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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 7 (9): 1631-1635, 2004
ISSN 1028-8880

Production and Some Properties of Protease Produced by *Bacillus licheniformis* Isolated from Tihamet Aseer, Saudi Arabia

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Abstract: Culture conditions affecting protease production by *Bacillus licheniformis* isolated from Tihamet Aseer, Saudi Arabia were investigated. The bacterium strain gave a maximum protease production of 221 U mL^{-1} when growing in a casein broth medium after 36 h compared with gelatin broth medium. Optimum pH and temperature of protease production were 8 and 50°C, respectively. The highest level of protease production in the presence of soybean meal as a carbon source and the peptone as a nitrogen source was obtained. The protease was optimally active at pH 9 and 55°C . The enzyme was stable at temperature range of 60-65°C during the period tested (1h) and retaining more than 85% of its activity at 70°C. These properties make the enzyme suitable for detergent industry.

Key words: *Bacillus licheniformis,* protease, production, properties

INTRODUCTION

Proteolytic enzymes represent a very important group of industrial enzymes. They have a wide array of biotechnological applications such as the manufacture of food, leather, detergents, protein hydrolysate and pharmaceuticals.[1-3] Microbial alkaline proteases dominate the worldwide enzyme market, accounting for a two-thirds share of the detergent industry[4]. Alkaline proteases useful for detergent applications were mostly active in the pH range 8-12 and at temperatures between 50 and 70°C[5]. A great interest has been brought to the proteases production by a number of bacteria[3,5,6] and fungi[7-9]. *Bacillus licheniformis* and *Bacillus pumilus* were the species which have been used in industry for alkaline protease production[10,11]. However, in spite of much researches of this subject, little studies have been done in Saudi Arabia[12]. The present work was carried out to investigate the protease production by *Bacillus licheniformis* isolated from soil samples of Tihamet Asser in Saudi Arabia and the culture conditions affecting their production. Some properties of the enzyme have also been studied.

MATERIALS AND METHODS

Microorganism: *Bacillus licheniformis*, used in this study was isolated from soil samples collected from Tihamet Aseer Saudi Arabia. The identification was made according to the key of Bergey`s manual of determinative bacteriology[13].

Media: Media used for growth and protease production were:

1. Casein broth (C.B): Casein, 3%; $NaNO_3$ 0.5%; K_2HPO_4 0.5%; $MgSO_4$. $7H_2O$ 0.02%; Na_2CO_3 1%.
2. Gelatin broth (G.B.): Gelatin, 3%; K_2HPO_4 0.2%; Glucose 0.1%; peptone 0.5%

Culture conditions for protease production: Enzyme production was carried out in 250 mL Erlenmeyer flasks containing 50 mL production medium (pH 7.0). A 1 mL inoculum was added and incubated at 45°C and 100 rpm for different periods up to 72 h in a shaking incubator. The culture was centrifuged at 8000xg for 10 min at 4°C and the supernatant was used as enzyme solution. The effect of incubation periods, initial pH, temperature, carbon and nitrogen sources were investigated.

Enzyme properties: Optimum temperature and pH for enzyme activity were determined by performing the standard assay at different temperature ranging from 40 to 70°C and different pH in the range of 6 to 12. Thermal stability of the enzyme was also determined by measuring the residual enzyme activity after enzyme incubation at various temperatures ranging from 40 to 80°C for 1 h.

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Enzyme assay: Proteolytic activity in culture supernatant was determined using the spectrophotometric method[14]. Enzyme solution (1 mL) was incubated with 1 mL of 2% casein in phosphate buffer (50 mM, pH 7) at 50°C for 10 min. and then the reaction was terminated by the addition of 5 mL (5%) trichloroacetic acid. After 30 min, the mixture was filtrated and 2 mL of filtrate was added to 4 mL 0.1 N NaOH and 0.5 mL diluted Folin-ciocalteus reagent, the absorbency was 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.

