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Original Article

Production and Characterization of Keratinolytic Protease of *Bacillus licheniformis* MZK-03 Grown on Feather Mill

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Keratinolytic protease is an inducible specific proteolytic enzyme, which is produced by *Bacillus licheniformis* MZK-03 in presence of keratin (feather mill) as sole carbon and nitrogen source in growth medium. Maximum level of keratinolytic protease was produced after 42 h at 37°C over a wide range of initial pH (5.0 to 12.0) under continuous agitation (200 rpm). Keratinolytic protease worked best at 37°C and at pH 8.5. The enzyme was quite stable over a wide range of pH (7.0 to 11.0) but activity dropped drastically beyond this level (enzyme activity dropped to 8.8% and 19.3% at pH 6.5 and 12.0, respectively). Half-life of keratinolytic protease at 70° and 60°C were found to be 3 and 7 min respectively. The enzyme showed highest stability at 40°C (>90% after 3 h). The half-life at 4°C was 34 days. The presence of metal ions (5 m*M*) like Mg^{2+}, Mn^{2+}, Ca^{2+} and K^{+} had no remarkable effect on the keratinolytic protease activity but the activity decreased in presence of Hg^{2+} and Cu^{2+}. The enzyme may belong to serine protease group as it is inhibited by serine protease inhibitor phenyl methyl sulphonyl fluoride (PMSF). The enzyme is as compatible as other commercially available enzymes used in leather industry with tannery chemicals. It was completely incompatible with Na_2S and CaO for their high alkalinity (pH >13.0), which was also observed for other commercial enzymes except the commercial enzyme supplemented with ammonium sulphate.

Keywords: *Bacillus licheniformis* MZK-03, Keratinolytic protease, Keratin, Production, Characterization

Introduction

Proteases are catabolic enzymes that catalyze the complete hydrolysis of protein. They constitute one of the most important groups of industrial enzymes, accounting for nearly 60% of the total worldwide enzyme sale[1]. Keratinolytic protease is a specific protease that has immense commercial importance. It acts on keratin of the hides thus can be used in dehairing[2]. This enzyme along with protease can be effectively used in leather industry as dehairing agent instead of chemicals or with chemicals thus reducing the use of harsh chemicals in leather processing. In addition, it offers considerable opportunities for a low-energy consuming technology for bioconversion of poultry feathers to protein enriched feedstuff for livestock[2-3].

Only few bacterial species have been reported till date able to produce keratin enzyme. Many of the *Bacillus* spp. are found to be useful in enzyme, amino acids, essential metabolites and life saving drug production[4]. Isolation of keratinolytic protease producing strains of *Bacillus licheniformis* were previously reported by us but elaborate characterization was not carried out[5]. One of the major drawbacks affecting the stability at alkaline pH of enzymes recovered from thermophiles is that enzymes from alkalophiles confer stability to a wide range of pH but are usually thermolabile[6]. In the present study we have optimized the physical conditions of keratinolytic protease production and partially characterized the keratinolytic protease produced by *Bacillus licheniformis* MZK-03.

Materials and Methods

Microorganism

Bacillus licheniformis strain MZK-03 was previously isolated from feather-decomposed soil and identified by 16S rRNA typing[5]. Stock culture of the organism was maintained at -70°C in nutrient broth containing 10% glycerol.

Production of seed culture

A single colony from a freshly subcultured nutrient agar plate was transferred into 250-ml conical flask containing 50 ml of nutrient broth. The flask was incubated for 6-10 h at 37°C and 200 rpm in an orbital shaker until it reached to an absorbance of 0.5-0.8 at 600 nm.

Fermentation and separation of culture filtrates

The seed culture (5 ml) was transferred to 95 ml of feather mill medium in a 500-ml Erlenmeyer flask. Feather mill medium contained 0.075% NaCl, 0.21% K_2HPO_4, 0.105% KH_2PO_4, 0.015% $MgCl_2.6H_2O$, 0.009% $CaCl_2$, 0.15% molasses and 0.75% feather mill (initial pH 7.5). The inoculated flasks were placed in a thermostated orbital shaker for 48 h, at 37°C and 150 rpm. Samples were withdrawn at regular intervals and centrifuged at 5,000 rpm for 20 min. The cell free supernatant was preserved at 4°C and used for enzyme assay and protein estimation (in duplicate).

