

RESEARCH NOTE

PRODUCTION OF EXTRACELLULAR PROTEASE AND DETERMINATION OF OPTIMAL CONDITION BY BACILLUS LICHENIFORMIS BBRC 100053

Z. Ghobadi Nejad, S. Yaghmaei* and R. Haji Hosseini

Biochemical and Bioenvironmental Research Center, Sharif University of Technology
P.O. Box 11155-1399, Tehran, Iran

z_ghobadi@mehr.sharif.edu - yaghmaei@sharif.edu - reza_hajihosseini@yahoo.com

*Corresponding Author

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Abstract The production of protease by *Bacillus licheniformis* BBRC 100053 was studied. The most appropriate medium for the growth and protease production is composed of: lactose 1%, yeast extract 0.5%, peptone 0.5%, KH_2PO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02%. Enzyme production corresponded with growth and reached a maximum level (589 U/ml) during the stationary phase at 35°C, pH equivalent to 10 and with 150 rpm after 73 hours. Protease activity was highest at pH 8 and 45°C. The best carbon sources are respectively lactose and maltose and the best nitrogen source is peptone. The protease was highly active and stable from pH 7.0 to 11.0 with an optimum at pH 7-8. Thermo stability of the enzyme was considered in the presence and absence of 2mM CaCl_2 . Enzyme is non stable at temperatures higher than 50°C while the thermal stability was enhanced in the presence of Ca^{2+} . The enzyme retained 15 and 8% of its initial activity after heating for 60min at 60°C in the presence and absence of 2mM CaCl_2 , respectively and retained 7 and 3% of at 70°C in the presence and absence of 2mM CaCl_2 .

Keywords Protease, *Bacillus licheniformis*, Thermo Stable Protease, Enzyme, Optimization

چکیده در این تحقیق تولید آنزیم پروتئاز با باکتری باسیلوس لیکنی فرمیس مطالعه شده است. مناسبترین محیط جهت تولید آنزیم عبارت است از: لاکتوز ۱٪، عصاره مخمر ۰/۵٪، پپتون ۰/۵٪، KH_2PO_4 ۰/۱٪، $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ۰/۰۲٪. شرایط بهینه تولید آنزیم، دمای ۳۵°C، pH برابر ۱۰، دور شیکر ۱۵۰ rpm، بعد از ۷۳ ساعت در فاز سکون تعیین شد. آنزیم تولیدشده در دامنه pH برابر ۷-۱۱ پایدار است و بیشترین پایداری را در pH برابر ۷-۸ نشان داده است. پایداری دمایی آنزیم نیز در حضور غیاب یون Ca^{2+} بررسی شد. آنزیم تولیدشده در دمای بالاتر از ۵۰°C ناپایدار است و پایداری دمایی در حضور یون Ca^{2+} افزایش می‌یابد.

1. INTRODUCTION

Proteases are the most important enzymes accounting for about 60 % of the total industrial enzyme market [1]. They have diverse applications in a wide variety of industries, such as in detergent, food, leather tanning and processing, fiber, pharmaceutical, silk and for recovery of silver from used x-ray films [1,2].

Many bacteria belonging to the genus *Bacillus* excrete large amount of the enzymes into the culture medium [3]. Microbial proteases dominate

the world wide enzyme market, accounting for a two-third share of the detergent industry [4].

Although protease production is an inherent property of all organisms, only those microbes that produce a substantial amount of extracellular protease have been exploited commercially [4].

Bacillus licheniformis strains are listed in the third edition of food chemical codex (1981) as sources of protease enzyme preparations used in food processing [5].

The reason for this is their high pH and temperature stability. Alkaline proteases belong to

the group of proteases, which have either a serine center or are of metallo type, exhibiting a wide pH range of pH 6-13 [6,7].

Given the wide application of this enzyme, it is reported that in year 2005 the global proteolytic enzyme demand will increase dramatically to 1.0-1.2 billion dollars [6].

The aim of the present work was to identify the culture conditions that supported protease production by the strain *Bacillus licheniformis* 100053 using different carbon and nitrogen source, various temperature and pH range and some properties of the enzyme produced were determined.

2. MATERIALS AND METHODS

2.1. Bacterial Strain The organism used was *Bacillus licheniformis* BBRC 100053, obtained from Biochemical and Bioenvironmental Research Center, a Local Culture Collection in Sharif University of Technology, Iran. The culture was maintained on nutrient agar medium at 30°C for 7 days and stored at 4°C.

