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Purification, Characterization, and Application of Alkaline Protease Enzyme from a Locally Isolated *Bacillus cereus* Strain

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Abstract: Among the microbial enzymes protease and amylase are the most valuable enzymes which have been has diversified applications and used extensively because of their capabilities in the degradation of organic wastes, application in biofuels, agricultural, pharmaceuticals, chemical and biotechnological industries. The aim of the current research work was the purification, characterization and application of alkaline proteases extracted from *Bacillus cereus* AUST-7. Various concentrations of ammonium sulphate were applied for enzyme precipitation. Sephadex-G 100 was used in FPLC system for separation of protease from other proteins. SDS-PAGE was used to measure the molecular weight of required alkaline protease. Relative activities were determined against different pH, temperature, and incubation period to measure the enzymes activity. Stability of pH, temperature and various metal ions and inhibiter were also studied. Purified enzymes were applied on the goat skin to explore the dehairing efficacy. A 6.5 purification fold and 1163.50 U/mg of specific activity were obtained at 70% saturation and 35. 91 purification fold and 8902 U/mg of specific activity were observed after FPLC separation. The 35 kDa molecular size of protease enzyme was exhibited on the SDS-PAGE. The purified enzyme was stable at pH 10, temperature 55 °C and 35 min of incubation period. The purified enzyme was found to be stable at pH 8–11, thermo-stability at 50 °C and phenyl methyl sulphonyl fluoride (PMSF) and di-isopropyl fluorophosphates (DFP) inhibited the enzyme activity. The enzyme has good potential as dehairing agent in leather industries.

Keywords: protease enzyme; *Bacillus cereus*; purification; FPLC; SDS-PAGE; characterization

1. Introduction

As one of the most extensively used enzyme, proteases (EC 3.4) are considered the most valuable and powerful enzymes counting for approximately 60% of the global enzyme usage because of their competency to break down complex protein compounds into amino acids and peptides [1–5]. Bacteria is considered as the most excellent source to produce three major types of proteases i.e., acid, neutral and alkaline. Amongst them, neutral and alkaline proteases have a great application potential in the enzyme detergent and leather industry because of the increasing trend of development of ecofriendly technologies [6,7]. Proteases or peptidases are classified as proteolytic enzymes. A proteolysis is a procedure

by which the hydrolysis of peptide bonds takes place [8]. The proteases have dynamic industrial applications and alkaline proteases amongst which occupies 52% of total stokes between the main segments of this enzyme group [9]. Those groups of enzymes which remain active at highly alkaline pH array from 7–11 with optimal pH 9 are the alkaline proteases [10].

The world environment is changing by different socio-economic factors. The major factors are enormous production of different industrial sectors including food processing, leather, waste management and dairy processing industries [11]. Many industrial processes do not have acceptance by society as they cause many environmental problems. Leather industry is one of the industries that refused by many societies as it because air pollution and bad odor [12]. They need chemicals in different stages of its process to eliminate bad odor and air pollution. Enzyme can contribute to this role by efficiently removing bad odors and reduces air pollution. Enzymes contribute a great role in various applications [1]. The enzymatic procedure is one of the maintainable and favorable alternatives to conventional procedures [13].

The use of low-cost energy and reducing the environmental pollution are main advantages of enzymes [14]. Proteases contribute about 60–65% of the whole enzyme worth due to their countless applicability in various industries like leather, pulp and paper, detergents, food, meat processing, making of cheese, bioremediation, and silver recovery from photographic film. Similarly in therapeutic treatments these enzymes can also be used to cure harmful lesions and inflammation [15,16]. Several proteases have been purified and characterized from various sources. Bacteria and fungi are the most important from all other sources of proteases. The microbial proteases exhibit proteolytic activities in an extensive range of pH and temperature as compared to proteases isolated from animals and plants sources [17]. Mostly the microbial enzymes are extracellular and are excrete directly into the production media; hence the separation of microbial cells from the culture media is usually conceded out by process of centrifugation or in some situation by filtration techniques, leaving the crude enzyme in the form of supernatant of filtrates.

The culture supernatant is concentrated by salting out. The solubility of desired proteins are lowers by ammonium sulphate are the usual agents employed for precipitation [18]. The crude protease enzymes specific activities increasing to many folds at 70% of Ammonium sulfate saturation level [19]. At commercial level *B. subtilis* amongst all the *Bacillus* sp. regarded as a chief producer of alkaline proteases [20]. The alkaline proteases have a diverse molecular mass from the range of 20 to 130 kDa [20,21]. The optimal pH assortment of alkaline proteases is usually found in range of pH 8–11 [22]. In few cases with an optimal pH 12 reported [23]. Alkaline proteases from microbial sources have the optimal temperature ranges from 50–70 °C [24]. The present study was conducted on the purification, characterization, and application of alkaline protease from *Bacillus cereus* produced under submerged fermentation. The industrial skin (tannery) waste (defatted) was used as a substrate.

