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Purification, Characterization and Applications of Proteases Produced by *Bacillus amyloliquefaciens* 35s Isolated from Soil of the Nile Delta of Egypt

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The current study aimed at purifying and determining general characteristics of proteases produced by *Bacillus amyloliquefaciens* 35s isolated from the soil of Nile Delta of Egypt and to study its applications in environmental and industrial purposes.
Study Design: The produced proteases were purified by ammonium sulfate precipitation method followed by dialysis. The effects of some physical and chemical factors on the activity and stability of the partially purified proteases were determined. The produced proteases were tested in various environmental and industrial applications.
Place and Duration of Study: Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, between July 2014 and September 2014.
Methodology: Proteases produced in optimized medium and conditions were extracted by centrifugation, purified by ammonium sulfate precipitation and followed by dialysis. The effects of pH, temperature, metal ions, inhibitors, organic solvents, H_2O_2 and surfactants on enzyme's activity

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and stability were determined. Protease's ability to hydrolyze gelatin was employed to recover silver from used X-ray films, and used a detergent additive for stain removal from cloths. Both proteases and the producing isolate were used to digest chicken feathers to produce amino acids, peptides and soluble proteins. All generated data were analyzed using one-way ANOVA with post hoc multiple comparison analysis using Tukey's HSD.

Results: Ammonium sulfate at 70% had the best effect on precipitating the produced protease increasing specific activity to 5143 (u/mg), a 2.6 fold of that of the crude enzyme (1408.5 u/ml). Characterization experiments showed optimum conditions for activity to be pH 9 and 60ºC. Ca^{2+}, Mn^{2+}, Pb^{2+}, Mg^{2+}, Co^{2+}, Zn^{2+} and Ba^{2+} negatively affected the enzyme activity at 5mM. The enzyme was stable at low concentrations of oxidizing agent and surfactants and retained nearly 100% and 65% activity in presence of 0.1% and 0.5% of SDS, respectively. Purified proteases was 23 fold active than the commercial Savinase® under the standard assay conditions. As a detergent additive, it was effective in removing blood and egg stains from cotton fabric when combined with detergent. In digesting chicken feather, the isolate showed maximum caseinase activity of 645 u/ml in the initial screening process and an ability to degrade raw feather to free protein hydrolysates under optimal conditions (after 96 h of fermentation in 30 g/l chicken feather and pH 8.0).

Conclusion: Optimum pH indicated alkalinity class and optimum indicated thermal classification. Purified proteases was 23 fold more active than the commercial Savinase® under the standard assay conditions. Applications of the partially purified proteases proved it to be a good profitable industrial tool.

Keywords: Bacillus amyloliquefaciens; proteases; purification; characterization; silver recovery; detergent additive; chicken feather hydrolysis.

1. INTRODUCTION

A number of proteases from different sources have been purified and characterized. Microbial enzymes are mostly extracellular and are released into the fermentation media; thus the separation of cells from the media is generally carried out by centrifugation or in some cases by filtration, leaving the enzyme in the supernatant of filtrate. Then, the culture supernatant is concentrated by salting out. Ammonium sulphate, which lowers the solubility of the desired proteins are the usual agents employed for precipitation [1].

The properties of proteases from different microbiota have been studied with respect to the effect of pH and temperature on activity and stability, substrate specificity, effect of various additives as well as metal ions. The properties of the enzyme identified during these studies help in determining the areas of its possible application [2]. The optimum pH range of alkaline proteases is generally found to be between pH 9 and 11 [3] with a few exceptions of higher (12) pH optima [4]. The optimum temperature of alkaline proteases from microbes generally ranges from 50-70ºC [5]. Alkaline proteases from *Bacillus* sp. isolated from the soil of Veraval coast of the Gujarat (India) had a temperature optima of 37ºC [6] while an unusually high temperature optimum of 75ºC was reported for the proteases from *B. laterosporus*-AK1 [7].