2.2. Inoculum Preparation and Protease Production Inocula were prepared by adding a loop full of pure culture into 25 ml of sterile Luria-Bertani (LB) broth medium (composed of (g/l): peptone 10; yeast extract 5, NaCl 5 [1]).

Broth cultures were diluted with the appropriate broth to obtain 10^7 cfu/ml as estimated by absorbance at 600 nm. Target absorbance to obtain these populations was 0.2 for Gram-positive bacilli, based upon preliminary testing [8].

A 10% inoculum from this culture was added to protease production medium containing: Maltose 1%, yeast extract 0.5%, peptone 0.5%, KH_2P_4 0.1%, $\text{Mg SO}_4 \cdot 7 \text{H}_2\text{O}$ 0.02% and pH 8 [6]. Media were autoclaved at 120°C for 20 min.

After incubation of 73 h at 37°C under shaking condition (150 rpm), the cultures were centrifuged and the supernatants were used for estimation of proteolytic activity. The growth of the microorganism was determined by measuring absorbance at 660 nm. All experiment were carried out in duplicate and repeated at least twice.

2.3. Assay of Proteolytic Activity Protease

activity was determined by modified method [9,10] using casein as substrate.

0.5 ml of enzyme solution was added to 4.5 ml of substrate solution (1% V/V, casein with 50 mM Tris-HCl buffer, pH 8.0) and incubated at 30°C for 30 min independently with respective controls. The reaction was stopped by adding 5ml of 5% TCA mixture (5% TCA, 9% Na-acetate, 9% acetic acid) followed by 30min holding at room temperature followed by centrifugation at 8000 rpm for 20 min.

The precipitates were removed by filtration through whatman-1 filter paper and absorbance of the filtrate was measured at 280 nm. One unit of protease activity was defined as the amount of enzyme liberating 1 μg of tyrosine/min under assay conditions. Enzyme units were measured using tyrosine (0-100 mg) as standard.

2.4. Growth Kinetics and Protease Production

The kinetics of growth and enzyme production was followed at different time intervals. The microbial culture was inoculated in production medium and incubated at 37°C under shaking conditions (150 rpm). Culture samples were withdrawn aseptically every 4 h and cell density along with enzyme activity was monitored, as describe above [4].

2.5. Optimization of Production Medium and Protease Production Condition

2.5.1. Effect of pH on protease production The effect of pH on protease production was determined by growing bacteria in fermentation media of different pH (7-12) using appropriate buffers, 100 mM sodium acetate buffer for pH 5.0, potassium phosphate buffer for pH 6.0-8.0, Tris-HCl buffers for pH 8.0-9.0, glycine-NaOH buffer for pH 9.0-12.0 [1,11,12].

After incubation of 73h at 37°C under shaking conditions at 150 rpm, growth and protease activity were quantified.

2.5.2. Effect of temperature on protease production The effect of temperature on protease production was studied by growing bacteria in fermentation media set at different temperatures (25-55°C).

Protease productions were measured after 73h fermentation period through assay of protease activity.

2.5.3. Effect of agitation speed The effect of the agitation speed on the protease production was studied, by incubation bacteria in fermentation media at 37°C under shaking condition at 100, 130, 150, 180, 200 rpm, and protease productions were measured after 73h fermentation period through assay of protease activity.

2.5.4. Effect of different carbon sources The effect of different carbon sources on the protease production was studied, by replacement of maltose (1%) in the basal medium by various carbon sources included: glucose, lactose and casein.

Protease productions were measured after 73 h under same conditions.

2.5.5. Effect of different nitrogen sources Organic nitrogen sources included tryptone and peptone, while inorganic nitrogen sources included ammonium nitrate and urea, were added in the basal medium (0.5%). Protease productions were monitored after 73h at 37°C.

2.6. Characterization of the Protease Enzyme

2.6.1. Effect of pH on activity and stability of protease The effect of pH on the proteolytic activity was determined by assaying the enzyme activity at different pH value ranging from 6.0 to 11.0 with casein 1 % (w/v), as substrate using appropriate buffers (mentioned 2.5.1). The relative activities were based on the ratio of the activity obtained at certain pH to the maximum activity obtained at the range and expressed as percentage [6].