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Estimation of extracellular soluble protein

Soluble protein in the culture supernatant was estimated according to the Bradford method[7] using bovine serum albumin (BSA) as standard.

Determination of keratinolytic protease activity

Keratinolytic protease activity was measured by modified azocasein (Sigma, USA) digest method[8]. The enzyme (400 ml) was incubated with 200 ml of 1% azocasein in 0.05 m*M* Tris-HCl buffer (pH 8.5) at 37°C for 30 min in a shaking water bath. The reaction was terminated by adding 1.4 ml of 10% trichloroacetic acid (TCA) and the mixture was kept at 4°C for 15 min. After centrifugation at 10,000 rpm for 10 min, 1 ml of the supernatant was decanted to a fresh tube and mixed with 1 ml of 0.5 *M* NaOH. The absorbance of the reaction mixture was read at 440 nm against a control prepared in the same way, except that the TCA was added before addition of enzyme. One unit of enzyme activity was defined as an increase of 0.01 absorbance units per min under the given conditions.

Results and Discussion

The optimum incubation time for keratinolytic protease production by *B. licheniformis* MZK-03 was determined by carrying out the fermentation in 500-ml Erlenmeyer flask containing 95 ml feather mill medium at 37°C and 150 rpm in an orbital shaker. Samples were collected at 3-h interval up to 48 h. Time course data revealed that maximum level of keratinolytic protease was produced after 42 h of cultivation period, which represented 95 U/ml enzyme activity and 0.28 mg/ml protein concentration (Figure 1). Protein concentration decreased with time while enzyme activity increased as feather mill consists of protein (keratin) that was hydrolyzed more with elapsed time. An initial peak of keratinolytic protease activity was observed at 21 h of the cultivation (84 U/ml). This could be explained by Type II fermentation where two maxima were observed (Figure 1). Other possible explanation would be cytoplasmic membrane bound longer secretion phase or the excretion of other proteases rather than primary protease[9-10]. It was reported previously that *B. licheniformis* PWD-1 exhibited maximum keratinolytic protease activity (35 U/ml) by azo-keratin method after 30 h of incubation period[11].

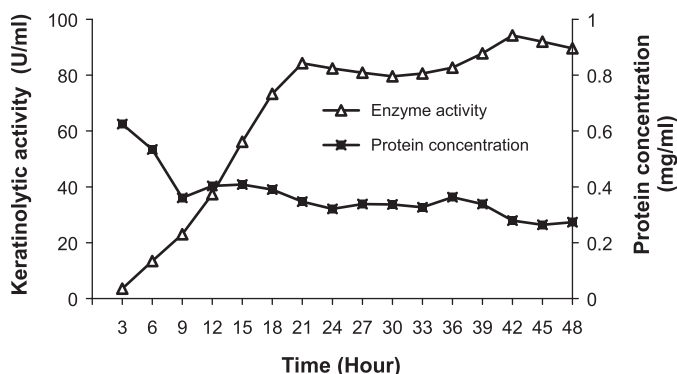

Figure 1. *The time course of keratinolytic protease production by Bacillus licheniformis MZK-03.*

The effect of different initial pH on the production of keratinolytic protease by *B. licheniformis* MZK-03 was investigated by adjusting the initial pH of feather mill broth to different pH ranging from 5.0 to13.0 separately with 0.1 *N* HCl and 1 *N* NaOH in 500-ml Erlenmeyer flask containing 95 ml medium. It was found that the strain efficiently produced keratinolytic protease over a wide range of initial culture pH (5.0 to 12.0). The enzyme production was drastically reduced at pH 13.0 that corresponded to the enzyme activity ca. 14% of the maximum (Figure 2) and high protein concentration.