Alkaline proteases often require a divalent cation like Ca^{2+}, Mg^{2+} and Mn^{2+} or a combination of these cations for maximum activity. These cations are believed to protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at high temperatures [3]. Several workers have reported the role of Ca^{2+} in enzyme stabilization, by increasing the activity and thermal stability of alkaline proteases at higher temperatures [8].

Inhibition studies give insight into the nature of the enzyme, its cofactor requirements and the nature of the active site [9]. Effect of inhibitors such as phenlymethylsulfonyl fluoride (PMSF), iodo acetic acid (IAA), ethylene-diamine tetraacetic acid (EDTA), 1, 10-phenanthroline and pepstatin on proteases have routinely been studied to determine the class of proteases [10].

Alkaline proteases added to detergent formulations enable the release of proteinaceous dirt (blood, sweat, egg) that appear as stains on fabric [11]. Most of the proteases that have been reported to be capable of stain removal and/or compatible with commercial detergents or surfactants have been from the different species of *Bacillus* [12]. A highly bleach stable and halo-

tolerant alkaline proteases has been reported from *B. pumilus* JB05 [13]. A novel species of alkalinophilic *Bacillus* that produces oxidatively stable serine proteases were isolated by Saeki et al. [14].

Methods adopted for silver recovery from waste films include enzymatic hydrolysis of gelatin to release the silver [15]. The enzymatic hydrolysis of the gelatin layers on the X-ray film enables not only the recovery of the silver, but also the polyester base which can be recycled. Basically enzymatic processes are more specific and remove gelatin layer from X-ray film in a few minutes; without damaging the polyester film base [16]. Most of the proteases used so far for silver recovery are from bacteria such as *B. sphaericus* [17] and *B. subtilis* [18].

Feathers represent 5–7% (w/w) of the mature chickens total weight and are generated in large amounts as a waste product in commercial poultry-processing plants, reaching millions of tons per year worldwide [19]. Although the feathers are considered as wastages, it contains large and various types of proteins which can be converted into valuable materials [20]. Raw feathers, however, are very poorly digested by non-ruminant animals because they contain a high proportion of keratin [21]. Using microbial enzymes in feather degradation improves the nutritional value of feather wastes. Kumar et al. [22] produced 38.2 g/l of total amino acids and peptides using 30 g/l feathers in the production medium at pH 10 using a *Bacillus* sp. Srivastava et al. [23] also found that concentrations of total amino acids, cysteine, serine, and methionine were continuously monitored in the fermentation broth and reached to a maximum of 1.2 mg/ml, 46 µg/ml, 107 µg/ml and 17.52 µg/ml respectively, using 10 g/l feather in the production medium.

The aim of this work is to evaluate the characteristic properties of the purified proteases produced by *Bacillus amyloliquefaciens* 35s isolated from the soil of Nile Delta of Egypt and an investigation into the possible areas of agricultural applications of the enzyme were also determined.

2. MATERIALS AND METHODS

2.1 Microorganism

A novel proteolytic strain (*Bacillus amyloliquefaciens* 35s), previously isolated and identified by 16S rRNA gene sequencing analysis [24], was used in the present study to produce proteases and its production was statistically and practically optimized [25]. Culture of this strain was maintained on nutrient agar slants at 4ºC and sub-cultured at monthly intervals. Nutrient agar medium [26], was used for bacterial stock culture maintenance and standard inoculum preparation. Standard inoculum was prepared by inoculating 50 ml of nutrient broth medium in 250 ml Erlenmeyer flasks with a full loop of tested culture. The inoculated flask was incubated on a rotary shaking incubator (Lab-line Ltd.) at the rate of 120 rpm for 24 h at 30ºC and considered as the standard inoculum (7.0 x 10^5/ml viable cells).

2.2 Extraction of Crude Proteases

The culture containing proteases, obtained from the optimized cultivation of *Bacillus amyloliquefaciens* 35s in bioreactor [25], was centrifuged at 10000 rpm for 10 min at 4ºC to obtain a crude proteases supernatant. Cell pellet was discarded, the supernatant was preserved at 4ºC for analysis and the assay was done within 24 h [27].