The pH stability of the enzyme was investigated in the pH range of 6.0-12.0. Therefore, 2 ml of the enzyme was mixed with 2ml of the buffer solutions and incubated at 30°C for 2h. Afterwards, aliquots of the mixtures were taken to measure the residual protease activity (%) with respect to the control under standard assay conditions [6].

2.6.2. Effect of temperature on activity and stability of protease The effect of temperature on the enzyme activity was determined by assaying the enzyme activity at pH 8.0 at different temperatures ranging from 25-55°C. The relative activities (as percentage) were expressed as the ratio of the proteolytic activity obtained at certain

temperature, to the maximum activity at the given temperature range.

In order to determine the thermo stability of the enzyme, experiments were conducted by measuring the residual activity after incubation at various temperature ranging from 30-60°C, for 30 min, in the presence and absence of 2mM CaCl₂ [1].

Thermal inactivation was also examined by incubating the enzyme preparation at 60 and 70°C for 60 min in the absence or presence of 2 mM CaCl₂ [1]. Aliquots were withdrawn at desired time intervals to test the remaining activity at pH 8.0 and 47°C.

The non-heated enzyme was considered as control (100%).

3. RESULTS AND DISCUSSION

3.1. Protease Production and Kinetics of *Bacillus licheniformis* Growth By studying the growth kinetics of protease production, it was clear that the growth of organism increased with the increase of the length of the incubation period (Figure 1). The highest value of growth was achieved after 48 h of incubation.

The proteolytic activity was low during the first hours of cultivation, but increased with cultivation time, and reached the highest value (588.8 U/ml) after 73 h of incubation at 37°C and pH 10. Protease production nearly corresponded

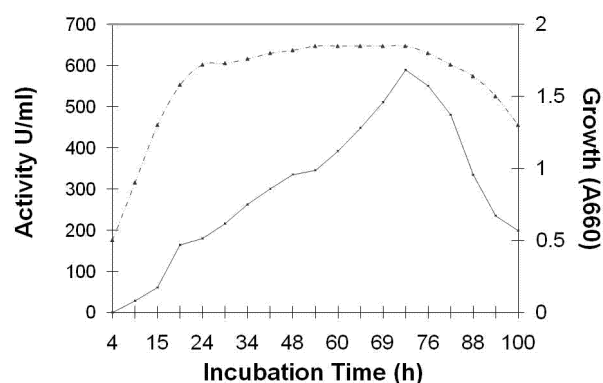


Figure 1. Growth kinetics of *Bacillus licheniformis* with references to protease production. Samples were withdrawn at 4 h interval for determination of cell growth (OD660) (▲) and protease activity (■).

with the growth and maximal in stationary phase. These results are in good agreement with previous results obtained for *Bacillus sp.* by other researchers [13,14].

At 37°C *Bacillus licheniformis* produced the maximal enzyme production after 73 h incubation and a higher temperature might face the risk of denaturation of the protein, as well as degradation due to the proteolytic activity of the protease produced (Figure 2).

By studying the effect of pH on protease production, it was revealed that maximum enzyme production was obtained at pH 10 (Figure 3).

The effect of the agitation speed on protease production showed that at 37°C and pH 10, 150 rpm was determined to be the optimal agitation speed. During the cultivation period, protease production and cell growth has been found to be negatively affected by variation in agitation rates

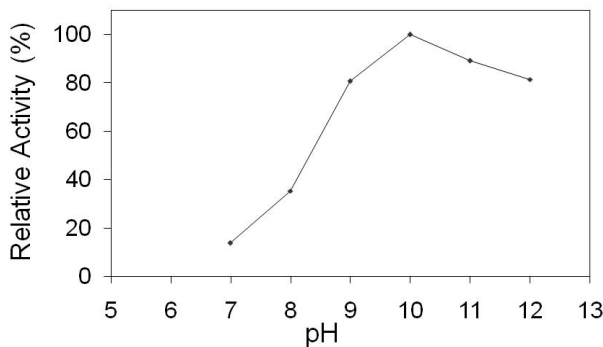


Figure 2. Effect of different pH on protease production.

