MTCC 7728 (Microbial Type Culture Collection) designated as isolate $C_3$, from the soil samples taken from leather factories in Nacharam industrial area, Hyderabad. A progressive research is

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currently on, on the industrial applications of this extracellular alkaline protease.

## MATERIALS AND METHOD

### Isolation and screening of microorganisms

Soil samples were taken from leather factories in Nacharam industrial area, Hyderabad. One ml of the thoroughly mixed sample was used for serial dilution. These serially diluted samples were plated on a high protein media containing 1.5% corn meal (w/v), 1.5% soya meal (w/v), 0.02% $Na_2HPO_4$ (w/v), 0.012% $KH_2PO_4$ (w/v), 0.01% $MgCl_2$ (w/v), 0.012% $CaCl_2$ (w/v), 0.012% $Na_2CO_3$(w/v), and 0.036% glucose, pH 7 and incubated for 24 h. Very few colonies appeared owing to the high protein in the media. The cultures were maintained on nutrient agar slants at 4°C and sub-cultured every fortnight. The basic biochemical tests were done and sent to IMTECH, Chandigarh for identification.

### Screening for proteolytic activity

The bacterial isolates were inoculated onto the casein and gelatin agar plates and incubated at 37°C for 24 h. A clear zone of hydrolysis gave an indication of proteolytic microorganisms. Depending on the zone of clearance, one isolate *C3* showing maximum activity was selected for further experimental studies.

### Production media and culture conditions

The following media has been used through out the study for protease production. The composition of yeast extract casein (production) media - 1% glucose (w/v), 0.5% casein (w/v), 0.5% yeast extract (w/v), 0.2% $KH_2PO_4$(w/v), 0.2% $K_2HPO_4$ (w/v) and 0.1% $MgSO_4.7H_2O$ (w/v). The culture was grown for 72 h in a shaking incubator (120 rpm) at 37°C. The pH of the media was adjusted to 9. At the end of fermentation period, the broth was centrifuged at 10,000 rpm, 4°C for 20 min and the clear supernatant was used as the crude enzyme.

### Protease assay

Proteolytic activity in the culture supernatant was determined by using the spectrophotometric method (Chopra and Mathur, 1985) with slight modification. 1 ml of enzyme solution was incubated with 1 ml of 2% casein in phosphate buffer (50 mM, pH 7) at 40°C for 10 min and the reaction was terminated by the addition of 5 ml trichloroacetic acid (5%). After 30 min, the mixture was filtered and 2 ml of filtrate was added to 4 ml 0.1 N NaOH and 0.5 ml diluted Folin-Ciocalteau reagent. Absorbance was then measured at 670 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µg of tyrosine/min under standard conditions.

### Effect of pH on protease production

The effect of pH on protease production from the isolate *C3* was determined by growing the isolate in production media with different pH in the range of 4 -12 using appropriate buffers, citrate buffer (pH 3 - 5), phosphate buffer (pH 5 - 6), 25 mM $KH_2PO_4$/NaOH (pH 6.0 - 8.0), Tris-HCl buffer (pH 7.0 - 9.0) and borate buffer 25 mM $H_3BO_3$/NaOH (pH 8.0 - 12.0).

### Effect of temperature on protease production

The effect of temperature on protease production by the isolate *C3* was determined by growing the isolate in production media at varied temperatures (20 – 55°C). Protease production was Monitored at regular intervals.

### Growth curve

The isolate *C3*, was inoculated in yeast extract casein media (production) at 37°C, pH 9 and observed for growth by reading the optical density at regular intervals for 72 h. The optical density was measured at 630 nm. The alkaline protease activity was monitored at regular intervals.

### NaCl (sodium chloride) tolerance

The effect of salt on the isolate *C3* was studied by growing it in yeast extract casein (production) media supplemented with varied concentrations of NaCl (1, 2, 3, 4, 5, 6 and 7%) at 37°C in an incubated shaker at 120 rpm. Protease production was monitored at regular intervals.

## RESULTS AND DISCUSSION

The soil samples were collected from the Leather factories in Nacharam industrial area, Hyderabad. Among the isolates, *C3* was selected for further studies because of its potential as a good extracellular alkaline protease producer. The Biotechnological application of the isolate in the valorization of keratin and in the leather industry where it can elaborate the non-polluting process is under study. The organism *C3* was isolated on protein rich media constituting soyameal and cornmeal as the nitrogen and protein sources by serial dilution and spread plate techniques. Few cultures appeared owing to the high protein content of the isolation media. The isolate was screened for extracellular proteolytic activity on casein as well as gelatin agar media by observing the zone of hydrolysis. The isolate also has the ability to hydrolyse starch indicating the production of amylase. The photographs showing the hydrolyzed zones are presented in Figure 1.