2.3 Partial Purification of Proteases

Supernatant containing crude proteases were purified as follows: To the chilled crude supernatant, solid ammonium sulphate (40–80% saturation) was added as per standard chart to precipitate out the enzyme [28]. Precipitation was done at 4ºC. The precipitate was collected by centrifugation (10000 rpm, 4ºC for 15 min.) and re-suspended in 0.9% NaCl and then dialyzed against phosphate buffer at 4ºC in the refrigerator to prevent contamination of the final preparation before dialysis [29]. This dialyzed fraction was made up to a known volume and referred to as partially purified proteases. The partially purified enzyme was di-filtered using Amicon UF Stirred Cell (Model 8010) with 10 kDa cut off membrane against Tris-HCl buffer (pH 8.5) to remove ammonium sulfate [30]. Proteases activity was determined according to the modified method of Anson [31]. The protein content of different enzyme preparations was determined by the method of Lowry et al. [32]. Specific activity of the sample was calculated by dividing the enzyme units (U) on the total protein content (mg/ml). The activity of the sample is expressed in units (U) and wherever necessary as specific activity (U/mg).

2.4 Characterization of Partially Purified Proteases

2.4.1 Effect of pH and temperature on enzyme activity and stability

The effect of pH on proteases activity was evaluated in the range of 7–12, using buffers of 0.1 M sodium phosphate (pH 7), 0.1 M Tris-Cl (pH 8–9) and 0.1 M Glycine NaOH (pH 11–12) in the reaction mixture. 1 ml of crude enzyme was mixed with 5 ml casein substrate (0.65% in buffer at the tested pH). Enzyme activity was expressed in terms of relative activity calculated according to the proteases stability at different pH values. Various pH values were studied by pre-incubating the enzyme in buffers of different pH (7–12) for 1h and the residual enzyme activity (%) was determined after the incubation period. Residual activity is the percentage of enzyme activity of the sample with respect to activity of the control (untreated sample). Residual activity = Activity of sample (U) x 100 / activity of control (U) [33].

The effect of temperature on the proteases activity was assessed by determining the activity at temperatures ranging from 30 to 80°C. The percentage relative activity was calculated considering the activity at 60ºC as 100%. The temperature stability of the enzyme was determined by pre-incubating the enzyme at different temperatures ranging from 30 to 80ºC for one hour and then assaying the residual activity (%) under the standard assay conditions. Activity of untreated enzyme (control) was considered 100% as mentioned by Abu-Sayem et al. [27].

2.4.2 Effect of metal ions and inhibitors on enzyme activity

The influence of various metal ions on the partially purified proteases were studied by incubating the enzyme in the presence of various metal ions ($ZnCl_2$, $CaCl_2$, $MgCl_2$, $MnCl_2$, $PbCl_2$, $CoCl_2$, $HgCl_2$, $BaCl_2$, and $CuSO_4$) at final concentration of 1mM and 5mM at 60°C for 30 min. The percentage of relative activity was calculated by considering the activity of enzyme (in absence of metal ions) at 60ºC and pH 9 as 100% activity. To study the effect of different proteases inhibitors on the purified enzyme, aliquots of enzymes were pre-incubated with the different enzyme inhibitors such as phenylmethylsulphonyl fluoride (PMSF) (5 mM), iodo acetic acid (IAA) (1mM), ethylene-diaminetetraacetic acid (EDTA) (5 mM) and 1,10 phenanthroline (5 mM) for 30 minutes at room temperature. Residual activities (%) were measured and calculated. Control treatments were placed without inhibitors as mentioned by Abu-Sayem et al. [27].

