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Research Paper

Characterization of an alkaline protease with high quality bating potential in leather processing from *Bacillus licheniformis* MZK05M9 mutant

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Abstract

An alkaline protease from *Bacillus licheniformis* MZK05M9 (*Bl*M9), a mutant strain developed in our laboratory, has been partially purified and characterized for its robustness and eco-friendly application potential in processing of hides and skins for leather manufacturing and detergent industries. The enzyme was purified 2.70 fold with specific activity of 1624U/mg in comparison to crude enzyme extract by using ammonium sulfate precipitation, dialysis and Sephadex G-75 column chromatography. The molecular mass of the enzyme was 27.2 kDa as judged by SDS–PAGE. The purified protease had a pH optimum of 8.5 and temperature optimum of 55°C. According to the inhibition profiles obtained with the various protease inhibitors, it was confirmed that the partially purified protease belongs to the serine protease type. The activity of partially purified enzyme was enhanced by calcium, magnesium, barium, potassium and manganese ions and strongly inhibited by mercury ion. In addition, the protease showed remarkable stability in the presence of 1% SDS; 1, 3 and 5% Triton X-100 and H_2O_2, which comprise the common bleach-based detergent formulation. The enzyme was found equally efficient to a commercial enzyme Oropon K (one of the commercial enzymes imported into Bangladesh for bating purpose) in bating of animal hide as proved by different comparative qualitative tests such as tensile strength, percent of elongation, stitch tears strength, water vapor permeability, grain crack strength and tongue tear strength tests. In addition, the stability profile (pH, temperature and surfactants) and blood stain removal data also revealed its suitability for application in detergent industry.

Keywords: *Alkaline Protease; Bacillus licheniformis MZK05M9 (BlM9); Bating; Characterization; Eco-Friendly.*

1. Introduction

Proteases are the most valuable enzymes (Kaur *et al*. 2001) and accounting for more than 65% of the total industrial enzyme market (Rao *et al*. 1998, Banik & Prakash 2004). Alkaline proteases occur widely in plants, animals and microorganisms (Kumar *et al*. 2008) but proteases from microbial sources are preferred to the enzymes from plant and animal sources since microbial alkaline proteases are extracellular nature and are directly secreted into the fermentation broth by the organism, thus simplifying the downstream processing of the enzyme as compared to the proteases obtained from plant and animals (Arulmani *et al*. 2007). Microbial proteases can be produced from bacteria, fungi and yeast through submerged and solid - state fermentation (Kumar & Takagi 1999, Anwar & Saleemuddin 2000, Haki & Rakshits 2003). In bacteria, protease enzyme is produced mainly by *Bacillus licheniformis*, *B. horikoshii*, *B. sphaericus*, *B. furmis*, *B. alcalophilus*, *B. subtilis* (Adinarayana & Ellaiah 2004).These enzymes are used in the detergent, leather industries, food, pharmaceutical and also have application in silver recovery from photographic plates and in peptide synthesis (Paliwal *et al*. 1994). The largest application of the proteases is in the laundry detergents, where they help in removing protein-based stains from clothing during washing (Najafi *et al*. 2005). In leather industry, the protease enzymes are used in soaking, dehairing and bating steps of leather processing (Zambare *et al*. 2013). In bating process the alkaline protease removes the non-structural proteins from hides and skins which render the leather produce soft, pliable and permeable. In this study, characterization of partially purified protease obtained from a mutant *Bacillus licheniformis* MZK05M9 (*Bl*M9) and its application in bating step of leather processing, and in washing to remove blood stain from cloth have been described.

2. Material and methods

2.1. Bacterial strain

Bacillus licheniformis MZK05M9 (*Bl*M9), a mutant strain developed in our laboratory, that produced an alkaline protease was used in the present study. The stock cultures of the strain were maintained as glycerol stocks (15% v/v) and stored at -80°C.