Cell growth determination: Cell growth was determined by diluting the culture supernatant to the appropriate concentration with 0.9% NaCl. Cell concentration was calculated as the difference in optical density values between the sample and blank at 560 nm[3].

RESULTS AND DISCUSSION

Time course of protease production using two different media: The results shows that the enzyme production determined in C.B. medium was increased up to 2.14 fold than that of G.B. medium (Fig. 1 and 2). This may be due to the fact that C.B medium contains less easily metabolizable carbohydrates, as reported by Schaffer[15]. Moon and Parulekar[2], Beg *et al.*[16] also mentioned that the depression of protease synthesis by excess of glucose led to 52% decline in protease production. From the profile of protease production and cell growth using C.B. medium (Fig. 1), it can be observed that, cell growth was parallel increased with enzyme production. Little synthesis of protease was achieved during the earlier part of logarithmic growth phase reaching 32 U mL^{-1} at 12 h.

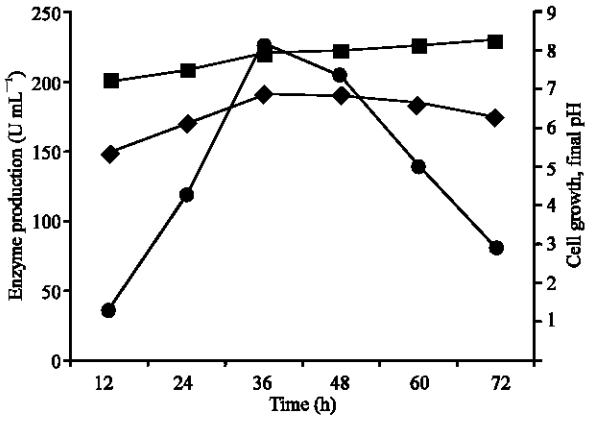

Fig. 1: Time course of protease production using casein medium. Enzyme unit (●) Cell growth (♦) Final pH (■)

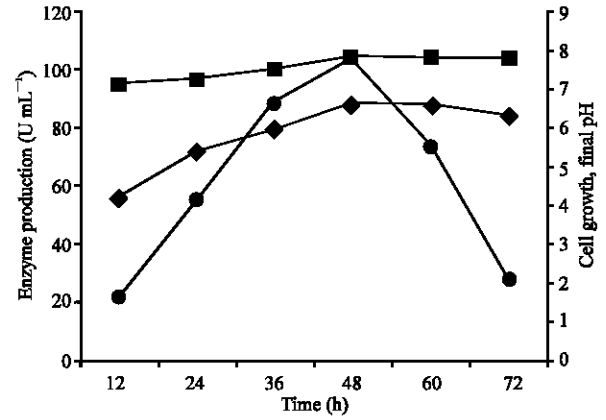

Fig. 2: Time course of protease production using gelatin medium. Enzyme unit (●) Cell growth (♦) Final pH (■)

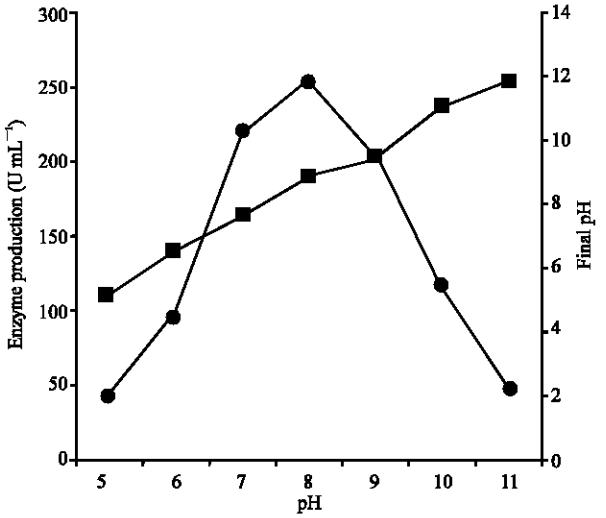

Fig. 3: Effect of initial pH on protease production, Enzyme unit (●) Final pH (■)

A rapid increase in protease production was recorded in the late stage of the logarithmic growth phase. It was increased gradually to maximum value (221 U mL^{-1}) after 36 h of cultivation. Thereafter protease production was decreased gradually with cell autolysis. The present results were supporting the findings of MacFarlane *et al.*[17] Feng *et al.*[3] and Kim *et al.*[18] where they reported that the maximum protease production usually occurs at the late logarithmic to the beginning of stationary phase of growth.