2. Materials and Methods

2.1. Microorganisms and Production of Enzymes

A newly strain of *Bacillus cereus* AUST-7 was isolated from soil and identified by 16S rRNA sequencing (unpublished data). The enzymes were produced in lab scale bioreactor, industrial skin (tannery wastes) were used as a substrate and the agro-industrial waste molasses was as a carbon source.

2.2. Purification of Protease Enzyme

All of the purification stages were conceded at 4 °C. Alkaline proteases produced by newly isolated strain of *Bacillus cereus* were purified and the SDS-PAGE electrophoresis were used for the determination of their molecular weight. The purification process was carried out via following steps.

2.2.1. Ammonium Sulphate $(NH_4)_2SO_4$ Precipitations

Approximately 1000 mL of cell free solution of enzymes (supernatant) were precipitated by the addition of different concentrations (40–80%) of ammonium sulphate. By increasing the concentration level of $(NH_4)_2SO_4$ by10% the supernatant was precipitated independently. The enzymes solutions were agitated for 1 h at 4 °C. Precipitates from the saturation level of ammonium sulphate at which maximum protein was precipitated, collected by centrifugation at 12,000 rpm at 4 °C for 20 min. The precipitates in pellet form were dissolved in 25 mL of 0.05 M tis-HCl buffers with pH 9.0 to obtain the concentrated enzymes suspension. To remove the ammonium sulphate ions the enzymes suspension was dialyzed in dialyzing membrane in the same buffer at 4–8 °C. Sealed membrane having the enzymes suspension immersed in buffer solution (0.05 M Tris-HCl with pH 9.0) in a large beaker for 24 h. To increase the efficiency of desalting the buffer was exchanged with fresh buffer solutions after every 6 h.

2.2.2. Preparation of Gel and Gel Filtration Chromatography

About 4 g of Sephadex G-100 (Sigma-Aldrich, St. Louis, MO, USA) gel were soaked in the de-ionized water for swelling at room temperature for 24 h. Subsequently, before packing into the gel slurry was defined. The gel slurry was transferred into the column (Bio-Rad 1.5 cm × 15 cm) and added water. The gel was mixed properly by flip-flopping the column many times and allowed to settle the gel. Later, 90–95% of the gel settled down, poured the supernatant added water several times to repeat the process.

Concentrated enzymes sample were purified on Sephadex G-100 column by using FPLC system (Biologic LP, Bio-Rad, Hercules, CA, USA). Tris-HCl buffer of 0.05 M with 9.0 pH were used to equilibrate the Sephadex column. The pre-dialyzed enzymes sample were loaded onto Sephadex G-100 columns independently and by the same buffers then it was eluted. The fraction of 4 mL was collected by the fraction collector (model, 2110 Bio-Rad) at the 30 mL/h of flow rate. The fraction showing optical density (absorbance) at 280 nm was evaluated for protease activity. The fractions having enzymes activities were collected dialyzed against distilled water and were stored at −20 °C for further research work.

2.2.3. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

A 12% of SDS-PAGE was performed followed the method mentioned by Laemmli [25] applying a small piece of glass plates (8 × 8 cm) gel apparatus. Molecular weights of purified, crude and pellets of ammonium sulphate precipitation were obtained by using standard molecular weight marker (fermentas).

2.3. Characterization of Alkaline Protease

2.3.1. Impact of pH on Activity of Protease

The protease activities were executed at various values of pH (6–12) by employing (w/v) of 1% casein solution as substrate prepared in different buffers (0.05 M), pH 6–7 phosphate, pH 8–9 tris-HCl and pH 10–12 glycine-NaOH were used independently. The reactions of mixture were incubated for 30 min at 40 °C. Then the activities were measured according to the enzymes assay procedures.

2.3.2. Impact of Temperature on Protease Activity

The temperature Impact on the purified protease enzymes activities were explored by incubating the reactions mixtures at distinct temperature range (30, 35, 40, 45, 50, 55, 60, 65 and 70 °C) with 1% casein as a substrate. The activities were checked as discussed previously.

2.3.3. Impact of Induction Period on Protease Activity

Influence of induction period on the protease activity were measured, the reaction mixture was incubated at 10 pH and 55 °C temperature for various time periods ranging

from 15–50 min. then the comparative activities were measured according to previous mentioned method.

2.4. Stability Exploration of Alkaline Protease

2.4.1. pH Stability

The stability of pH was determined by pre-incubating the enzyme solution without substrate at various pH values from 6–12. Different solutions of buffers such as (pH 6, 7) of sodium phosphate, (pH 8, 9) of Tris-HCl and (pH 10–12) of glycine NaOH were prepared. The enzyme solution was mixed separately in the ratio of 1:1 in the above-mentioned buffers and incubated at 40 °C for 12 h in water bath (Eyela, Tokyo, Japan). According to the standard assay procedure the enzymes activities were then studied.

