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EFFECT OF TEMPERATURE, pH AND METAL IONS ON THE ACTIVITY AND STABILITY OF ALKALINE PROTEASE FROM NOVEL *BACILLUS LICHENIFORMIS* MZK03

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Abstract: The effect of temperature, pH and metal ions on the activity and stability of crude protease from *Bacillus licheniformis* MZK03 was studied. The fermentation in shake culture revealed that maximum level of enzyme was produced at 37 ° C and pH 8.5 after 39 hr at 120 rpm. It lost its activity rapidly above 50 ° C and half-life of the protease at this temperature was 50 min with optimum activity at 40 ° C. It was most stable at pH 8.5 and lost its activity rapidly above pH 10.0, and at pH 11.0 reached 30% of the activity obtained at pH 9.0. The enzyme lost its activity completely at pH 13.0. Optimum proteolytic activity was found at 40°C and pH 9.5. The enzyme activity was accelerated by the addition of Mg^{2+}, Ca^{2+} and Mn^{2+}, whereas it was inhibited by Hg^{2+}.

***Keywords*:** *Bacillus licheniformis,* alkaline protease, fermentation, half life, proteoytic activity

Introduction

Proteases represent the class of enzymes, which occupy a pivotal position in respect to their physiological roles as well as their commercial applications [1]. The current estimated value of the worldwide sales of industrial enzymes is US $1 billion; among them 75% are hydrolytic [2,3]. The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications [1]. Proteases have a long history of application in the food and detergent industries, and a relatively new development is the use of protease in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals in the leather industry [4]. Biotechnological production of this enzyme locally can at best save foreign currency each year but its successful application in the leather industry and bioremediation is bound to replace the usage of harsh chemicals thus curtailing environmental pollution. In view of the recent trend of developing environmentally friendly enzymes, proteases are envisaged to have extensive applications in leather processing and in several bioremediation processes. Preliminary study of this cell-free enzyme preparation proved to be very promising in its application in pre-tanning and feather solubilization (to be published elsewhere). Proteases occur ubiquitously in animals, plants and microbes. However, microbes are the goldmine of protease production and the most significant source compared with animal or plant proteases [5]. Proteases from *Bacillus stearothermophilus, B. subtilis* and *B. licheniformis* have been marketed but most of the development of these works is patented. So to develop our own strain, we investigated the protease of *B. licheniformis* MZK03 that was isolated and identified from the local environment [6].

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Materials and Methods

Microorganisms and growth conditions

Bacillus licheniformis MZK03 was isolated and identified by 16 S rRNA gene sequence analysis [6]. The organisms were then studied for their ability to grow on casein containing solid medium to determine their caseinolytic activity as protease enzyme potential. In this medium, they were cultured at 45ºC for 48 hr. The strain was preserved at 70ºC in the Department of Microbiology, University of Dhaka. A single colony was tansferred into screw cap test tube containing 5.0 ml of nutrient broth to obtain fresh inoculums, and was incubated for 24 hours at 37°C in an orbital shaker.

Fermentation

A 5.0 ml portion of the above inoculums was transferred to 100 ml of alkaline protease-producing broth (APPB) consisting of 10.0 g of glucose, 5.0 peptone g, 5.0 g yeast extract, 5.0 g K_2HPO_4, 0.1 g $MgSO_4.7H_2O$ and 1000.0 ml distilled water in several 500 ml Erlenmeyer flasks. The inoculated medium was placed in a thermostatic orbital shaker for 42 hrs at 37°C and 120 rpm. Samples were withdrawn at different time intervals and centrifuged at 4000 ´ g for 25 minutes. The cell free supernatant was used as a crude enzyme preparation. Protease activity was determined with azocasein as substrate by a modified procedure described by Kreger and Lockwood [7]. One unit of protease activity was defined as the amount of enzyme that produces an increase in absorbance of 0.01 under the above assay condition. Soluble protein in the culture supernatant was estimated according to the Bradford method [8].

Optimization of the Cultural Conditions for the Production of Alkaline Protease by Bacillus licheniformis MZK03 in shake culture

To determine the optimum incubation time for alkaline protease production by the strain, *B. licheniformis* MZK03, fermentation was carried out at 37°C and 120 rpm in an orbital shaker and the samples were collected at different time intervals. The supernatant was separated by centrifugation for 30 min at 4000 xg for the determination of alkaline protease activity as well as soluble protein estimation.

To detect the effect of different initial pH on the production of alkaline protease by *B. licheniformis* MZK03, the pH of the fermentation broth was adjusted to 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 and 10 separately with NaOH in 500 ml Erlenmeyer flasks. The fermentation was carried out for 42 hrs at 37°C and 120 rpm in an orbital shaker.