2.4.3 Effect of organic solvents on proteases activity and stability

To determine the effect of organic solvent on proteases activity, 1 ml of five organic solvents (benzene, xylene, toluene, butanole or pyridine) was added to 1 ml of proteases then 5 ml of casein solution were added and activity was determined as described above. For determining the effect of these organic solvent on the enzyme stability, 1 ml of proteases solution was pre-incubated with 1 ml of one of the above five organic solvents at room temperature for 30 min., then the enzyme activity was determined as previously described. The stability was expressed as residual activity relative to the non-solvent containing control.

2.4.4 Effect of H_2O_2 and surfactants

To investigate the effect of the oxidizing agent (H_2O_2) and three surfactants (SDS, Triton X-100 and Tween-80) on the enzyme stability, the partially purified proteases were pre-incubated with different concentrations of H_2O_2 (0.1 and 0.5%), SDS (0.1 and 0.5 %), and Tween-80 (0.5 and 1 %) for 30 min and then their residual activities were measured as mentioned by Barindra et al. [34].

2.4.5 Comparison of the partially purified proteases with commercial proteases

Activity of the partially purified proteases was compared to that of commercial proteases (SAVINASE ® P3111, Sigma Co; USA). Comparison was done by measuring the proteases activity under the standard assay conditions (pH 9 and temperature 60ºC of the partially purified proteases and proteases SAVINASE and specific activity was calculated.

2.5 Applications of the Crude Proteases

2.5.1 Silver recovery from used X-ray films (decomposition of gelatin and release of silver)

Used X-ray films were washed with distilled water and wiped with cotton impregnated with

ethanol. The washed film was dried in an oven at 40°C for 30 min [35]. One gram of X-ray film (cut into 2 x 2 cm pieces) was then incubated with 100 ml diluted partially purified alkaline proteases in buffer (Tris–Cl, pH 9) with continuous shaking at 50ºC. Turbidity of the reaction mixture increased with time (as the hydrolysis progressed). Hence, the progress of hydrolysis was monitored by measuring the absorbance at 660 nm [5]. Samples were removed at regular intervals and time required for complete removal of gelatin layer was noted. Visual examination of films after complete hydrolysis was also done. Repeated utilization of enzyme was studied by changing the X-ray films every 30 min. Each 30 min., change of X-ray sheets was recorded as one run and was carried out in total four runs. The weight of X-ray films before and after each run was recorded. The extent and time of decomposition was also noted.

2.5.2 Detergent additive

2.5.2.1 Wash performance with proteases solution

Wash performance analysis of partially purified alkaline proteases (PPAP) was done on square (dimension 4 x 4 cm) pieces of cotton fabric stained with human blood and egg yolk. After staining, fabric was air dried followed by oven drying [36]. The following sets of treatments to the stained fabric pieces were prepared:

A) 100 ml detergent (7 mg/ml Persil, Procter and Gamble Co. Ltd) + stained fabric piece.
B) 100 ml detergent (7 mg/ml Persil, Procter and Gamble Co. Ltd) + 500 µl PPAP solution (final dilution 250 U) + stained fabric piece.
C) 100 ml tap water + 500 µl PPAP solution + stained fabric piece.
D) 100 ml tap water + stained fabric piece (control).

Two such sets of treatments of human blood and egg yolk stained fabrics (each set in triplicates) were prepared. One set was incubated at 50ºC for 30 min while the other was kept at room temperature. Later the cotton fabric was taken out rinsed in tap water, dried and evaluated for stain removal [37].

2.5.2.2 Compatibility with commercial detergents

The commercial detergents Persil, Ariel and Tide (Procter and Gamble Co. Ltd) and Sunlight (Hindustan Unilever) were dissolved in tap water to give a final concentration of 7 mg/ml to stimulate washing conditions [37]. The endogenous proteases contained in these detergents were inactivated by incubating the detergent solutions at 65ºC prior to the addition of exogenous purified proteases from *B. amyloliquefaciens* 35s [38]. After the addition of exogenous proteases in detergent solution (in a ratio 1:4) [39], the mixture was incubated for 1 h at 40°C and the residual activity was determined under standard assay conditions. The enzyme activity of the control sample (without detergent) was taken as 100% [40].