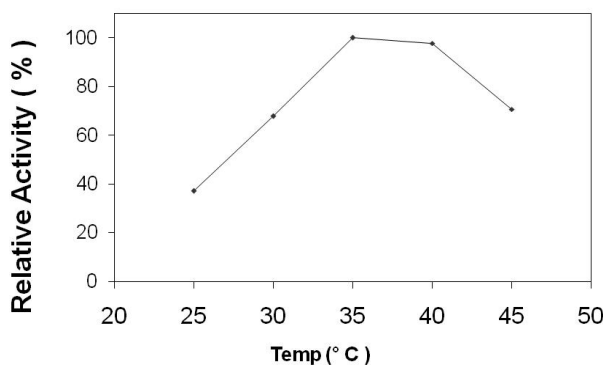


Figure 3. Effect of different temperature on protease production.

beyond 150 rpm and so is agitation speed less than 150 rpm (Figure 4).

3.1.1. Effect of different carbon sources The effect of replacement of maltose in the basal medium by various carbon sources, on protease synthesis is shown in Figure 5. As shown, *Bacillus licheniformis* exhibited higher productivity of protease in culture media containing lactose as carbon source. Both lactose and maltose enhanced the enzyme productivity and protease production decreased when the organism was grown in the present of glucose and casein. On the other hand, glucose and casein reduced the enzyme activity to 77 % and 89 % respectively of the control. Similar to our results, several studies have reported that glucose repressed protease formation [4,15].

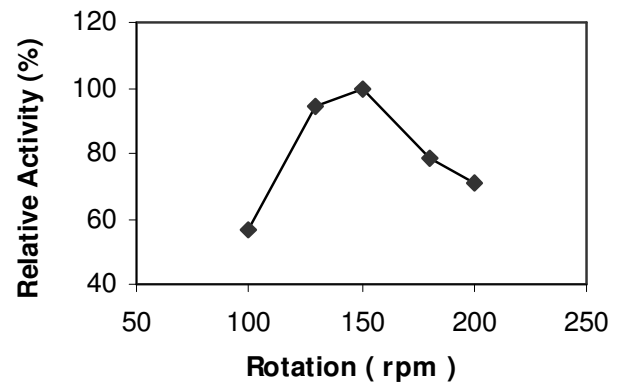


Figure 4. Effect of different agitation speed on protease production.

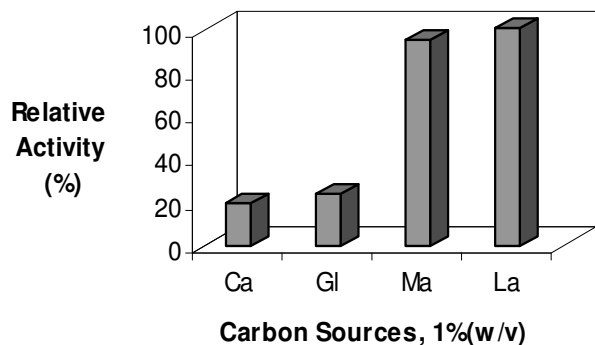


Figure 5. Effect of different carbon sources (1% w/v) on protease production. Ca, casein; Gl, glucose; Ma, maltose; La, lactose.

3.1.2. Effect of various nitrogen sources In microorganism, nitrogen (both organic and inorganic forms) is metabolized to produce primarily amino acids, nucleic acids, proteins and cell wall components.

Alkaline protease production heavily depends on the availability of both carbon and nitrogen sources in the medium [16,17].

The organic nitrogen sources used in our study supported growth, while the maximum effect on growth was observed with a combination of peptone and yeast extract (Figure 6).

These results indicated that a mixture of peptone and yeast extract (0.5,0.5%) were the best nitrogen source and with maximal production of protease (588 u/ml), on the other hand, the enzyme activity decreased dramatically when using urea. These results were in accord with those reported for an alkaline protease from *Bacillus sp.* (Fujiwara and Yamamoto, 1987). Tryptone and ammonium nitrate followed by yeast extract also proved favorable nitrogen source for protease production.

3.2. Characterization of the Crude Enzyme Obtained from *Bacillus licheniformis*

3.2.1. pH activity and pH stability profiles The effect of pH on the protease activity toward casein was examined at various pH values at 37°C (Figure 7a). The enzyme was active between pH 7.0 and 10.0 with an optimum at pH 8.0.