The isolate *C3* was identified as *B. firmus* MTCC 7728 by IMTECH, Chandigarh. The morphological, physiological and biochemical tests are presented in Table 1. They are slender gram-positive rods, 3 – 4 µ long and ~ 1.0 µ wide. The colony is circular, opaque with a rhizoid border, slightly raised and has a granular shiny surface. They are Spore forming and motile.

The culture supernatant of *B. firmus* 7728 was used as the crude enzyme which was tested at varying pH values (4 – 12) and temperature (20 – 55°C). Though the enzyme was active within the pH range of 4 – 12 and a temperature range of 20 – 55°C, maximum proteolytic activity was observed at pH 9 and temperature 40°C. The alkaline protease produced by the isolate was found to be mesophilic (Figures 2 and 3).

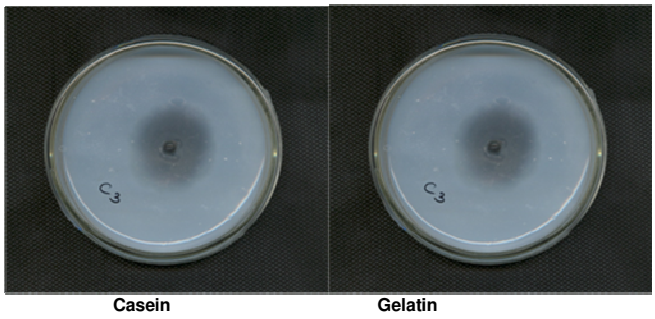


**Figure 1.** Casein and gelatin hydrolysis by *Bacillus firmus* MTCC 7728

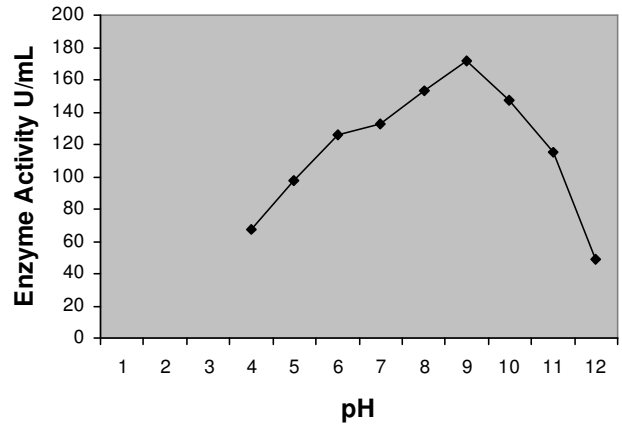


**Figure 2.** Protease activity of *Bacillus firmus* MTCC 7728 in culture media at different pH after 72 h in a shaking incubator (120 rpm) at 37°C.

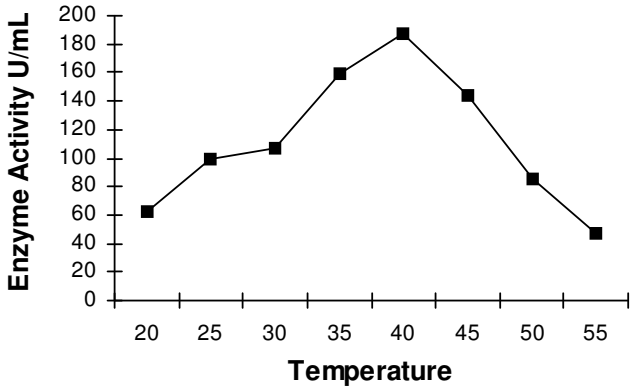


**Figure 3.** Protease activity of *Bacillus firmus* MTCC 7728 in culture media at different temperatures after 72 h in a shaking incubator (120 rpm) at 37°C.

Yeast extract casein media was proved to be the best for protease production by *B. firmus* MTCC 7728. By growing the culture at optimum pH and temperature, the

**Table 1.** The morphological, physiological and Biochemical tests as given by IMTECH, Chandigarh.

| Test | *Bacillus firmus* |
|---|---|
| **Colony morphology** | |
| Configuration | Circular |
| Margin | Rhizoid |
| Elevation | Slightly raised |
| Surface | Granular Shiny |
| Pigment | --- |
| Opacity | Opaque |
| Gram's Reaction | Positive |
| Cell Shape | Rods |
| Size (µm) | Length: 3-4 µ, Width: approx.1.0 µ |
| Arrangement | Short chains |
| Spore(s) | + |
| Endospore | + |
| Position | Central |
| Shape | Oval |
| Sporangia bulging | --- |
| Motility | + |
| **Physiological tests** | |
| Growth at 25 - 42˚C | + |
| Growth at pH4 – 10) | + |
| Growth in NaCl (2 – 7%) | + |
| **Biochemical tests** | |
| Growth on MacConkey Agar | - |
| Indole Test | - |
| Methyl Red Test | + |
| Voges Proskauer Test | - |
| Citrate Utilization | - |
| Gas from Glucose | - |
| $H_2S$ Production | - |
| Casein Hydrolysis | + |
| Esculin Hydrolysis | - |
| Gelatin Hydrolysis | + |
| Starch Hydrolysis | + |
| Urea Hydrolysis | - |
| Nitrate Reduction | + |
| Nitrite Reduction | ND |
| Catalase Test | + |
| Oxidase Test | + |
| Lysine decarboxylase | - |
| Arginine dihydrolase | + (W) |
| Ornithine decarboxylase | - |
| Tween 20 hydrolysis | - |
| Tween 40 hydrolysis | + |
| Tween 80 hydrolysis | - |
| Lecithinase Test | - |
| ONPG Test | - |
| Acid production from carbohydrates | NIL |