2.4.2. Thermo-Stability

Thermo-stability of purified enzyme solution was explored after employing the incubation of 1 h at various temperature ranges from 30–80 °C in a water bath. The enzyme activities were then measured according to the standard assay protocol.

2.4.3. Effect of Metal Ions and Inhibitors

Effect of different metal ions such as (Na^{+}, Mg^{2+}, Ca^{2+}, Hg^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+}, Al^{3+}, Co^{2+} and Cd^{2+}) and the inhibitors like phenyl methyl sulphonyl fluoride (PMSF), pepstatine, 1, 10 phenonthroline, di-isopropyl fluorophosphates (DFP), cysteine inhibitors ethylene diamine tetra acetic acid (EDTA), p-chloromercuric benzoate (pCMB) were explored on the relative activity of purified enzyme. The overall enzyme solutions were incubated for 35 min at 40 °C before the addition of substrate and routinely the enzyme activities were measured.

2.5. Commercial Application of Alkaline Proteases

Dehairing Effects

Dehairing characteristics of alkaline proteases were perceived by the method recommended by Wang et al. [26]. A salted skin of goat was brought from the market and washed thoroughly with de-ionized water. The skin hides were drenched in the alkaline protease and incubated in water bath shaker at 100 rpm of shaking speed for various time intervals (5–12 h) at 40 °C. The skin piece was scraped gently with fingers after the completion of required period to confiscate the unfastened hairs. The dehairing efficiency was considered according to the cleared area of the skin and the dehaired skin quality was assessed according to the physically appearance that were observed by naked eyes.

2.6. Analytical Methods

The enzymes activity (one unit of enzyme activity is equal to 1 micro mol of amino acid released by the per ml/per minute of enzyme source) was checked according to the slightly modified methodology of Yang and Haung [27]. Crude enzyme (1 mL) mixed with 2 mL of 1% casein solution in a glycine-NaOH buffer (pH 10) incubated for 30 min at 40 °C. Then 3 mL of 10% tri-chloro acetic acid (TCA) solution was added to stop the reaction. The mixture was then centrifuged at 9000 rpm for 10 min. The absorbance was measured at 280 nm against the blank. Tyrosine standard curve of different concentration were used to calculate standard factor. Total estimation of proteins was assessed by using bovine serum albumin "BSA" as a standard protein, a recommended method of Lowery et al. [28].

2.7. Statistical Analysis

All the data obtained was statistically analyzed using SPSS software and the data presented were mean with standard error of triplicates samples.

3. Results

3.1. Precipitation by Ammonium Sulphate

The precipitation of $(NH_4)_2SO_4$ was executed as the initial phase of enzymes purification. Suspension precipitated from 70% saturation level confined the highest proteolytic activity as described in Table 1. Maximal protease activities were found at 70% of saturation level and the activities above that range were found approximately similar to the previous saturation level. Total proteins were determined as 765.25 mg at 70% of saturation level. Therefore 70% of saturation level was applied for further studies.

Table 1. Purification of alkaline protease produced by *Bacillus cereus* AUST-7.

Steps of Purification	Total Volume (mL)	Total Activity (U)	Total Proteins (mg)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Crude enzyme	1000	1,815,000	6980	260.02	1	100
$(NH_4)_2SO_4$ Precipitation (70%)	20	890,368	765.25	1163.50	6.5	62
Sephadex G-100	20	208,591	24.04	8902	35.91	10.73

3.2. Gel Filtration Chromatography

From elution profiles of filtration chromatography, it was perceived that the protease activities and the total proteins were confined in the fractions got from peaks as represented in the (Figure 1a–c). Figure 1a, two peaks, one large peak represent protease and the next are other proteins, Figure 1b, single large peak was obtained by the precipitates are the alkaline protease and Figure 1c, an open wide small peak was achieved from supernatant of precipitated are the mixture of ions and small number of enzymes. This stage resulted 35.91 folds of purification and 10.73% enzymes yield having specific activities of 8902 U/mg as compared with solution of crude enzyme denoted in Table 1. The fractions of gel filtration having hydrolytic activity were preserved at −20 °C for further study.