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2.2. Enzyme production

Production of alkaline protease by *Bl*M9 was carried out in Molasses soya meal medium containing (g/l): soybean meal 10, molasses 5.0, K_2HPO_4 3.0, $MgSO_4.7H_2O$ 0.5, NaCl 0.5 and $CaCl_2.2H_2O$ 0.5. Fermentation was performed in a laboratory scale bio-reactor (7 liter) at 37°C and pH 7.5 for 34 hrs. The dO_2 concentration was maintained at 30% by cascade control system.

2.3. Enzyme separation and partial purification

The fermented culture was centrifuged at 6000 rpm for 15 minutes for solid-liquid separation and the supernatant (crude enzyme) was used for further purification. The proteins from the cell free supernatant were precipitated with ammonium sulfate (60% saturation). The protein is re-suspended in 0.02M Tris-HCl buffer, pH 7.5 and dialyzed against same buffer for desalting. The dialyzed fraction was subjected to gel filtration on a Sephadex G-75 column (1.5×60 cm), which has been equilibrated previously with 25 mM Tris–HCl buffer, pH 7.5. The same buffer was used to collect the fractions of 1.5 ml at a flow rate of 1 ml/min. Protein concentration and protease activity were determined. Fractions showing protease activities were pooled.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using a 4% (w/v) stacking gel and 10% (w/v) separating gel as described by Laemmli (1970), under reducing conditions. The sample for SDS-PAGE was prepared by mixing the enzyme with sample buffer in a ratio of 1:1. The mixture was heated at boiling water for 5 minutes. After complete electrophoresis, the gel was transferred into a staining solution of Coomassie Brilliant Blue R-250 for overnight with gentle agitation at room temperature. After staining, the gel was destained using a destaining solution until the background had been satisfactorily removed and protein bands became clear.

2.5. Protease assay

Protease activity was determined by modified Kreger & Lockwood (1981) method with azocasein as a substrate. 400 µl of appropriately diluted enzyme was added to 400 µl of 1% azocasein solution in 0.05 M Tris-HCl buffer (pH 8.5) and incubated in a water bath at 37°C for 60 minutes. The enzyme reaction was stopped by the addition of 135 µl of 35% TCA and then kept at 4°C for 15 minutes. The solution was then centrifuged at 13,000 rpm for 10 minutes. In the 750 µl of supernatant 750 µl of 1 N NaOH solution was added and mixed well. Absorbance was measured at 440 nm immediately. In control, TCA was added before the incubation. One unit of protease activity was defined as the amount of enzymes that produces an increase in the absorbance of 0.01 under the above assay condition.

2.6. Protein content determination

Soluble protein in the culture supernatant was estimated according to the Bradford method (Bradford 1976) using Bovine Serum Albumin (BSA) as a standard. Protein concentration at various steps of purification was estimated by taking absorbance at 595 nm. The specific activity is expressed as units/mg of protein.

2.7. Characterization of partially purified protease

2.7.1. Effect of temperature on enzyme activity and stability

The optimum temperature for partially purified protease activity was measured by incubating the enzyme with 1% (w/v) azocasein as a substrate at various temperatures ranging from 30°C to 75°C for 60 minutes. The thermostability of the protease was determined by pre-incubating the enzyme in absence and presence of 5mM of Ca^{2+} at temperature of 30, 40, 50, 60, and 70°C for 60 minutes following the determination of relative activity under standard assay condition. The untreated enzyme was taken as control.

2.7.2. Effect of pH on enzyme activity and stability

The optimum pH for partially purified protease was determined with 1% (w/v) azocasein as the substrate dissolved in different buffers (potassium phosphate, pH 5~7.5; Tris-HCl, pH 8.0~9.5; and glycine-NaOH, pH 10~11). The pH stability was determined by measuring the relative activity of the enzyme after 60 minutes of pre-incubation in the buffers of various pH values (5~11) at room temperature.

2.7.3. Effect of metal ions and enzyme inhibitors

Effect of various metal ions (Ca^{2+}, Ba^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, Na^{+}, K^{+} and Zn^{2+}) on the protease activity was studied by pre-incubating the partially purified enzyme at room temperature in a specified ion (5mM final concentration) containing buffer solution for 60 minutes. The enzyme assay was carried out under standard assay condition. The effect of enzyme inhibitors was studied using ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF) and β-mercaptoethanol. The partially purified enzyme was pre-incubated for 60 minutes at 45°C with each inhibitor (5mM). The relative activity was measured using azocasein as substrate at 37°C.