The relative activities at pH 7.0 and 9.0 were about 88 and 87 %, respectively, of that at pH 8.0. As seen in Figure 7a, protease activity decreased

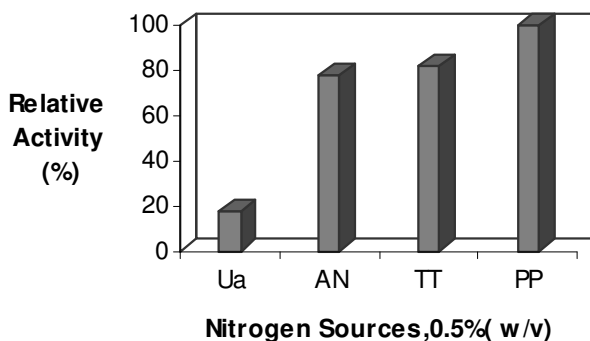
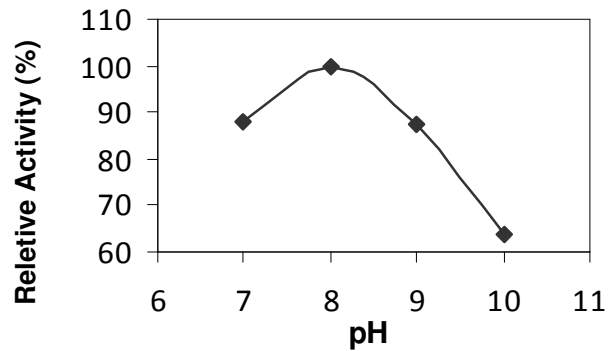


Figure 6. Effect of different nitrogen sources (0.5% w/v) on protease production. Ua, urea; AN, ammonium nitrate; TT, tryptone; PP, peptone.

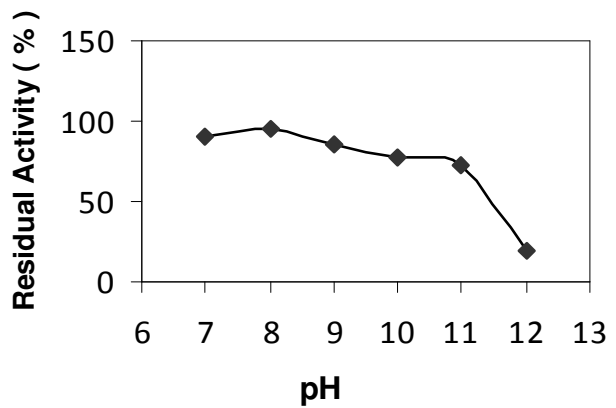
above pH 9.0 and was 60 % of the maximum enzyme activity at pH 10.0. The maximum activity obtained at pH 8.0.

Very large number of alkaline protease isolated from *Bacillus* has a high optimal pH for their activity. The important detergent enzymes, subtilisin Carlsberg produced by *Bacillus licheniformis* showed maximum activity at pH values of 8.0-10.0 [18].

The pH stability was determined by incubating the enzyme in buffers of different pH in the range of 7.0-12.0 for 2 h at 37°C, followed by activity estimation at pH 8.0 and 47°C.



(a)



(b)

Figure 7. Effect of pH on the activity (a) and stability (b) of the protease. The pH profile was determined in different buffers of varying pH values. The maximum activity obtained at pH 8.0 was considered as 100 % activity. The pH stability of the protease was determined by incubating the enzyme in different buffers for 2 h at 37°C and the residual activity was measured at pH 8.0. The activity of the enzyme before incubation was taken as 100 %.

As shown in Figure 7b the enzyme was stable between pH 7.0-11.0, and higher stability was observed in the range of pH 7.0-8.0. Enzyme stability decreased significantly at pH 12.0 and residual activity retained 19 % of its initial activity.

3.2.2. Effect of temperature on enzyme activity and stability The effect of temperature on the protease activity using casein as substrate was examined at various temperature for 10min at pH 10.0. As shown in Figure 8a the maximum enzyme activity was obtained between 40 and 50°C. Above 50°C, protease activity decreased. The relative activity at 55°C was 59 %.

The thermostability of protease was estimated by incubating of enzyme in different temperature in the range of 30-60°C for 30 min at presence or absence of 2mM CaCl₂.

The thermal stability profiles showed that Ca²⁺ was required for enzyme stability, and the enzyme unstable above 50°C. The enzyme retained 63, 61 and 19 % of its initial activity after 30 min incubation at 40, 50 and 60°C, in the presence of Ca²⁺, respectively, while in the absence of Ca²⁺ the enzyme retained 48, 40 and 12 % of the initial activity (Figure 8b.)