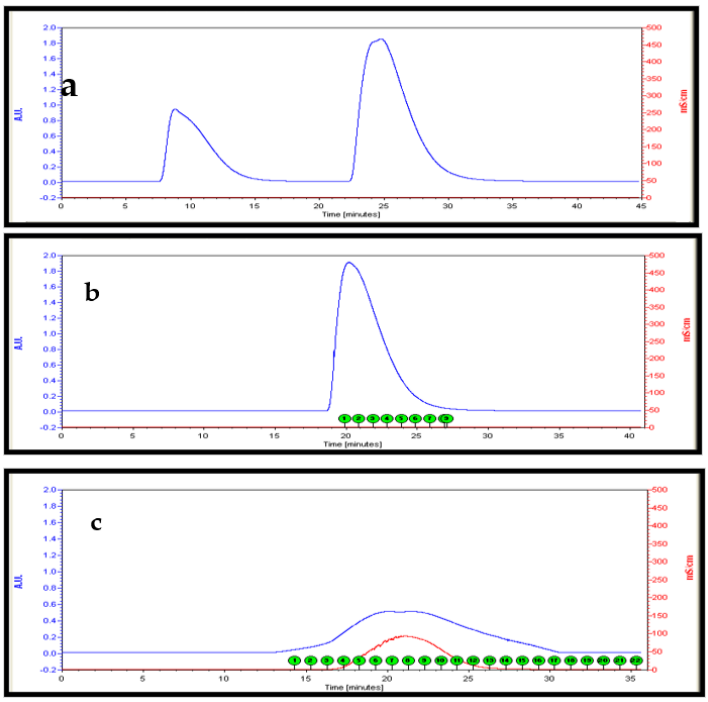

Figure 1. (**a**) Elution profiles of crude alkaline protease, (**b**) elution profiles of ammonium sulphate precipitates, (**c**) elution profile of supernatant.

3.3. SDS-PAGE

Purified sample of protease from FPLC were examined on 12% of SDS-PAGE by running under the denaturing condition. Purified proteases exhibit single band, the crude and precipitates showed numerous bands after with commissive blue R-250 staining Figure 2. From Figure 2, we understand that the crude and pellets have numerous bands on SDS-PAGE represents multi proteins in the mixtures and the fraction filtrate have a single band of 35 kDa.

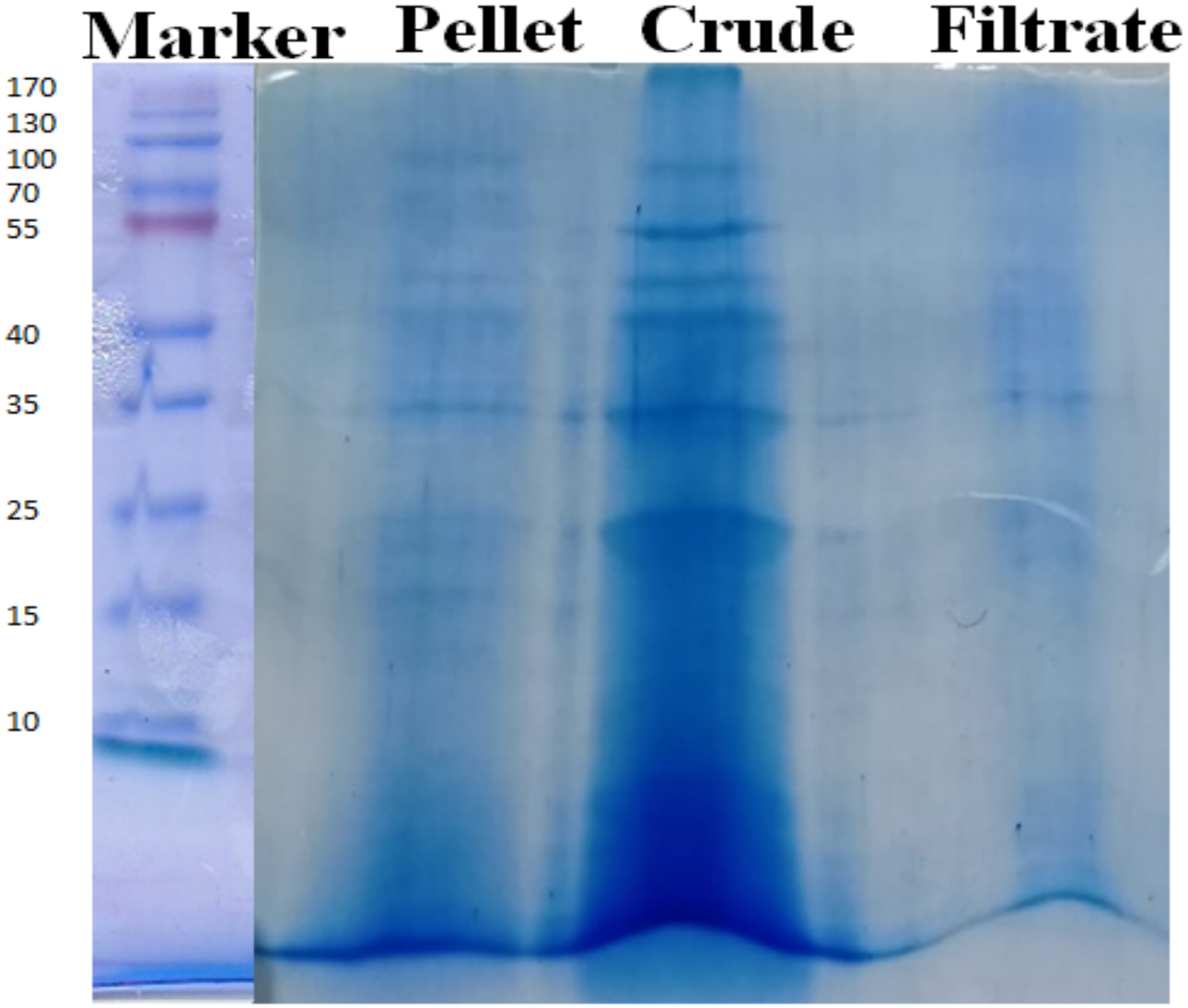

Figure 2. SDS-PAGE of the purified protease, crude, and precipitates.