2.7.4. Effect of bleach, surfactants and detergents on enzyme activity

The effect of some surfactant and oxidizing agents (SDS, Tween-80, Triton X-100 and H_2O_2) at 1%, 3% and 5% concentration on partially purified enzyme was studied by determining the relative activity of enzyme after pre-incubation for 60 minutes at 37°C. The activity of enzyme without additive was taken as 100%.

The compatibility of partially purified protease with local laundry detergents was studied using Surf Excel, Wheel, Chaka, Tibet, Jet and Fast Wash. The tap water was used to dilute the detergents to give a final concentration of 7 mg/ml to simulate washing conditions. The endogenous proteases contained in these detergents were inactivated by incubating the diluted detergents at 65°C for 60 minutes prior to the addition of enzyme. Protease was incubated in various detergents solutions for 60 minutes at room temperature and the relative activity was determined under standard assay condition. The enzyme activity of a control (without detergent), incubated under the similar conditions, was taken as 100%.

2.8. Washing performance test

For the determination of applicability of protease enzyme as detergent additive clean cotton cloth pieces (5 cm × 5 cm) were soiled with blood and dried for 7 days at room temperature. The cloth pieces stained with blood were taken in separate flasks. The following three sets were prepared and studied.

1) Flask containing tap water (50 ml) + cloth piece stained with blood.
2) Flask containing tap water (50 ml) + cloth piece stained with blood + Surf Excel (7 mg/ml).

3) Flask containing tap water (50 ml) + cloth piece stained with blood + Surf Excel (7 mg/ml) + 1.5 ml (902 U) of crude enzyme solution.

The above flasks were incubated at room temperature for 30 minutes. After incubation, the cloth pieces were taken out of flasks and rinsed with water and dried. Visual examination was performed and difference was checked. Untreated cloth piece stained with blood were taken as control.

2.9. Bating activity of the protease

For determining the bating activity of *Bl*M9 protease, crude enzyme (2% of hide weight; 100 ml equivalent to 60,100 U =34,841 LVU for 5 kg hide) was applied to the cow hide in presence of water. Cow hide (after deliming) emerged in the enzyme preparations were rolled in a drum for about 60 minutes. Then the bubble, cross section, thumb, tensile strength, percent of elongation, stitch tear strength, water vapor permeability, grain crack strength and tongue tear strength tests were performed. In parallel, as a control, a commercial enzyme (Oropon K) as 0.5% (w/w) of leather weight was also used in bating experiment.

3. Results

3.1. Protease purification

The protease produced by *Bacillus licheniformis* MZK05M9 (*Bl*M9) was partially purified by ammonium sulfate precipitation, dialysis and size exclusion chromatography on Sephadex G-75. The partially purified protein showed a specific activity of 1624 U/mg and the purification fold was 2.70 (Table 1). SDS-PAGE analysis of purified enzyme showed a molecular mass of about 27.2 kDa (Fig. 1).

Table 1: Purification of Alkaline Protease from *Bl*M9.

Purification steps	Specific activity (U/mg)	Purification (fold)
Crude extract	601	1
Ammonium Sulfate (60%) precipitation	743	1.23
Sephadex G-75 Column Chromatography	1624	2.70

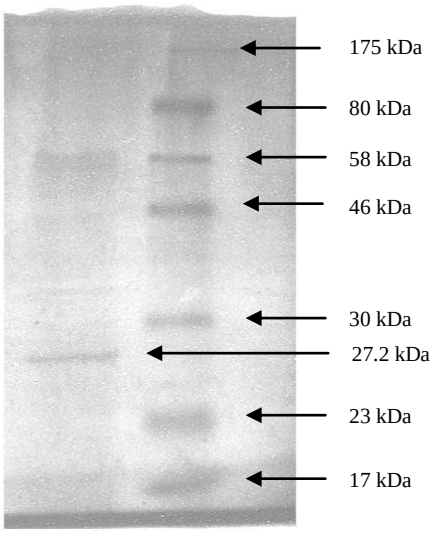

Fig. 1: SDS-PAGE Analysis of Partially Purified Alkaline Protease.