To investigate the effect of different temperatures on the production of alkaline protease, fermentation was carried out at different temperatures (30°C, 35°C, 37°C, 40°C, 45°C and 50ºC) in an orbital shaker at 120 rpm for 42 hr.

Characterization of alkaline protease

The thermostability of crude enzyme was measured by incubating the enzyme preparation at 30°C, 40°C, 50°C, 60°C, 70°C and 80°C and pH stability was measured by incubating the enzyme at pH 6 to 12 in different buffers (0.1M) such as KH_2PO_4-K_2HPO_4 (6.0-7.5), Tris -HCl (8.0 -9.0), Glycine-NaOH (9.0 –13.0) and Na_2HPO_4-NaOH (11.0-12.0) and the residual activity was measured.

To detect the effect of metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+} and Hg^{2+}) on crude enzymes, metal salt solutions were prepared in a concentration of 10 mM, and 1.0 ml of metal solution was mixed with 5.0 ml of crude enzymes and was incubated for 2 hr. Initial and final enzyme activities were measured by the azo-casein digest method.

Results and Discussion

B. licheniformis MZK03 demonstrated a large zone of hydrolysis around the large colony on skim milk agar medium (SMA) (Fig. 1). This indicated their good growth with the ability of producing extracellular proteolytic enzymes at high temperature. From the industrial viewpoint, it is necessary to select a strain with high yield of alkaline protease in a short fermentation period. The study of the time course for alkaline protease revealed that *B. licheniformis* MZK03 produced maximum level of alkaline protease after 39 hr (98 U/ml; Fig. 2). Almost similar results were obtained by Frankea *et al.* [9] and Manachini *et al*. [10]. Some earlier studies have shown that the Optimum incubation period for protease production by *B. licheniformis is* about 36 hr [11], 24-36 hr for *B. stearothermophilus* [12] and 48 hr for *B. subtilis* [13]. Kunamneni *et al.* [14] have found that the highest activity is after 48-hr of incubation with *B. subtilis* PE-11.

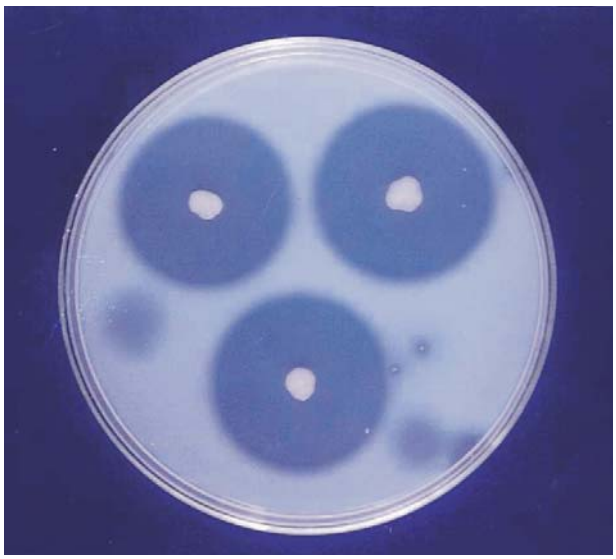

Figure 1
Zone of clearance due to hydrolysis of casein by *B.licheniformis* MZK03 on SMA medium.

The production of enzyme is greatly influenced by the initial culture pH. The optimum pH for production of alkaline protease by *B. licheniformis* MZK03 was 8.5 (98 U/ml)(Fig. 3). The earlier reported optimum pH for *B. licheniformis* is 8.0 and for *B. licheniformes* MIR-29 it is 7.5 [15]. For *B. subtilis* and *B. cereus* it has been shown to be 8.2 and 9.0, respectively [13,16]. In the present study, however, the most significant level of growth and production of protease was supported by pH ranging between 8.0 and 10.

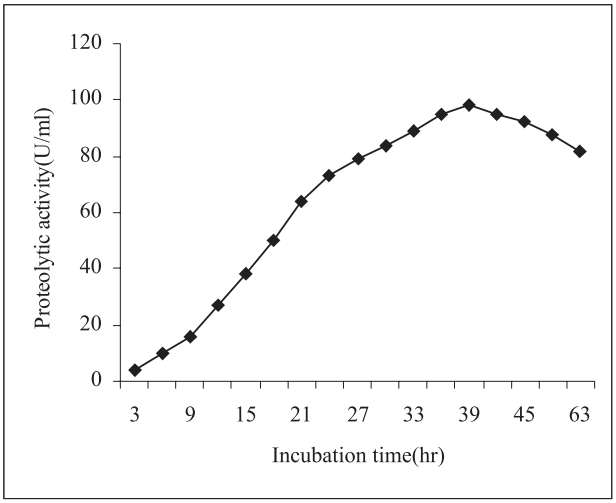

Figure 2
Time course for the production of alkaline protease by *B.licheniformis* MZK03.