2.5.3 Application for waste management

2.5.3.1 Feather protein hydrolysates (FPH) production

Chicken feathers, obtained from a local poultry were washed three times with tap water and then with distilled water. The washed feathers were then cooked until boiling for 20 min and pressed to remove water. The resulting pressed product was minced with a blender, dried at 90ºC for 22 h, minced again to obtain a fine powder and stored at room temperature. For feather protein hydrolysis medium preparation, 250 ml Erlenmeyer flasks containing 100 ml tap water and chicken feather powder ranged from 10 to 60 g/l were added to each flask. After adjusting the pH to 8, all media were autoclaved at 121ºC for 20 min. After sterilization, flasks were inoculated by 5% (v/v) of *B. amyloliquefaciens* 35s standard inoculum. After inoculation, flasks were incubated for 144 h at 37ºC with shaking at 120 rpm. After incubation, the cultures were centrifuged at 12,000 rpm for 15 min at 4ºC and the cell-free supernatants were used for determination of proteases activity, soluble proteins and amino acid concentration. Productivity was determined as soluble protein concentration per time [41]. Amino acids, peptides and soluble proteins were determined according to Kumar et al. [22].

In an additional treatment to evaluate the direct effect of the partial purified alkaline proteases (PPAP) on feather hydrolysis, 0.1 ml of PPAP was added to test tube containing one feather in 10 ml tap water. Feather hydrolysis was monitored during 144 h of incubation at 37ºC.

2.6 Statistical Analysis

Data generated from the above experiments were analyzed using one-way ANOVA with post

hoc multiple comparison analysis performed using Tukey's HSD. In shake flask experiments, mean of three replicates were compared using SPSS 16.0 for windows at a significance level of $p<0.05$.

3. RESULTS AND DISCUSSION

3.1 Partial Purification of Proteases Produced by *Bacillus Amyloliquefaciens* 35s

Cell-free supernatant of *B. amyloliquefaciens* 35s culture was used as the source of the crude enzyme. Proteases were purified by ammonium sulfate precipitation followed by dialysis (Table 1). 40–80% ammonium sulfate saturation fractions were tested for proteases precipitation, and 70% showed to have the best effect on precipitating the produced proteases. Precipitated proteases were dialyzed and concentrated. Ammonium sulfate increased the activity by 2.6 fold (3626.2 u/ml), while dialysis doubled the activity by 6 fold (9024 u/ml). Padmapriya and Williams [42] produced proteases by *B. subtilis* and was able to purify it first by 75% ammonium sulfate precipitation, increasing its activity by 3.12–fold, then dialyzed increasing 5.14 fold. Borah et al. [43] revealed that the culture supernatant of *B. altitudinis* contained an initial proteases activity of 105.5 u/ml and used 80% (w/v) ammonium sulfate to precipitate the enzyme. This treatment increased specific activity to 2366.15 (u/mg) and purification folds 4 times the specific activity 9464 (u/mg) comparing with the crude proteases on partial purification of proteases enzyme.

3.2 Characterization of the Partially Purified Proteases

3.2.1 Effect of pH and temperature on proteases activity

pH activity profile of partially purified proteases of *B. amyloliquefaciens* 35s was determined using different buffers with varying pH values. Statistical analysis revealed that pH had significant ($p<0.01$) influence on the activity of proteases whereas, in the pH range 7–9, more than 78 % of activity was retained by the enzyme with no significant difference ($p>0.05$) in the residual activity at pH 7 and 8 (Table 2). The purified enzyme was active in pH range of 6.0–11.0, with an optimum at pH 9 and reached a relative activity of 100%. Activity of the enzyme increased proportionally with the increase in pH from 6 to 9 then dropped beyond this point. With respect to the activity determined at pH 9, proteases exhibited 71.22%, 90.83%, 60.34% of the maximal activity at pH 7, 8 and 10, respectively. The relative activity of enzyme at pH 6 (26.2%) and pH 12 (6.5%) were minimal. These results clearly indicate that the proteases are alkaline proteases. Other investigators recorded optimum proteases activity at different pH values, such as pH 7.8 [44], 8.0 [45], 9.8–10.2 [4], 10.5 [44] and 12–13 [46].