3.4. Characterization of Alkaline Protease

3.4.1. Impact of pH on Protease Activity

The maximal proteolytic activity was measured at 10 pH and the control was taken as 100% of relative activity Figure 3. The purified proteases were active in the pH range from 6–12, with an optimal activity at 10 pH. The activity of enzymes increases continuously from 6–10 then decreases further from this point. The change in pH with respect to acidic condition (pH 6.0) the relative activity decreased progressively. Protease enzymes exhibited the activity of 52.03%, 69.23%, 91.6% and 71.26% at pH 8, 9, 10, 11 respectively.

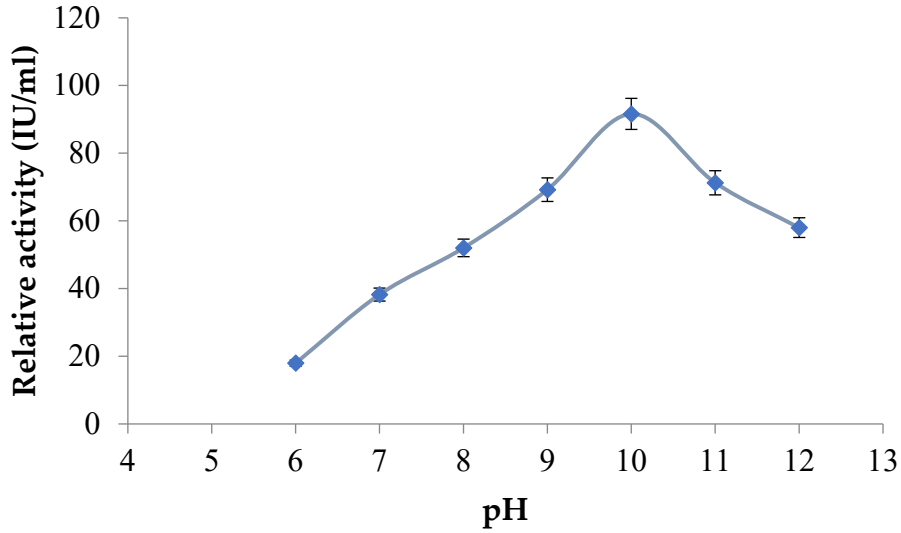

Figure 3. Effect of pH on protease activity.

3.4.2. Impact of Temperature on Protease Activity

Study the effects of different temperature observed that the increase in temperature the activity of enzymes increased. The maximal activity of enzyme was obtained at 55 °C and subsequently the activity reduced steadily as shown in Figure 4. The maximum relative activity was taken 100%. Therefore 55 °C temperature was cogitated optimum for proteolysis by alkaline proteases. The relative activity was lesser below optimum temperature (55 °C) and become decreased above the optimal temperature. Relative activities were 55.06%, 72.8%, 93.62%, 69.37% observed on 45, 50, 55 and 60 min respectively.

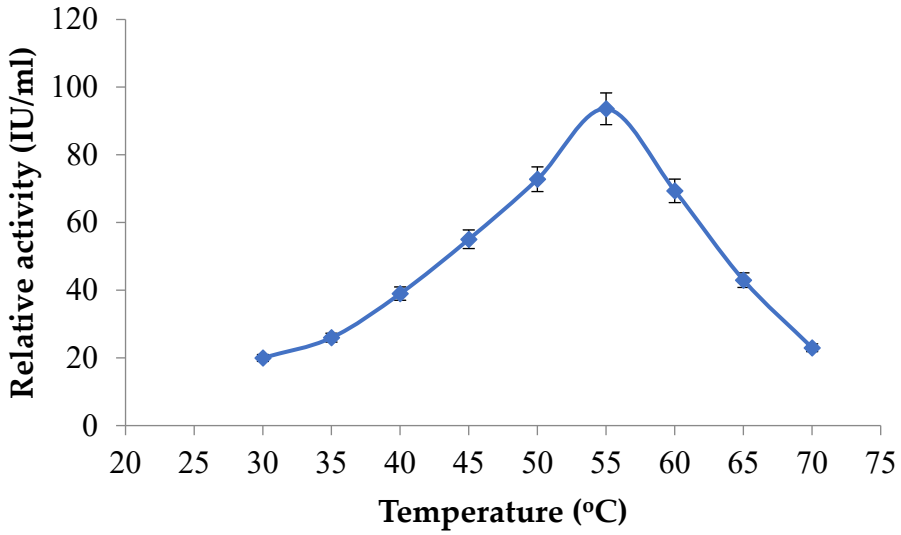

Figure 4. Effect of temperature on enzyme activity.