Effect of initial pH: Results obtained in Fig. 3 revealed that the protease production was particularly sensitive to acidic pH. Maximum protease production was obtained at alkaline condition of pH 8 reaching 255 U mL^{-1}. The enzyme production was gradually decreased to 18.04% at

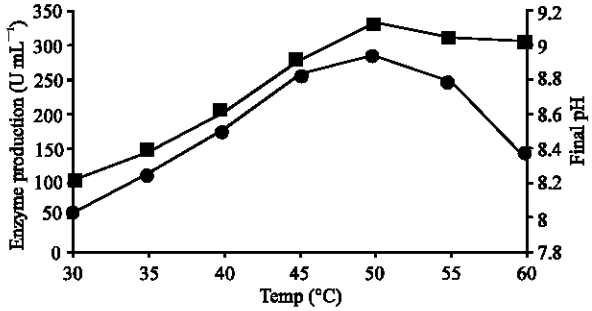

Fig. 4: Effect of incubation temperature on protease production, Enzyme unit (●) Final pH (■)

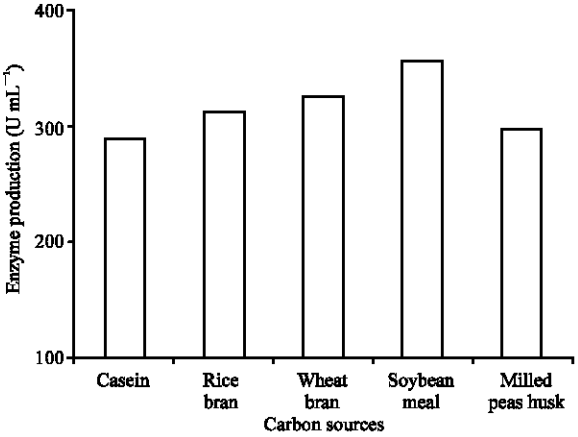

Fig. 5: Effect of carbon sources on protease production

pH 11. These observations were in agreement with the result of El-Hawary and Ibrahim[19], Joo *et al.* [20] They concluded that the optimum pH must meet the requirements of the protease producing gene and the bacteria were more sensitive to pH when used for the production of enzymes.

Effect of incubation temperature: Temperature is one of the most important factors affecting the enzyme production. The results illustrated in Fig. 4 referred to a positive relationship between protease production and incubation temperature up to 50°C. However, about 84% of the original enzyme production was gained at 55°C. These results were in harmony with the findings of Atalo and Gashe[21], Johnvesly *et al.*[22].

Effect of carbon sources: The influence of agro-natural products as carbon sources on protease production was (Fig. 5) revealed that enzyme production was increased with all natural carbon sources compared with casein. The most efficient natural carbon source for protease production was soybean meal, which yielded 345 U mL^{-1}. The superior effect of natural carbon sources in enzyme production may be due to the presence of growth