Lane (Left) = partially purified enzyme, Lane (Right) = Marker.

3.2. Effect of temperature on enzyme activity and stability

The enzyme was active in a large temperature range, with an optimum at 55°C (Fig. 2). The relative activities at 50 and 60°C were about 83% and 75%, respectively, of that at 55°C.

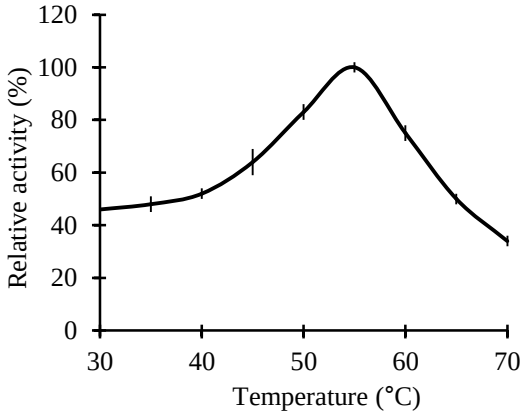

Fig. 2: Temperature Activity of the Protease from *Bl*M9.

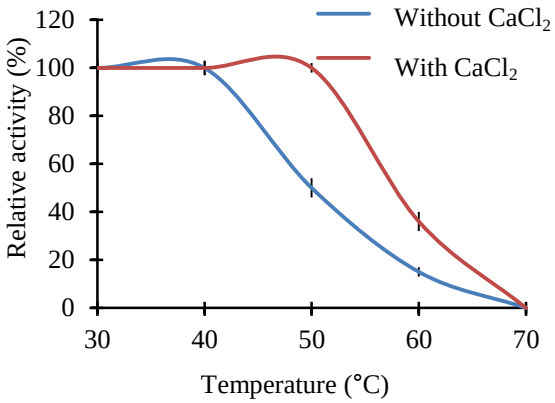

Fig. 3: Thermostability of the Protease from *Bl*M9.

The purified enzyme showed different temperature stability than its direct activity at various temperatures (30-70°C) (Fig. 3). Results indicate that the enzyme was stable at 40°C without loss of any activity. Whereas at 50°C the relative activity become half (50%) of the original activity and at 70°C it becomes zero (0%). However, addition of $CaCl_2$ stabilizes the enzyme up to 50°C.

3.3. Effect of pH on enzyme activity and stability

The partially purified enzyme was found highly active between pH 7.0 and 9.5 with an optimum at pH 8.5, indicating its alkaline nature. The relative activities at pH 6.5 and 10.0 were about 74% and 69%, respectively, of that at optimal pH (Fig. 4). The pH stability profile of alkaline protease showed that the partially purified enzyme was stable between 7.0 and 8.0 but in presence of $CaCl_2$, the enzyme showed stability in wide pH ranges 6.5-9.0 (Fig. 5).

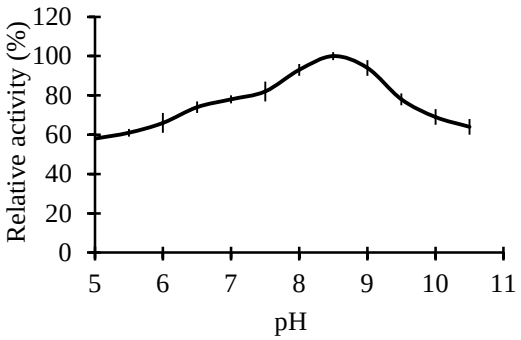

Fig. 4: pH Activity of the Protease from *Bl*M9.