3.4.3. Impact of Induction Period on Protease Activity

From the study of various induction periods, it was declared that the proteolytic activities altered along with induction periods. The maximum enzymes activities were found at 35 min as represented in Figure 5. Further increasing in induction period, the enzyme activity decreased gradually. The relative activity was found high 88.82% at 35 min if incubation period, however below the optimum induction period tiniest relative activity were observed and by increasing the incubation period above optimum range relative activity going to be decreased.

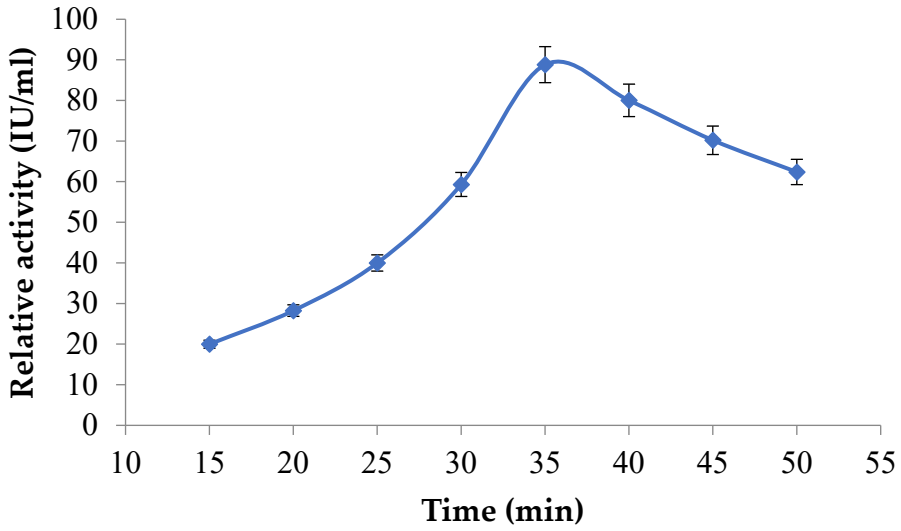

Figure 5. Effect of induction period on protease activity.

3.5. Stability Studies

3.5.1. pH Stability

The purified alkaline proteases were found stable over a wide range of pH 8–11 and retained 86.25% activity at pH 11 Figure 6 the enzymes activities become decreased with increased in pH and 40% of activities were perceived at pH 12.

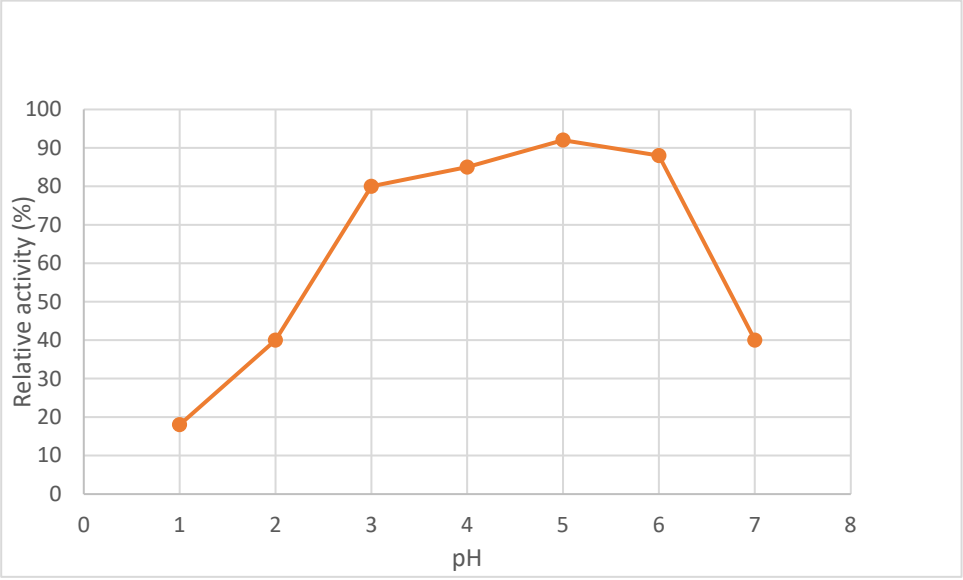

Figure 6. pH stability of alkaline protease.

3.5.2. Thermo-Stability

The thermo-stability of purified proteases was assessed, and the enzymes retained its whole activities at 50 °C for 1 h Figure 7. Though, 46% relative activities were determined after disclosure at 60 °C which were reduced consequently 0% at 80 °C.