When enzyme stability was determined in different pH buffers, it can be deduced from the data in Table 2 that proteases were mostly stable at pH range from 7 to 10 with the highest residual activity reached 89.1 %, observed in the sample incubated at pH 9. In a similar study, Jellouli et al. [33] revealed that the optimum pH was 10, and it was stable at alkaline pH ranges.

With regards to the optimum temperature for proteases activity, the proteases of *B. amyloliquefaciens* was active at all temperatures (30–80ºC) tested, with a maximum activity recorded at 60ºC, qualifying it to be designated as a moderately thermo-active proteases. A sharp decline in activity at temperatures above 60ºC was noted as illustrated in Table 2. Within the temperature range 40–60ºC proteases retained more than 85% of its maximum activity. Even at temperatures 30 and 80ºC, it exhibited 39.25 and 30.68% relative activity, respectively with optimum temperature at 60ºC recorded relative activity of 100%. The statistical analysis

Table 1. Summary of the partially purification of *B. amyloliquefaciens* 35s proteases

Purification step	Proteases activity (u/ml)	Protein content (mg/ml)	Specific activity (u/mg)	Purification fold
Crude extract	1408.5	0.936	1504	1
70 % Ammonium sulphate fraction	3626.2	0.712	5143	2.6
Dialysis	4521	0.501	9024	6

Specific activity (u/mg) of the sample was calculated by dividing the enzyme units (U) on the total protein content

revealed that temperature had significant (p<0.01) influence on the activity of the proteases. Borah et al. [43] found that the effect of temperature on enzyme activity of purified proteases from *B. altitudinis* showed maximum production at 40°C (4800 u/ml) and minimum production at 30ºC (3700 u/ml). Different optimum temperatures were determined by various investigators, e.g. 30ºC [47], 35ºC [48], 50ºC [49] and 70ºC [50].

Temperatures ranged from 30 to 70ºC were experimented to explore their effect on proteases stability. Proteases temperature stability (Table 2) revealed a great deal of stability approximated to 92.46 to 93.04 % in the temperature range 30–50ºC and the stability exhibited in this range did not vary significantly (p>0.05). However, proteases were unstable at its optimal temperature activity (60ºC). It retained 28.99 % of its activity. Moreover the alkaline proteases were almost completely inactivated when incubated at 70ºC for 1 h. Similarly, Jellouli et al. [33] revealed that the optimum temperature was 70ºC, using casein as substrate. On the other hand, Patel et al. [6] found that proteases produced by newly isolated haloalkaliphilic *Bacillus* sp. was highly stable at 37ºC and retained 50 % of its activity at 45ºC. However, the enzyme was quite unstable at temperatures beyond 55ºC but stable at higher temperature in the presence of NaCl and $CaCl_2$.

3.2.2 Effect of metal ions, inhibitors, oxidizing agent and surfactants on alkaline proteases activity

This experiment was designed to study the effect of chloride, sulfate and nitrate ions using concentrations of 1 and 5 mM on alkaline proteases activity. As illustrated in Table 3, in presence of either Co^{2+} (1 mM) or Mn^{2+} (1 mM), the activity of the enzyme was similarly affected (p>0.05). Even in the presence of Mn^{2+} (85.64%), Pb^{2+} (72.22%), Mg^{2+} (91.6 %) and Ba^{2+} (98.22%) ions, the enzyme showed a great activity. At both concentrations (1 mM and 5 mM), Hg^{2+} and Cu^{2+} were found to be inhibitory, while Zn^{2+} had a negative effect at 5 mM concentration. All the metal ions at 5 mM have a negative influence on the activity of alkaline proteases produced by *B. amyloliquefaciens* 35s. In a study by Nadeem et al. [51], the effect of metal ions on proteases activity, Mg^{2+} and Ca^{2+} ions enhanced the activity by 128% and 145%, respectively. In a similar study by Tari et al. [52] on proteases characterization by *Bacillus* sp. L21, Ca^{2+} ions had a positive effect on proteases activity by 17.94%. The stimulating effect of $CaCl_2$ was also reported by Mabrouk et al. [53] who related this stimulation to the stabilizing effect of $CaCl_2$ on alkaline proteases. However, higher concentrations of $CaCl_2$ (1 g/l) possessed an inhibitory effect on proteases activity that reached down to 77.7 u/ml.