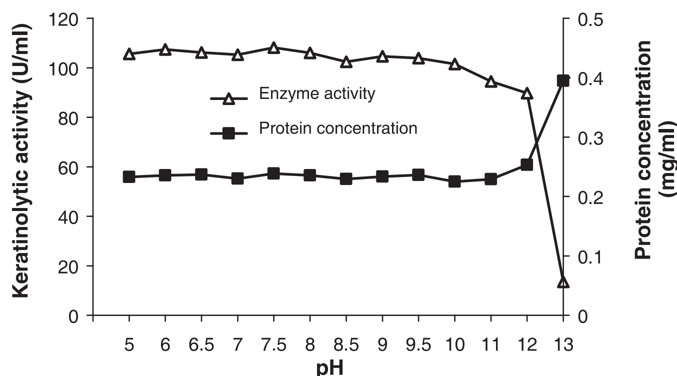

Figure 2. *Effect of initial pH on keratinolytic protease production by Bacillus licheniformis MZK-03.*

The effect of cultivation temperature on the production of keratinolytic protease by *B. licheniformis* was determined between 30° and 50°C in an orbital shaker at 150 rpm for 42 h as it is a mesophilic bacterium[23]. Keratinolytic protease production rate was highest (96 U/ml) at 37°C, which was also been reported for the production of keratinolytic protease from other *Bacillus* isolates[5,12].

The fermentation was carried out at different agitation rates ranging from 100 to 250 rpm in an orbital shaker to find out the appropriate agitation rate for keratinolytic protease production. It exhibited a gradual increase of keratinolytic protease production with increased agitation rates (data not shown) and the production was maximum at 200 rpm (109 U/ml).

The optimum pH for keratinolytic protease activity was determined using substrate solutions prepared in different buffer systems (0.05 *M*) like citrate-phosphate buffer (pH 5.0-6.0), phosphate buffer (pH 6.5-7.5), Tris-HCl buffer (pH 8.0-9.0), carbonate buffer (9.5-10.0) and glycine-NaOH buffer (11.0-13.0). Reaction mixtures were incubated at 40°C for 30 min and residual keratinolytic protease activity was measured. The enzyme showed maximum activity at pH 8.5 and the activity was about 75% of that of the maximum over a wide range of pH 7.0 to 9.5 (data not shown). Activity declined gradually with more alkalinity (at pH 11.0, the activity was below 30%) as well as in more acidic condition (at pH 6.0, activity was below 35%).

The pH stability of the keratinolytic protease was determined by pre-incubation of the enzyme in various buffers of different pH values (5.0 to 13.0). The enzyme was 10-fold diluted in respective buffers and incubated in a 10°C cooler for 24 h. Control was made by 10-fold dilution of the enzyme with distilled water. Residual activity was measured taking control as 100% activity. The enzyme remained stable over a wide range of pH from 7.0 to 11.0 (data not shown). The result indicated that residual activity at pH 6.5 and 12.0 were about 8 and 19% of the control, respectively and remained over 70% up to pH 11.0. Maximum stability of keratinolytic protease was found at pH 8.5. Therefore, the keratinolytic protease is not able to work efficiently in high alkaline condition with the association of chemicals used in the dehairing step. These findings are in accordance with several earlier reports showing pH optima of 9.0-9.5 of keratinase and proteases from other organisms[13-15]. A higher alkalinity of the protease produced by *B. licheniformis* MIR 29 has been reported having an optimum pH of 13.0[16].

The optimum temperature for the keratinolytic protease activity was determined by incubating the reaction mixture at different temperatures ranging from 20° to 50°C and assayed at pH 8.5 for 30 min. The optimum temperature for keratinolytic protease activity was recorded 37°C (data not shown). The activity was below 30% at temperature 20°C and ca. 50% at 50°C recorded. The optimum temperatures of extracellular proteases produced by *B. cereus* MZK-09 and *B. licheniformis* S-40 were reported to be 40°C and 50°C, respectively[17-18].