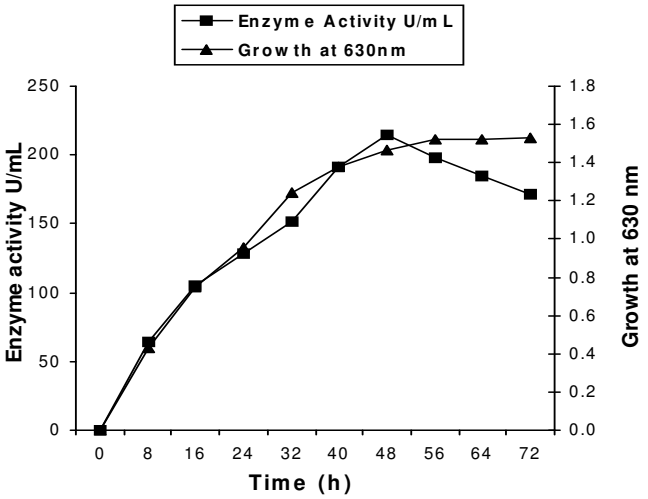


**Figure 4.** Protease activity and growth of *Bacillus firmus* MTCC 7728 in a shaking incubator (120 rpm) at 37°C.

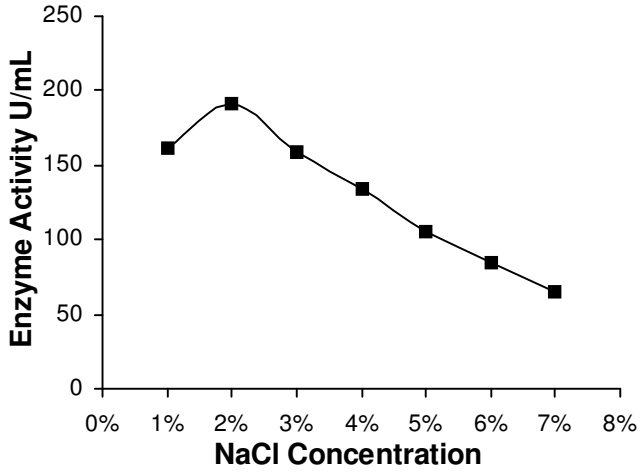


**Figure 5.** Protease activity of *Bacillus firmus* MTCC 7728 in culture media at different salt level after 72 h in a shaking incubator (120 rpm) at 37°C**.**

protease activity was estimated at different intervals. *B. firmus* MTCC 7728 was observed to be very fast growing. The maximum proteolytic activity (215 U/ml) was observed after 48 h of growth where it reached stationery phase. The alkaline protease activity was stable even after 72 h of growth (Figure 4).

The alkaline protease production by *B. firmus* MTCC 7728 was studied within the range of 1 - 7% NaCl concentration, but maximum production was observed at 2% concen-tration (Figure 5). The alkaline protease activity of *B. firmus* MTCC 7728 was studied over a pH range of 4 – 12 and the optimum was found to be 9. The alkaline protease activity of *B. firmus* MTCC 7728 was observed starting from 20 – 55°C and the optimum was found to be 40°C.

The enzyme activity of *B. firmus* MTCC 7728 was studied in relation to growth. The enzyme activity was maximum after the isolate reached stationery phase around 48 h of growth. The maximum enzyme activity was found to be 215 U/mL. 2% NaCl concentration was found to be optimum for this mesophilic alkaline protease produced by *B. firmus* MTCC 7728.

## Conclusion

The extracellular alkaline protease produced by *B. firmus* MTCC 7728 could have great potential in various industries such as detergent, degumming and several processes like silver recovery, bioremediation and protein hydrolysate production, as the isolate is capable of various functions like casein, gelatin and starch hydro-lysis. The organism is active at mesophilic range of temperatures, which is the prerequiste for developing environment friendly technologies. The enzyme may have an important role to play in the food industry because of the GRAS status of *Bacillus* sp.

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