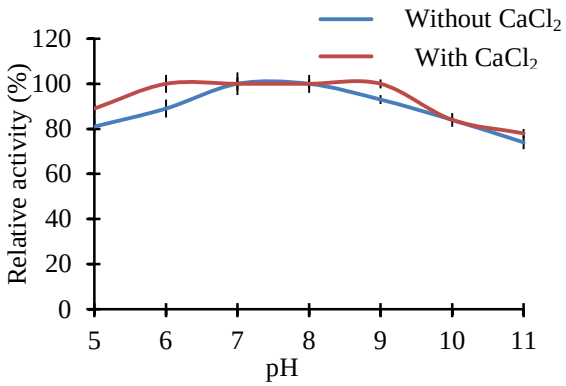

Fig. 5: pH Stability of the Protease from *Bl*M9.

3.4. Effect of metal ions and enzyme inhibitors

The results recorded (Fig. 6) indicating that in presence of Ba^{2+}, K^{+}, Ca^{2+}, Mg^{2+} and Mn^{2+} the proteolytic activity resulted in the discreet increase, while due to inhibitory effect it lost almost total activity in presence of Hg^{2+}. Metal like Na^{+} restored the enzyme activity as 97% of the original activity. Whereas other metals like Zn^{2+} and Cu^{2+} showed an appreciable inhibitory effect on enzyme with relative activity of 38 and 33% respectively. Inhibitors like PMSF, EDTA and β-mercaptoethanol inhibited the partially purified protease with 76, 29 and 17% inhibition respectively.

3.5. Effect of bleach, surfactants and detergents on enzyme activity

The partially purified protease was stable at low concentration (1%) of SDS with relative activity of 62%. While it was found stable in Triton X-100 and H_2O_2, it was unstable in Tween-80 (Fig. 7). The enzyme retained activity at room temperature with Surf Excel more than 65%, in Wheel 86%, in Tibet 113%, in Keya 87%, in Jet 70% and in Fast Wash 98% after 60 minutes incubation.

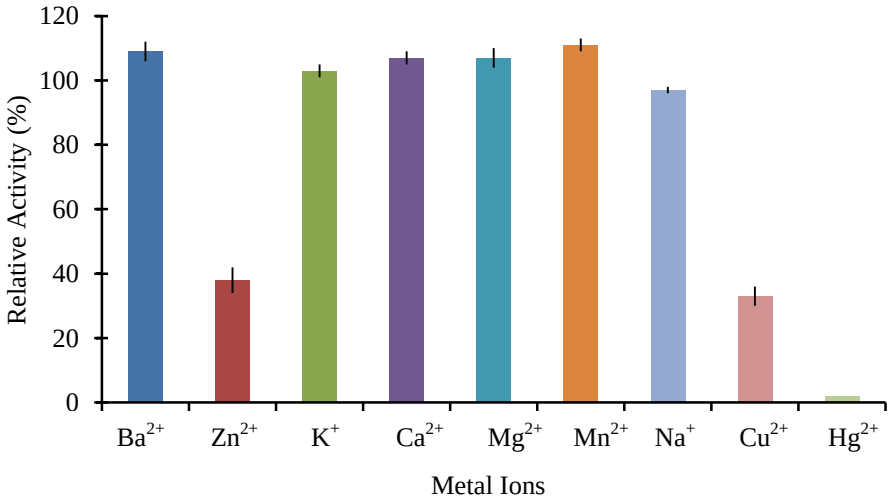

Fig. 6: Effect of Metal Ions on the Protease from *Bl*M9.

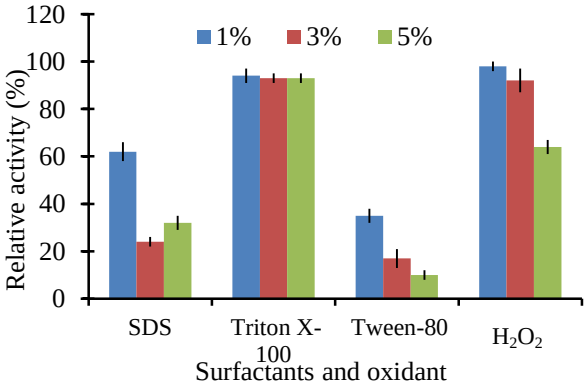

Fig. 7: Effect of Surfactants and Oxidant on the Protease.