Temperature is an important environmental factor affecting the growth and production of metabolites by microorganisms. The optimum initial temperature for production of alkaline

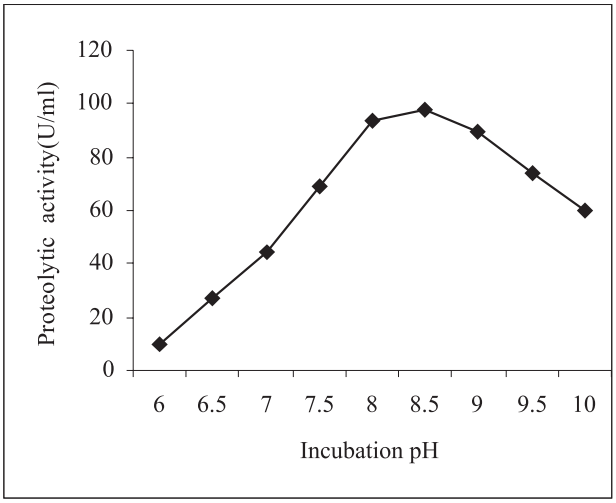

Figure 3
Determination of optimum initial pH on the production of alkaline protease by *B.licheniformis* MZK03.

protease by *B. licheniformis* MZK03 was 37° C, although appreciable amount of the enzyme production occurred at temperatures ranging between 30 ° C and 50 ° C (Fig. 4). Hameed *et al.* found that 36 ° C to be the optimum temperature for *B. licheniformis* [13].

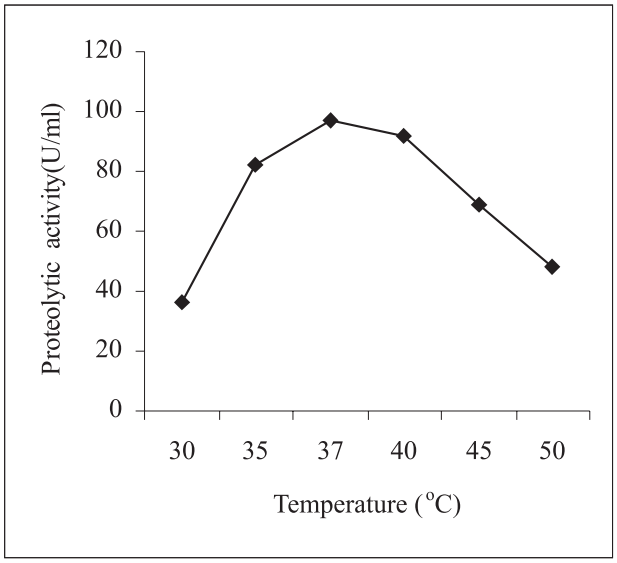

Figure 4
Determination of optimum temperature in the production of protease by *B.licheniformis* MZK03.

Protease from B. *licheniformis* MZK03 remained stable over a wide pH range (6 to 11). The residual activity at pH 6.0 was about 20% and remained above 70% up to pH 10.0, but elevation of pH beyond 10.0 caused rapid decrease in enzyme activity (about 29% at pH 11.0 and almost zero at pH 12.0). Maximum activity was found at pH 8.5 (Fig. 5). The enzyme studied by Malathi and Dhar [17] and Manachini *et al*. [10] had a pH optimum range of 9.0-9.5. This indicates that the enzyme studied by them was alkaline in nature. The present results show it to be slightly less alkaline even when compared with the protease produced by *Bacillus* No. 221 [18] and B. licheniformis MIR 29 [15] with an optimum pH of 11.5, 12.0 and 13 respectively.

The effect of the reaction pH value on the activity of protease was investigated in Tris–HCl buffer (pH 7.0–9.0) and Glycine–NaOH buffer (pH 10.0-11.0). The results in Fig. 4 show that the protease of *B. licheniformis* MZK03 was optimally active at pH 9.5, below or above which the activity decreased gradually. Protease of *Bacillus pseudofirmus* AL-89 exhibited optimum activity at pH 11 and showed low activity at pH values below pH 10 and above 11.5 [19].