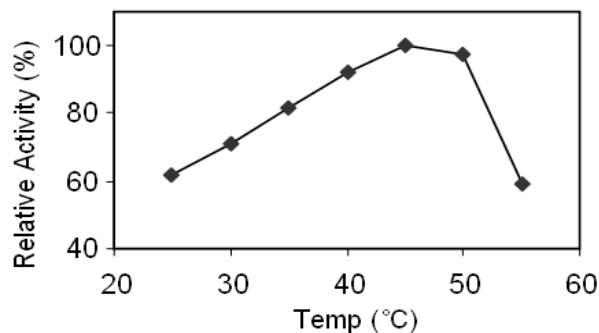
The thermal inactivation was also studied by incubating the enzyme, in the absence or presence of 2mM CaCl₂ at 60 and 70°C for 1h, and followed by measuring the residual activity under standard conditions (see Table 1)

After 1 h incubation at 60 and 70°C, the enzyme retained 16 and 6% of its initial activity, in the presence of Ca²⁺, respectively, while in the absence of Ca²⁺ the enzyme retained 8 and 3 % of initial activity.

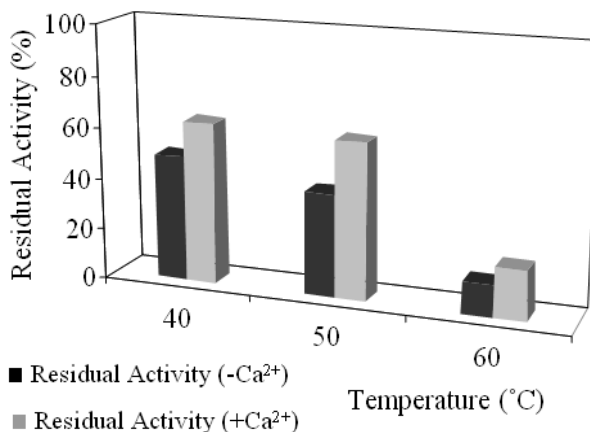
These finding are in line with earlier reports showing that calcium was required for the stability of protease.

Ghorbet, et al [19] reported also that calcium ions play a major role in stabilization at high temperature of a Ca²⁺ dependent metalloprotease from *B. cereus*.

The improvement in protease thermostability against thermal inactivation in the presence of Ca²⁺ may be explained by the strengthening of interactions inside protein molecules and by the binding of Ca²⁺ to autolysis sites. Indeed metal ions often act as salt or ion bridges between adjacent amino acids [20].



(a)



(b)

Figure 8. Effect of temperature on the activity (a) and thermal stability (b) of the protease. The temperature profile was determined by assaying enzyme activity at various temperature values using 50 mM Tris-Hcl buffer, pH 8.0. The thermal stability of the protease was determined by preincubating the enzyme 15 min in the absence or presence of 2 mM CaCl₂ at various temperatures. The original activity before preincubating was taken as 100 %.

4. CONCLUSIONS

This work describes the production and the characterization of alkaline protease from *Bacillus licheniformis*, and their fermentation conditions (temperature, agitation speed, incubation time and initial media pH) were optimized. Based on the results, protease production was optimum at 37°C, 150 rpm, pH 10 and incubation time of 73h. The most appropriate medium for the growth and protease production is composed of (%): lactose1,

TABLE 1. Thermal Inactivation of the Protease with Presence (+) and Absence (-) of 2 mM CaCl₂.

Temperature (°C)	Residual Activity (-Ca ²⁺) (%)	Residual Activity (+Ca ²⁺) (%)
60 (1 h)	8.40	16.93
70 (1 h)	3.36	6.94

peptone 0.5, KH₂PO₄ 9.1, MgSO₄.7H₂O 0.02. The protease was highly active from pH 8.0, and higher stability was observed in the range of pH 7.0- 11.0, with an optimum at pH 7.0-8.0. The enzyme was optimally active at 40-50°C. CaCl₂ (2mM) increase the temperature stability.

Considering the properties of this enzyme (such as high activity and stability in alkaline pH) the *Bacillus licheniformis* (BBRC-100053) may find potential application in various industrial fields. Further works should be done concerning the performance of this enzyme.

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