3.6. Washing performance test

In case of removing blood stain from cloth, it was observed that the protease enables the removal of blood stain very efficiently in association with detergent (Fig. 8).

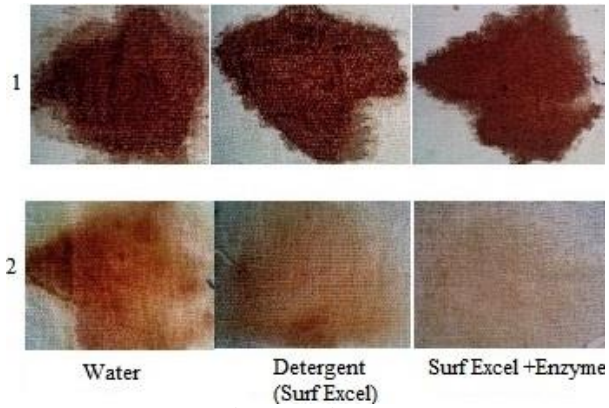

Fig. 8: Washing Performance of *Bl*M9 Alkaline Protease. 1 = Before Treatment, 2 = After Treatment.

3.7. Bating activity of the protease

Enzymatic bating in leather manufacturing offers eco-friendly process. For determining the efficiency in bating, *Bl*M9 protease was compared with a mostly used (in Bangladesh) commercial enzyme Oropon K (TFL, London) in prototype facilities. The results of different tests of the enzyme treated leather (crushed leather) such as tensile strength, percent of elongation, stitch tear strength, water vapor permeability, grain crack strength (Lastometer), and tongue tear strength tests indicated that *Bl*M9 bate was equally efficient to the commercial bate Oropon K (Table 2).

Table 2: Different Test Report of Crushed Leather Bated by *Bl*M9 Bate and Commercial Bate.

Name of the tests	0.5% commercial bate	2% *Bl*M9 bate	Standard Value*
Tensile Strength test	202.49 Kg/cm^2	254.12 Kg/cm^2	200Kg/cm^2 (minimum)
% of Elongation Test	44.66%	55%	80% (maximum)
Stitch Tear Strength Test	121.20 Kg/cm	173.07 Kg/cm	80 Kg/cm (minimum)
Water Vapor Permeability Test	10.96 mg/cm^2	11.32 mg/cm^2	10 mg/cm^2 (minimum)
Grain Crack Strength (Lastometer)Test	28 Kg	20 Kg	20 Kg (minimum)
Tongue Tear Strength Test	63.06 Kg/cm	55.55 Kg/cm	45 Kg/cm (minimum)

* (Dutta 1990)

Furthermore, the bubble, thumb and cross section tests of the treated leather (pelt leather) met the requirement of quality bating performance and comparable to the commercial enzyme (Fig. 9).

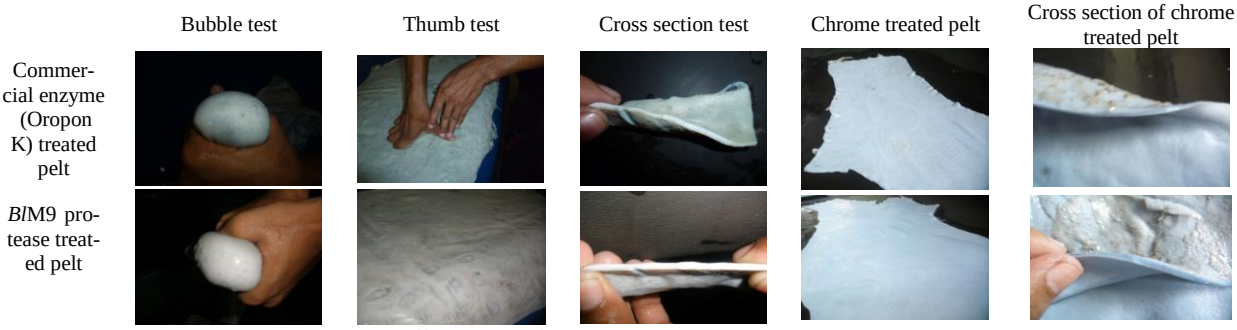

Fig. 9: Different Tests of Pelt Bated with *Bl*M9 and Oropon K Bate.