The thermostability of keratinolytic protease was measured by incubating the enzyme preparation at different temperatures between 30° to 70°C for different time intervals (10 to 60 min). The activity at 40°C was considered as 100% and the residual activity was measured. Half-life of the enzyme at different temperatures was measured from the graph (Figure 3). The result showed that at temperature 60° and 70°C the keratinolytic protease lost its activity rapidly and the half-life at 60° and 70°C was 7 and 3 min respectively for the *B. licheniformis* MZK-03 enzyme. The enzyme was stable at 40°C for more than 3 h (Figure 3). The serine alkaline protease produced by *B. subtilis* PE-11 was reported to be 100% stable at 60°C after 350 min of incubation[19].

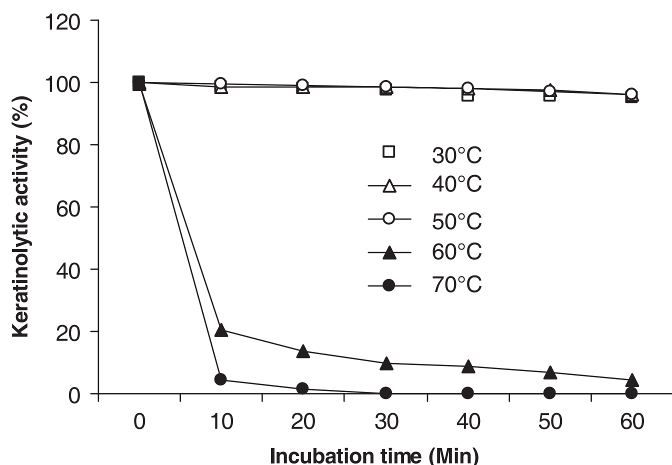

Figure 3. *Determination of the temperature stability of keratinolytic protease.*

The enzyme was dispensed in four 30-ml McCartney bottle and two were held at 4°C and others at 20°C to investigate the storage stability at those temperatures. Enzyme assay was performed every four days interval until the enzyme activity reached half of the initial enzyme activity. The keratinolytic protease was stable at 4°C for more than a month (Figure 4). Half-life of the keratinolytic protease from *B. licheniformis* MZK-03 at 20°C was about 20 days and at 4°C was about 34 days (Figure 4). Stability of keratinolytic protease at 20°C indicated that it could be used under ambient condition (25°-30°C) successfully.