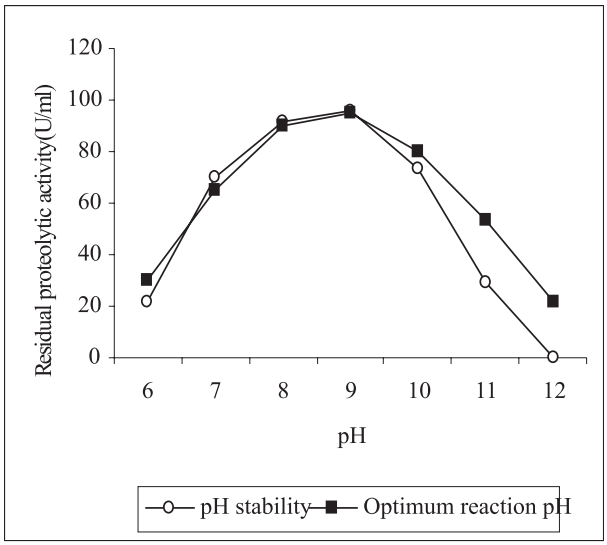

Figure 5
pH profile and pH stability of alkaline protease at 37°C.

Temperature also plays an important role in activation and inactivation of enzymes. Each enzyme has an optimum temperature for maximum enzyme activity. The present study has demonstrated that at temperatures of 60 ° C and 70 ° C, the enzyme lost its activity rapidly. Its half-life at 50° C was 50 min, at 60° C 17min, and at 70° C it was 8 min, with the optimum at 40° C (Fig. 6). For *B. licheniformis* S-40, it has been reported to be 50 ° C [20]. The results of the present study are not discordant with the results for *B. licheniformis* MIR-29 [15], in which case the enzyme retained 90% stability at 70 ° C followed by decrease.

The effect of the temperature on the enzyme substrate reaction was investigated using the Azo-casein digest method at pH 9.0. It was found that the activity of protease increased with the

increase of the reaction temperature up to 40° C (Fig. 6). However, further rise in the reaction temperature caused loss of proteolytic activity. Hossain *et al.* [17] have also obtained similar result.

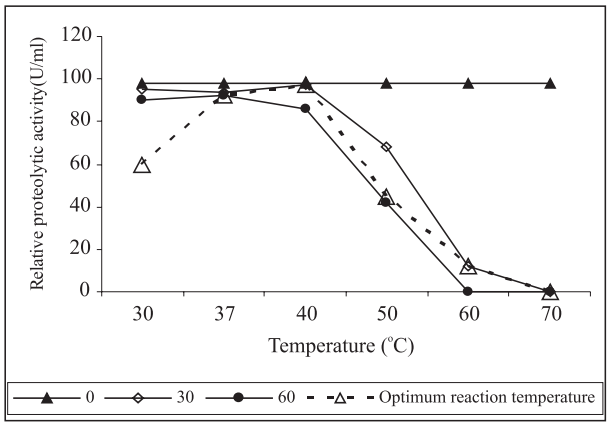

Figure 6
Temperature profile and thermostability of protease from *B.licheniformis* MZK03 at pH 9.0.

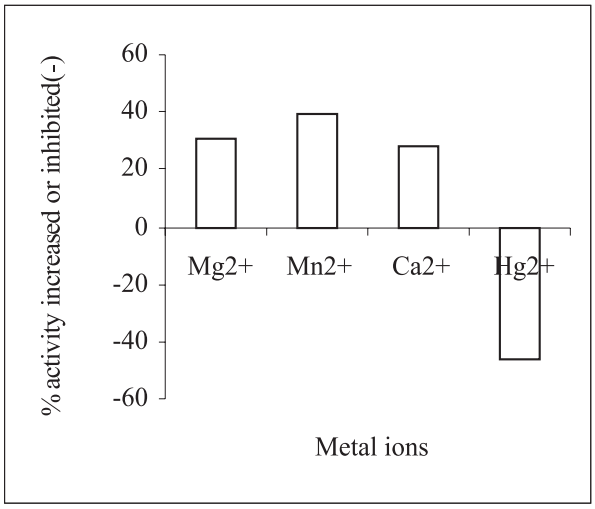

Figure 7
Investigation of the effect of metal ions on crude protease.

Storage stability is a prominent factor for commercialization of an enzyme. With the view of increasing the storage stability, the effect of various metal ions on crude protease was investigated. Fig 7 shows that the enzyme activity increased by 31%, 39%, 28% with Mg^{2+}, Mn^{2+}, Ca^{2+}, respectively, and decreased by 46% with Hg^{2+}. This phenomena indicates that the enzyme requires metal ions as cofactors. These results correlate with the observations of Kunamneni *et al.* [14] who found that Mg^{2+}, Ca^{2+} and Mn^{2+} increased the enzyme activity by 16%, 35% and 8%, respectively, while Hg^{2+} reduced the activity by 7% and has also been reported by Takeda *et al*. [19].

