CHEMICAL ENGINEERING TRANSACTIONS Volume 21, 2010
Editor J. J. Klemeš, H. L. Lam, P. S. Varbanov
 ISBN 978-88-95608-05-1 ISSN 1974-9791
DOI: 10.3303/CET1021242

Production of Extracellular Protease and Determination of Optimise Condition by Bacillus Licheniformis BBRC 100053

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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%, $MgSO_4.7H_2O$ 0.02 %. Enzyme production corresponded with growth and reached a maximums level (589 U/ml) during the stationary phase at 35 ^oC, pH equivalent to 10 and with 150 *rpm* after 73 hours.
Protease activity was highest at pH 8 and 45 ^oC. 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 2 *mM* $CaCl_2$. 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 60 min at 60 ^oC in the presence and absence of 2 *mM* $CaCl_2$ respectively and retained 7 and 3 % of at 70 ^oC in the presence and absence of 2 *mM* $CaCl_2$.

1. Introduction

Proteases are the most important enzymes accounting for about 60% of the total industrial enzyme market. 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 (Nedra ElHadj, et al., 2007).
Many bacteria belonging to the genus Bacillus excrete large amount of the enzymes into the culture medium (Mabrouk, et al, 1991) Microbial proteases dominate the world wide enzyme market, accounting for a two – third share of the detergent industry (Patel et al.,1995).
Bacillus licheniformis strains are listed in the third edition of food chemical codex (1981) as sources of protease enzyme preparations used in food processing (Bore et al., 1994).

Please cite this article as: Ghobadi Nejad Z., Yaghmaei S. and Haji Hosseini R., (2010), Production of extracellular protease and determination of optimize condition by bacillus licheniformis BBRC 100053, Chemical Engineering Transactions, 21, 1447-1452 DOI: 10.3303/CET1021242

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 .Broth cultures were diluted with the appropriate broth to obtain 10^7 *cfu/ml* as estimated by absorbance at 600 *nm* (Winniczuk, et al, 1997). A 10 % inoculum from this culture as added to protease production medium containing: maltose 1%, yeast extract 0.5 %, peptone 0.5 %, KH2P4 0.1%, MgSO4. 7 H2O 0.02 % and pH 8 (Genckal et al., 2006). under shaking condition (150 *rpm*), the cultures were centrifuged and the supernatants were used for estimation of proteolytic activity.

2.3. Assay of proteolytic activity

Protease activity was determined by modified method using casein as substrate (Kunitz, 1947). The amount of 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 5 *ml* of 5% TCA mixture, followed by 30 *min* 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*.

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 described above..

2.5. Optimization of production medium and protease production condition

The effect of pH on protease production was determined by growing bacteria in fermentation media of different pH (7-12) using appropriate buffers. The effect of temperature on protease production was studied by growing bacteria in fermentation media set at different temperatures (25-55 °C).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.*

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. Organic nitrogen sources included tryptone and peptone, while inorganic nitrogen sources included ammonium nitrate and urea, were added in the basal medium (0.5 %).

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. 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 2 *ml* of the buffer solutions and incubated at 30 °C for 2 *h*.

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. 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 2 *mM* CaCl2 for 60 *min* in the absence or presence of 2 *mM* CaCl2 (Nedra ElHadj, et al, 2007).

3. Results and discussion

3.1. Protease production and kinetics of B. licheniformis growth

The highest value of growth was achieved after 48h of incubation (Figure 1).

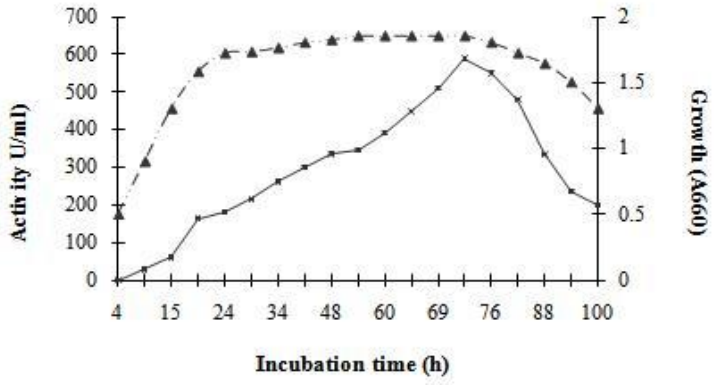


Figure 1: Cell growth (▲) and protease activity (■)

By studying the effect of pH, temperature and agitation speed it was revealed that maximum enzyme production was obtained at pH 10 (Figure 2), 37 °C (Figure 3) and 150 rpm -Figure 4 (Connelly, et al, 2004)