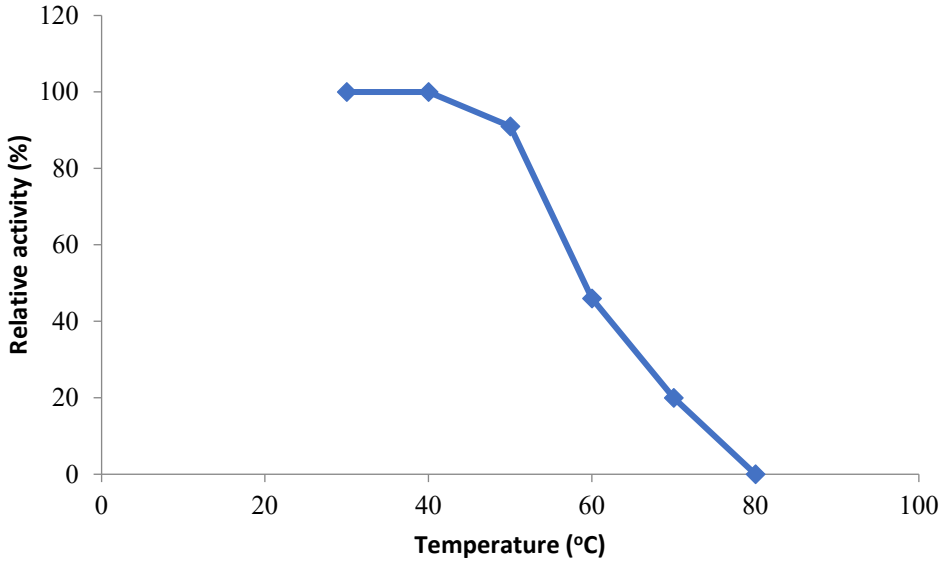

Figure 7. Thermo-stability of alkaline protease.

3.5.3. Effect of Metal Ions and Inhibitors

Effect of various metal ions and inhibitor were studied on the activities of purified proteases and given in Table 2 the activities after incubation with metal ions and inhibitors were stated relative to control (without treatment). It was perceived that Mg^{2+} and Ca^{2+} ions had substantial stimulatory effects on the protease activities. About 125% and 140% of relative activities were assessed in existence Mg^{2+} and Ca^{2+} metal ions respectively for the purified enzyme. Treatment with inhibitors it was observed that PMSF completely inhibited the enzymes however DFP exhibited up to 92% inhibition for protease as expressed in Table 2.

Table 2. Metal ions and inhibitors.

S.No	Metal Ions/Inhibitors	Relative Activity (%)
1	Control	100
2	Na^{+} ($NaCl_2$)	97
3	Mg^{2+} ($MgCl_2$)	116
4	Ca^{2+} ($CaCl_2$)	131
5	Hg^{2+} ($HgCl_2$)	90
6	Zn^{2+} ($ZnCl_2$)	93
7	Cu^{2+} ($CuCl_2$)	97
8	Ni^{2+} ($NiCl_2$)	92
9	Al^{3+} ($Al\ Cl_3$)	96
10	Co^{2+} ($CoCl_2$)	86
11	Cd^{2+} ($Cd\ Cl_2$)	92
12	phenyl methyl sulphonyl fluoride (PMSF)	02
13	pepstatine	102
14	1, 10 phenonthroline	95
15	di-isopropyl fluorophosphates (DFP),	11
16	ethylene diamine tetra acetic acid (EDTA),	96
17	p-chloromercuric benzoate (pCMB)	91

3.6. Commercial Application in Leather Industry

Alkaline proteases produced by *Bacillus cereus* were used for dehairing. Minimum hairs were removed after 5 h but with increasing in incubation period the dehairing activity become increased and after 12 h the hair removed partially. As the induction period was carried up to 15 h, brilliant clear holes along with hair exclusion were observed on the surface of skin. Complete dehairing of goat skin have a spotless hair opening with a clean structure were detected after 12 h of treatment and subsequently by increasing the time period up to 15 h small pores were formed on the skin surface which can be seen in Figure 8.

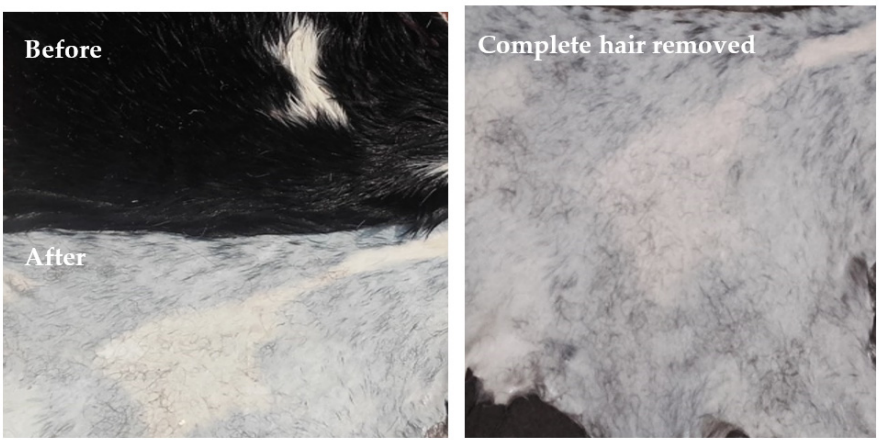

Figure 8. Dehairing activity of alkaline protease produced by *Bacillus cereus*.