References

1. **Fox, J.W., Shannon, J.D. and Bjarnason, J.B.** 1991. Proteinases and their inhibitors in biotechnology. Enzymes in biomass conversion. *ACS Symp Ser.* **460:62**–79.
2. **Godfrey, T. and West, S.** 1996. *Industrial enzymology.* 2nd ed. New York, N.Y: Macmillan Publishers Inc. p.3.
3. **Kalisz, H.M.** 1988. Microbial proteinase. In: *Advances in Biochemical Bioengineering,* Ed. Ficchter, A., Vol. 36, pp. 1-65, GBF. Frg.
4. **Rao, M. B. and Deshpande, V.V.** 1995. Proteases and their applications in biotechnology. In: *Microbes: for health, wealth and sustainable environment.* Ed. Varna, A. In Press, Malhotra Publishing House, New Delhi, India.
5. **Ward, O.P.** 1985. Proteolytic enzymes. In: *Comprehensive Biotechnology.* Eds. Blanc, H.W., Drew, S. and Wang, D., Vol 3, pp.789-818. Oxford, UK: Pergamon Press.
6. **Hoq, M.M., Siddiquee, K.A.Z, Kawasaki, H. and Seki, T.** 2005. Keratinolytic activity of some newly isolated *Bacillus* species. *J. Biological Sci.* 5:193-200.
7. **Kreger, A. and Lockwood, D.** 1981. Detection of extracellular toxins produced by *Vibrio vulnificas. Infect. Immun.* 33:583-590.
8. **Bradford, M.M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein-dye binding. *Appl. Biochem.* 72:G80-G85.
9. **Frankena, J., Verseveld, H.W.V. and Stouthmer, A.1.J.** 1985. A continuous culture study of the bioenergetics aspects of growth and production extracellular protease by *Bacillus licheniformis. Appl. Microbiol. Biotech.* 22:169-176.
10. **Manachini P.L. and Parini, C.** 1988. Thermostable alkaline protease produced by *Bacillus thermoruber,* a new species of *Bacillus. Appl. Microbiol. Biotech.* 28:409-413.
11. **Mao, W., Pan, R., and Fredman, D.** 1992. High production of alkaline protease by *Bacillus licheniformis* in a fed-batch fermentation using synthetic medium. *J. Indust. Microbiol.* 11:1-6.

12. **Fujio, Y. and Kume, S.** 1991. Characterization of a highly thermostable neutral protease produced from *Bacillus stearothermophilus*. *World J. Microbiol. Biotech.* 7: 12-16.
13. **Hameed, A.C., Natt, M.A. and Evans, C.S.** 1996. Production of alkaline protease by a new *Bacillus subtilis* isolate for use as a bating enzyme in leather treatment. *World J. Microbiol. Biotech.* 12:289-291.
14. **Kunamneni, A., Poluri, E., and Davuluri S.P.** 2003. Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. *AAPS PharmSci. Tech*. 4:56.
15. **Ferrero, M.A., Castro, G.R., Abate, C.M., Baigori, M.D. and Sineriz, F.** 1996. Thermo stable alkaline protease of *Bacillus licheniformis* MIR 29: isolation, production and characterization. *Appl. Microbiol. Biotech.* 45:327-332.
16. **Hossain, M.S., Iqbal, M.S. and Hoq, M.M.** 2004. Fermentative characteristics of alkaline protease by *Bacillus cereus* MZK-09. *Bangladesh J. Microbiol*. 21:86-89.
17. **Malathi, S. and Dhar, S.C.** 1987. Production of extracellular protease by an *Aspergillus flavus* isolate and its application in the depilation of skins. *Leather Sci.* 34:67-76.
18. **Horikoshi, K.** 1971. Production of alkaline enzymes by alkalophilic microorganisms, part-I. Alkaline protease produced by *Bacillus* No. 221. *J. Agri. Biol .Chem.* 3G:285-293.
19. **Takeda, M., Iohara, K., Shinmaru, S., Suzuki I. and Koizumi, J.** 2000. Purification and properties of an enzyme capable of degrading the sheath of *Sphaerotilus natans*. *Appl. Environ. Microbiol.* 66:4998–5004.
20. **Sen, S. and Saytyanarayana, T.** 1993. Optimization of alkaline protease production by *Bacillus licheniformis* S-40. *J. Ind. Microbiol.* 33:43-47.