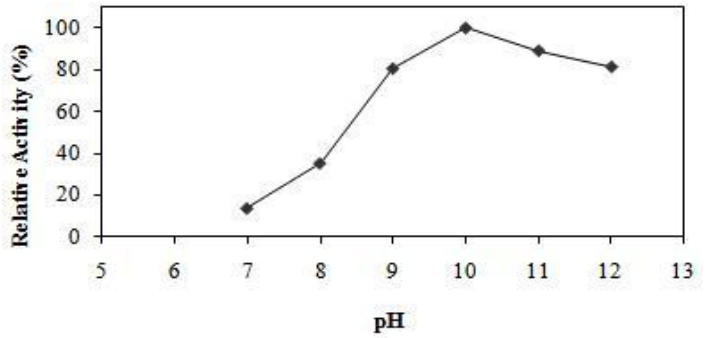


*Figure **2**: Effect of different pH on protease production*

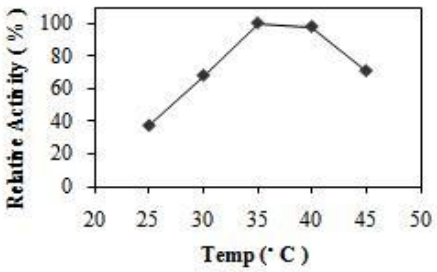


Figure 3: Effect of different temperature on protease production

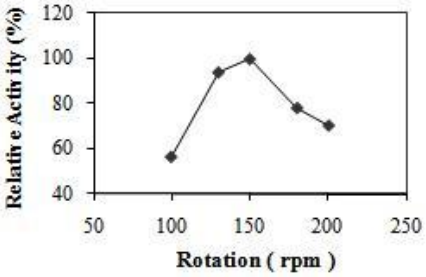


Figure 4: Effect of different agitation speed on protease production

3.1.1. Effect of different carbon and nitrogen sources

As shown in Figure 5, B.licheniformis exhibited higher productivity of protease in culture media containing lactose as carbon source. The best organic nitrogen sources observed with a combination of peptone and yeast extract (Figure 6)

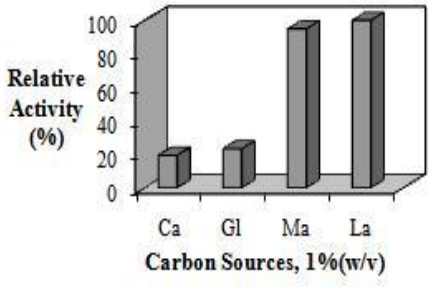


Figure 5: Effect of different carbon sources (1% w/v) on protease production. Ca, casein; Gl, glucose; Ma, maltose; La, lactose

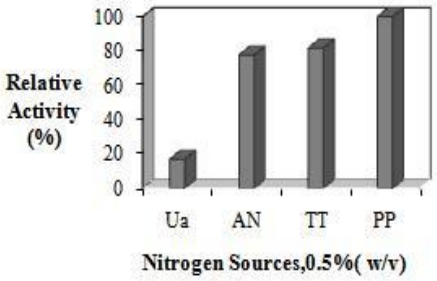
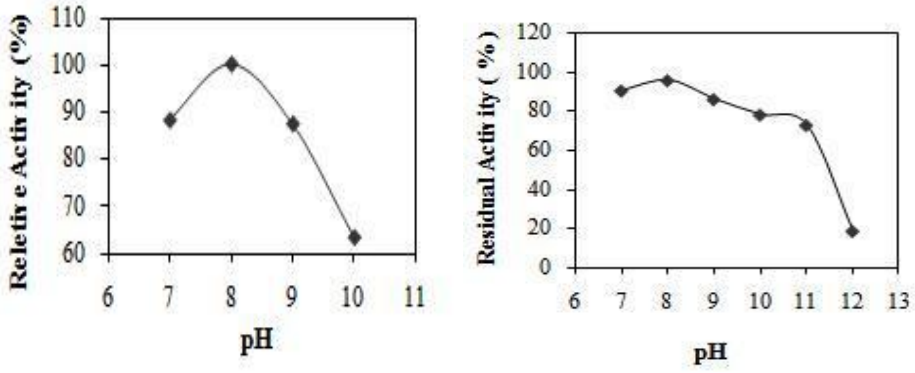


Figure 6: Effect of different nitrogen sources (0.5 % w/v) on protease production. Ua, urea; AN, ammonium nitrate; TT, tryptone; PP, peptone

3.2. Characterization of the crude enzyme obtained from B. licheniformis

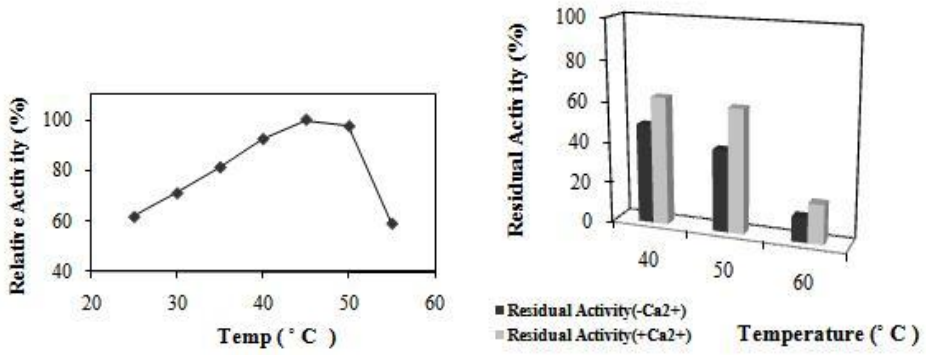
3.2.1. Effect of pH and temperature on enzyme activity and stability

The enzyme was active between pH 7.0 and 10.0 with an optimum at pH 8 (Figure 7a). As seen in Figure 7a, protease activity decreased above pH 9.0 and was 60 % of the maximum enzyme activity at pH 10.0. The maximum activity obtained at pH 8.0. As shown in Figure 7b,the enzyme was stable between pH 7.0-11.0 (Horikoshi, 1992).



(a) (b)

Figure 7: Effect of pH on the activity (a) and stability (b)



(a) (b)

Figure 8: Effect of temperature on the activity (a) and the thermal stability (b) of the protease

As shown in Figure 8a, the maximum enzyme activity was obtained between 40 and 50 ^{o}C. The enzyme retained 63, 61 and 19% of its initial activity after 30 *min* incubation at 40, 50 and 60 $^{\circ}$C, in the presence of Ca^{2+}-Figure 8b (Ghorbel, et al, 2003).

4. Conclusions

This work describes the production and the characterization of alkaline protease from B. Considering the properties of this enzyme –such as high activity and stability in alkaline pH, the B. licheniformis (BBRC-100053) may find potential application in various industrial fields.

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