4. Discussion

The present study has been carried out to partially purify and characterize the alkaline protease and as well as to check its applicability as crude enzyme in detergent and leather processing industries. The partially purified protease showed the molecular mass of 27.2 kDa. The optimum pH for the protease was recorded 8.5, which is lower than the pH optima values, viz pH 11.5 (Kumar 2002), pH 9.0 (Dhandapani & Vijayaragavan 1994), pH 10.0 (Gupta *et al*. 2008), pH 10.5 (Beg & Gupta 2003) and pH 11.0 (Margesin *et al*. 1992) reported earlier for other alkaliphilic *Bacillus* spp. However, the present enzyme with the optimum pH and stability obtained will suitably comply its application in bating step of leather manufacturing.

The present protease showed an increased activity in the presence of Ba^{2+}, K^{+}, Ca^{2+}, Mg^{2+} and Mn^{2+} but lost almost total activity in the presence of Hg^{2+}. Shimogaki *et al*. (1991) also found the stimulatory effect of Mn^{2+} on serine alkaline protease. Tsujibo *et al*. (1990) and Aretz *et al*. (1989) obtained best results with Ca^{2+} and Ba^{2+} with an increase in activity around two-fold. Many alkaline proteases were reported to be inhibited by mercury (Beg & Gupta 2003) which is harmonious to this study.

Results of sensitivity of partially purified protease to various protease inhibitors showed that strong inhibition (76% inhibition) was achieved by pre-incubation with PMSF indicating that the enzyme belongs to serine group proteases. However, EDTA, a metalloprotease inhibitor and β-mercaptoethanol, a cysteine protease inhibitor, slightly inhibited the protease with 29 and 17% inhibition respectively. Dhandapani & Vijayaragavan (1994) isolated *Bacillus stearothermophilus* strain AP-4 producing thermostable alkaline protease that was completely inactivated by PMSF, EDTA and β-mercaptoethanol and proposed a metal ion-dependent alkaline serine protease.

The feasibility of using alkaline protease as a laundry detergent-additive was evaluated by determining its stability towards some surfactants, oxidant and some laundry detergents available locally. A protease to be used in detergent formulation should be stable in presence of commercial detergents. Protease showed excellent stability and compatibility in the presence of locally available detergents (Surf Excel, Wheel, Tibet, Keya, Jet and Fast Wash) at room temperature. The enzyme retained higher activity 113% in Tibet and in Fast Wash 98% after 60 minutes incubation. Uddin *et al.* (2006) reported that protease from *Bacillus licheniformis* MZK05 retained 18, 24, 25, 54, 50, 32 and 27% of its original activity in presence of Jet, Surf Excel, Wheel, Keya, Chaka, Tide and Rain detergents respectively. Singh *et al*. (2001) reported that SSR1 protease retained 40-90% of its activity in presence of local detergents. Hence, the present protease may be suitably used as cleansing aid in detergent formulation like Tibet.

The results obtained using *Bl*M9 protease as a bating agent in leather processing, in a comparative study with bating agent Oropon K, reflected the unique property of the *Bl*M9 protease to selectively remove the non-structural proteins (eg. albumin, globulin, elastin etc.) from the hides and skins without affecting the collagen, the main composition of the leather itself.

5. Conclusion

In the present study an alkaline protease from *Bacillus licheniformis* MZK05M9 was purified, characterized and tested for its efficacy in bating of skins and hides processing in leather industry. The major application of the present protease was found in leather manufacturing industry where the results of different qualitative and quantitative tests suggested its potentiality in bating of skins and hides. The protease showed good stability towards surfactants and oxidants, which are usually used in the formulation of common bleach-based detergents. These results will be a useful basis for commercial production of the alkaline protease.

Acknowledgments

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