Studies on the effect of inhibitors on the alkaline proteases enzyme can help in determining the nature or the class of the proteases. *B. amyloliquefaciens* 35s proteases lost around 24% of its activity when treated with EDTA (5 mM), a metal chelator and completely by phenylmethylsulphonyl fluoride (PMSF), a serine inhibitor (Table 3), while IAA did not greatly affect the activity of the enzyme. Therefore, it can be concluded from the above results that the produced proteases could be serine proteases. Serine alkaline proteases are known to be completely inhibited by PMSF [38]. Most of the earliest studies reported that 1–5 mM concentrations of PMSF generally completely inactivated serine alkaline proteases [54]. Also, Nadeem et al. [51] found that proteases produced by *B. licheniformis* uv-9 were completely inhibited by PMSF suggesting that it was serine proteases.

Studies on the effect of oxidizing agents and surfactants on alkaline proteases activity help in exploring the application of proteases in detergent industry. H_2O_2, SDS and tween 80 were tested on proteases stability. The enzyme was stable at low concentrations of oxidizing agent and surfactants. At a concentration of 0.1 mM H_2O_2, (strong oxidizing agent) less than 20% inhibition in activity was observed (Table 3). The nature of surfactant seemed to influence its effect on proteases. SDS (anionic detergent) had negative effect on proteases while Tween 80 (nonionic detergent) had a slight enhancing effect on the enzyme. The enzyme retained nearly 91% and 65% activity in presence of 0.1% and 0.5% of SDS, respectively. Increasing concentration of SDS to 0.5 mM the inhibitory effect was increased. Jellouli et al. [33] revealed that proteases produced by *B. licheniformis* Mp1was stable towards non-ionic surfactants and SDS. Also Nadeem et al. [51] reported that the purified proteases showed extreme stability towards various surfactants such as Tween 80, Tween 45, Tween 65 and Triton X45. In addition, the proteases also exhibited 100% residual activity in the presence of oxidizing agents, H_2O_2 and sodium perborate.

3.2.3 Effect of organic solvents on proteases stability

The proteases produced by *B. amyloliquefaciens* 35s exhibited significant stability towards almost all the organic solvents tested in this study. Actually little enhancement in enzyme activity was observed with an increase in the incubation period from 1 to 72 h. However, the enzyme was least stable in the presence of pyridine, retaining only 22.61% of its activity, as illustrated in Table 4. The enzyme showed high degree of stability (almost 70%) in presence of benzene. Moreover, the longer the incubation in organic solvents, the greater was the residual activity. The stabilizing effect of organic solvents has also been reported by Sana et al. [55]. This stabilizing effect on proteases could be due to the replacement of some water molecules in an enzyme with organic molecules, which results in stabilizing the structure of the enzyme [56]. Peptide synthesis enhanced by the addition of organic solvents in the reaction mixture [57-59]. However, the reduced activity of enzymes under anhydrous conditions is a major shortcoming of this approach and this can be overcome only by the use of organic solvent tolerant proteases. The high degree of organic solvent stability of alkaline proteases from *B. amyloliquefaciens* 35s suggests its potential application in peptide synthesis.

3.2.4 Comparison of *Bacillus amyloliquefaciens* 35s alkaline proteases with commercial proteases

This experiment was carried out to compare the specific activities of commercial proteases with that of the produced proteases by *B. amyloliquefaciens* 35s. Purified *B. amyloliquefaciens* 35s proteases were 23 fold more active than the commercial Savinase® under the standard assay conditions (Table 5). Savinase® is a commercial enzyme that is claimed to be active over a wide range of pH.