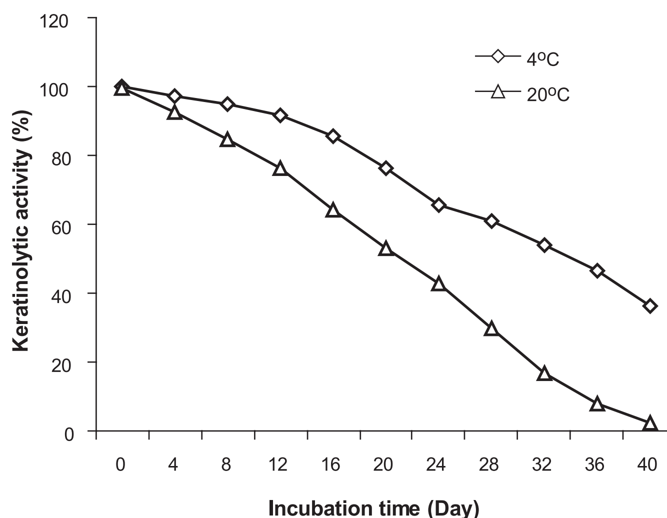

Figure 4. *Effect of storage temperature on the keratinolytic protease.*

With a view to increase the storage stability, investigation was carried out to observe the effect of different metal ions on keratinolytic protease. The effects of metal ions (Ca^{2+}, Mg^{2+}, Mn^{2+}, K^{+}, Cu^{2+} and Hg^{2+}) on the keratinolytic protease were investigated by adding the chloride salt of those metal ions with enzyme at concentrations of 1, 5 and 10 m*M*. Relative enzyme activities were measured considering the control (enzyme mixed with distilled water at respective quantity) as 100%. Mg^{2+}, Mn^{2+}, Ca^{2+} and K^{+} did not have any remarkable effect on the keratinolytic activity whereas Hg^{2+} and Cu^{2+} inhibited the enzyme activity. Pre-incubation of the enzyme with 5 m*M* of Hg^{+} and Cu^{2+} reduced the activity by 62 and 25% respectively (data not shown). These results correlate with other related studies on proteases where Mg^{2+}, Ca^{2+} and Mn^{2+} increased their activity by 16, 35 and 8%, respectively, and Hg^{2+} reduced the activity by 7%[19]. In case of Hg^{2+}, the reduction of the enzyme activity can be attributed to its affinity with the two -SH group present in the keratinolytic protease and disrupt the native polypeptide structure[20].

Inhibition studies primarily give an insight into the nature of an enzyme, its cofactor requirements, and the nature of active centre[21]. The effect of inhibitors like phenylmethylsulfonyl fluoride (PMSF) and ethylene diamine tetra acetic acid (EDTA) on the keratinolytic protease produced from *B. licheniformis* MZK-03 was investigated. Inhibitors were mixed with crude enzyme at various concentrations 10, 5 and 1 m*M*. The mixer was incubated

at 10°C for 24 h. Control was made by mixing respective amount of distilled water with crude enzyme. PMSF (10 m*M*) inhibited the keratinolytic protease activity by 80% (data not shown). It has been reported that PMSF combines with the essential serine residue in the active site of proteases and results in a complete loss of enzyme activity[22]. This result, therefore categorizes the keratinolytic protease produced by *B. licheniformis* MZK-03 as a serine protease. However, many *Bacillus* isolates are known to produce alkaline serine protease[23]. The presence of EDTA could also inhibit the keratinolytic protease activity partially (<30%).

The effects of various tannery chemicals on the activity of different commercial enzymes and the crude keratinolytic protease from *B. licheniformis* MZK-03 were examined. Solutions of tannery chemicals were mixed with enzyme preparations in a concentration likely to be used in different stages of hide processing [0.35% Na_2CO_3, 0.02% Na-hypochlorite, 2% CaO, 2% Na_2S and 2.5% $(NH_4)_2SO_4$] and the resultant pH was detected. After incubation, enzyme activity was measured at different time intervals. It was observed that commercial enzymes from NOVO (England) and the crude keratinolytic protease were completely unstable in presence of CaO and Na_2S while about 50% of their activities were retained in Na_2CO_3, Na-hypochlorite and were completely unaffected with $(NH_4)_2SO_4$ (Figure 5). Incompatibility of enzyme with lime-sulphide may be due to high alkaline condition (pH >13.0) when the enzymes' net electric charge might be changed along with subsequent modifications in its three-dimensional structure. On the other hand the stabilization effect of ammonium sulphate suggested that it might be used as a stabilizer in enzyme preservation. One of the commercially available enzymes was supplemented with ammonium sulphate