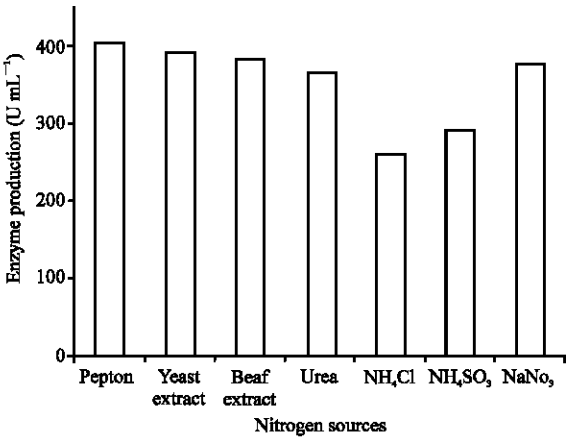

Fig. 6: Effect of nitrogen sources on protease production

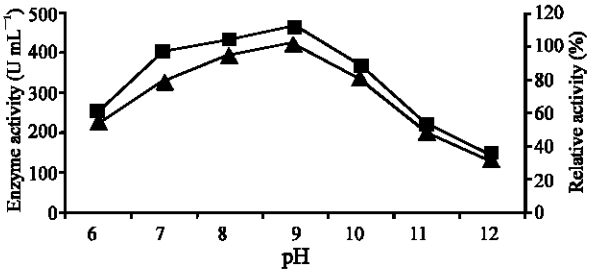

Fig. 7: Effect of pH protease activity. Enzyme activity (■), Relative activity (▲)

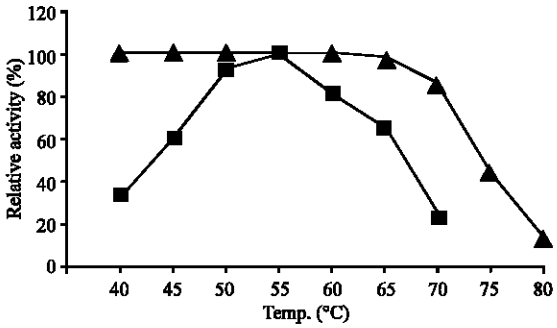

Fig. 8: Effect of temperature on protease activity (■) and stability (▲)

promoters in enough amounts covering the requirements of bacterial growth and enzyme production. These results were in agreement with the results of Chu *et al.*[6], Joo *et al.*[20] and Nilegaonkar *et al.*[23].

Effect of nitrogen sources: According to Fig. 6, it can be indicated that the inorganic nitrogen sources did not stimulate the protease production than organic nitrogen sources. Maximum protease production was recorded in culture medium containing pepton as nitrogen source reached to 401 U mL^{-1}. Final pH of the culture was found

10.24 and 10.85 when ammonium sulfate and ammonium chloride were used, respectively. It may have a relationship with the decreasing of protease production. These results were in accordance with Johnvesly and Naik[10] and Beg *et al.* [16]They reported that protease production was suppressed up to 90% when inorganic nitrogen sources were supplemented in the production medium.

Effect of pH on protease activity: Optimum pH of protease activity (Fig. 7) was determined by using 50 mM of phosphate buffer (pH, 6-8) and Glycin-NaOH buffer (pH, 9-13) in the assay system. Maximum enzyme activity was observed at pH 9. The enzyme activity was reduced to about 54.6% at pH 6 and lost 70.6% of its activity at pH 12. This enzyme was active in alkaline conditions, indicating its potential use in detergent formulations as reported by Towatana *et al.*[24] and Gupta *et al.* [4], who mentioned that alkaline protease which was useful for detergent applications are mostly active in the pH range 8-12.

Effect of temperature on protease activity and stability: The results shows that the optimum protease activity was achieved at 55°C with a sharp apparent decline below and above this value (Fig. 8). However, about 81.5% of the original activity was gained at 60°C. Comparing the present results with those of El-Hawary and Ibrahim[19] and Nilegaonka *et al.*[23] it could be concluded that the optimum temperature of proteolytic activity frequently exceeded the optimum temperature for enzyme production. The thermostability of enzyme was measured after the enzyme had been kept at different temperature for 1 h. The protease activity was relatively stable in the temperature range 60-65°C and retained 85.2% of its activity at 70°C. The stability of protease enzyme could be due to their genetic adaptability to carry out their biological activity at higher temperature, as mentioned by Gaur *et al.*[25]. Whittle and Bloomfield[26] and Kanekar *et al.*[27].

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