4. Discussion

With recent advanced technologies there is a lot of research studies going on the interest and demand of enzymes with novel properties [29]. In this regard substantial efforts have been made towards the selection of microbes producing enzymes with new physiological properties and tolerance to extreme conditions used in industrial processes for example temperature, salts, pH, and some others. About 75% of industrial enzymes have been observed as hydrolases while proteases comprise one of the most significant groups of hydrolytic enzymes which act upon native proteins to split them into small peptides and amino acids [30] and that is the reason that they are considered as of great commercial value. One of the most important proteases are the microbial proteases as they belong to the significant family of protease which have an advantage over animal and plant proteases because microbes can be cultured on a large scale in less time and the growth conditions can be simply optimized in the lab conditions [31,32]. Several proteases specifically the worldwide sales of alkaline proteases have been growing immensely and nearly 60% of the global protease market consists of alkaline proteases. Alkaline proteases demonstrate optimal enzyme activity at pH 7–11 and are consisted of metalloproteases and serine proteases [33]. Majority of the proteases available in the market today are secreted by bacillus strains. Other bacterial strains *Escherichia coli*, *Lactococcus*, *Streptomyces*, *Pseudomonas* and *Vibrio* spp. can produce proteases but Bacillus is preferred due to its heterotrophic nature, high yield capacity and ease in growth on a variety of substrates [31]

Obtaining purified enzymes comprises many steps and various techniques are involved in purification procedures. Numerous methods of purification are here but excellent techniques depend upon the source of production and the stability of operation steps [34]. A choice of techniques for enzyme production and the purification should be cost-effective and feasible economically [35,36].

The present study was presided on the purification, characterization, and application of alkaline protease from newly isolated *Bacillus cereus* AUST-7 by utilizing industrial skin (tannery) waste as a substrate. Maximal activity was observed at 70% of ammonium sulphate concentration. Similarly high activities of (2.6) folds increase was observed by Nassar et al. [12] at 70% of ammonium sulphate concentration level. Another group of

researchers purified alkaline proteases and maximum specific activity were archived at 80% of ammonium sulphate saturations [37]. These findings were in close agreement with the present study. From the SDS-PAGE the molecular weight of the purified protease is 35 kDa and it has appeared as single protein. The crude enzymes and precipitates appeared as multi-proteins. Mary et al. [38] and her colleagues detected 45 kDa of molecular weight by SDS-PAGE of alkaline proteases isolated from Pseudomonas sp. On the same way Chimbekujwo et al. [39] reported the molecular weight of purified alkaline proteases as 68 kDa. The purified alkaline protease indicated the optimum pH 10, temperature 55 °C and incubation period 35 min. Karray et al. [40] demonstrated that the optimum pH and temperature were 10, 55 °C respectively. Rejisha and Murugan [41] illustrated highest protease activity at pH 10 with 40 °C of temperature. It is also called serine proteases as it has the optimum pH in alkaline condition since they are sensitive to di-iso-propyle poosphoro-fluoridate which reacts a serine residue at active site [42]. According to the previous studies performed by Seifzadeh et al. [42], Al-Askar et al. [43] and Devi et al. [44] demonstrated that the optimal incubation period of alkaline protease activity was reported as 60 min.

The present alkaline protease enzymes were used as dehairing agent to explore its commercial application in leather industries. The alkaline proteases produced by *Bacillus cereus* were found advantageous due fast removal of hair from skin without damage. Past studies exhibit that alkaline protease produced by various bacterial strains dehaired goat skin in 18–24 h [45–47].

5. Conclusions

Alkaline proteases produced by Bacillus strains own great commercial value because of their great biochemical diversity and stability and various applications in different areas including food, medicine, detergents, silver recovery, waste treatment. These properties make alkaline proteases as the best and suitable candidate for industrial applications employing higher temperatures along with shorter reaction times and low risk of contamination. More precisely, the tolerance towards alkaline pH makes these isolates more significant to act as biocatalysts and make them more useful for the local industrial sector.

The findings of this study concluded that the protease enzyme produced by the locally isolated *Bacillus cereus* using ternary waste as a substrate was a thermostable alkaline protease that was successfully purified using the ammonium sulphate precipitation and sephadex G-100 chromatography. The obtained molecular size for the enzyme was 35 kDa. The present investigation exposed that the enzymes produced by *Bacillus cereus* is suitable for commercial exploitation as a dehairing agent in leather industries. It could be further explored for other applications also in many industries.

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