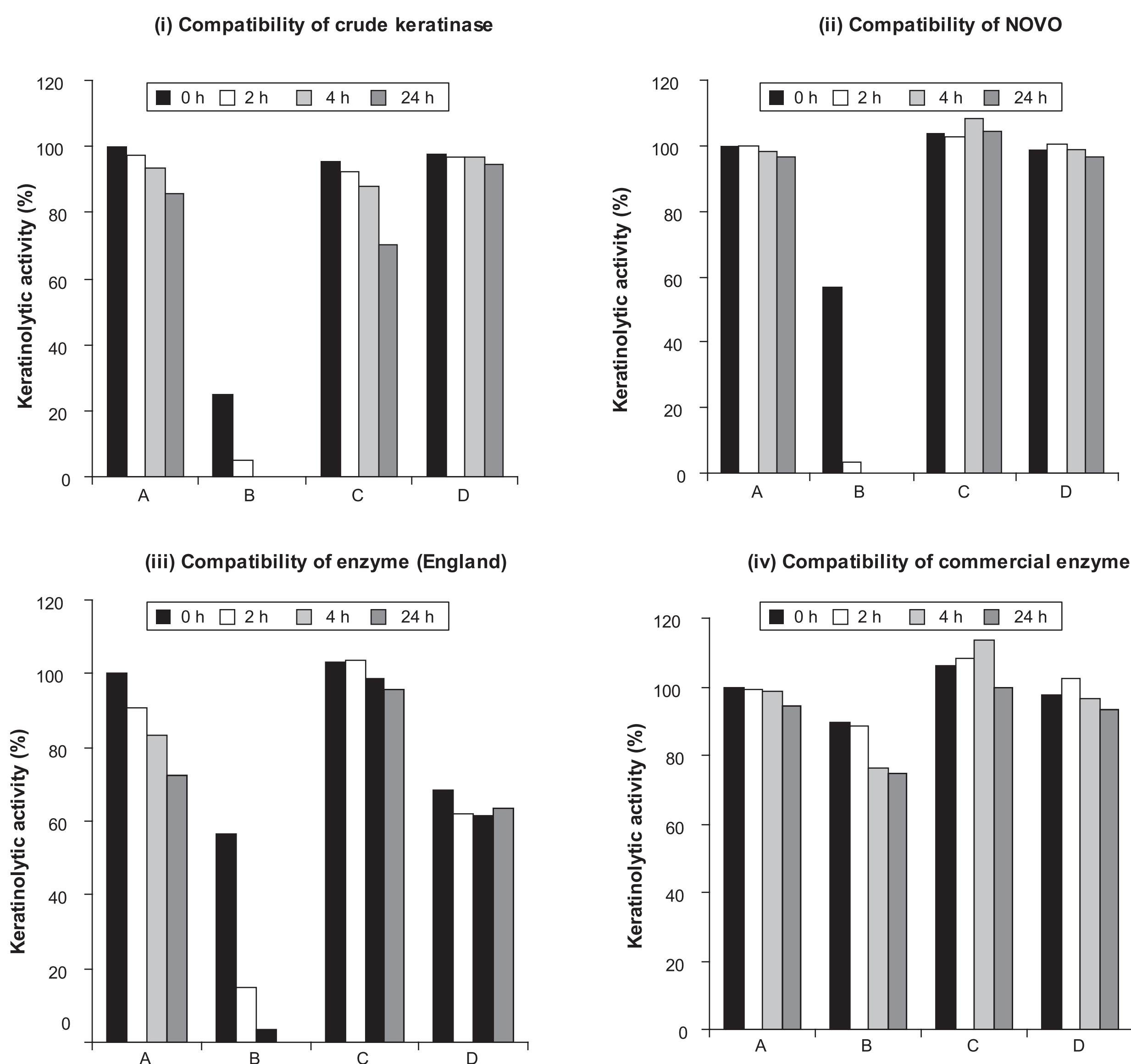

Figure 5. *Compatibility test of keratinolytic protease and commercial tannery enzymes with different tannery chemicals. A = Enzyme; B = Enzyme +* Na_2S *+ CaO; C = Enzyme +* Na_2CO_3*; D = Enzyme +* $(NH_4)_2SO_4$*)*

and it remained stable under high alkaline condition in presence of lime-sulphide.

The remarkable high production ability of keratinolytic protease by *B. licheniformis* MZK-03 validates the potential of using this strain in industries for large scale production of the enzyme. The broad pH stability (6.0-11.0) of the produced enzyme provides some advantages of using in many industrial processes. Its stability under ambient condition for few weeks allows it to be used and stored in crude form under limited facilities. The use of $(NH_4)_2SO_4$ as stabilizing agent provided a viable mechanism of increasing the storage stability of the enzyme which can make the enzyme more compatible with the commercially available chemicals used in tannery industry under high alkaline condition.

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