3.3 Applications of the Partially Purified Proteases

3.3.1 The recovery of silver from used X-ray films

As the natural resources are getting depleted rapidly all over the world; reuse or recycle remains a great solution to slowdown this exhaustion. Therefore, it was proposed in this study to test the produced alkaline proteases for recovery of silver from used X-ray films. Visual examination of the films clearly revealed the ability of proteases to hydrolyze the gelatin cover in X-ray films leaving a clean polyester sheet while releasing silver into the hydrolysate

Table 2. Effect of pH and temperature on the activity and stability of the partially purified proteases produced by *B. amyloliquefaciens* 35s

Factor	Levels	Relative activity (%)	Residual activity (Stability %)
pH*	6	26.20±0.30[b]	55.81±0.04[c]
	7	71.22±0.04[e]	79.45±0.05[d]
	8	90.83±0.03[f]	80.31±0.05[d]
	9	100.00±0.00[g]	89.1±0.20[e]
	10	60.40±0.11[d]	60.25±0.06[c]
	11	30.00±0.29[c]	43.53±0.09[b]
	12	6.50±0.14[a]	29.25±0.06[a]
Temperature (ºC)**	30	39.25±1.15[a]	92.46±1.94[c]
	40	83.26±0.10[c]	96.24±1.13[c]
	50	90.39±1.2[c]	93.04±0.02[c]
	60	100±0.00[d]	28.99±0.11[b]
	70	57.29±2.00[b]	0.159±0.01[a]
	80	30.68±1.13[a]	N.D#

Relative activity: activity measured relative to that obtained at pH 9 and 60°C, which is considered as 100%
Residual activity = (Activity of sample after incubation / activity of sample before incubation) x 100. Activity was determined after pre-incubating the crude enzyme in buffers of different pH (7–12) for 1h and the residual enzyme activity (%) was determined after the incubation period.
**Residual activity of pH experiment was determined at 60°C.*
*** Residual activity of temperature experiment was determined at pH 9.*
Values with same superscripts did not vary significantly, according to Tuky test at 5% level
The values are mean of three replicates ± standard deviation.
#N.D: Not detected

Table 3. Effect of various metal ions inhibitors, at 1 mM and 5 mM, oxidizing agent and surfactants on the activity of partially purified alkaline proteases produced by *B. amyloliquefaciens* 35s

		Residual activity (%)	
		1 mM	**5 mM**
Metal ions	Control*	100^{j}	100^{j}
	$ZnCl_2$	65.47 ± 0.96^{b}	7.9 ± 0.23^{c}
	$MnCl_2$	85.64 ± 1.02^{d}	30.90 ± 0.36^{e}
	$CaCl_2$	90.00 ± 1.21^{e}	71.58 ± 0.68^{h}
	$Pb(NO3)_2$	72.22 ± 0.90^{c}	12.27 ± 0.16^{d}
	$MgCl_2$	91.60 ± 0.99^{f}	72.31 ± 0.65^{i}
	$HgCl_2$	10.15 ± 0.72^{a}	1.35 ± 0.09^{a}
	$BaCl_2$	98.22 ± 0.62^{g}	65.28 ± 0.75^{g}
	Cu_2SO_4	10.43 ± 0.30^{a}	3.11 ± 0.08^{b}
	$CoCl_2$	85.08 ± 0.43^{d}	35.26 ± 0.59^{f}
Inhibitors	Indol acetic acid (IAA) cysteine proteases inhibitor)	98 ± 0.22^{ch}	81.3 ± 0.11^{cj}
	EDTA (metallo proteases inhibitor)	91 ± 0.51^{bf}	86 ± 0.45^{be}
	PMSF** (serine proteases inhibitor)	0.00 ± 0.00^{a}	0 ± 0.00^{a}
		Residual activity (stability %)	
		0.1 mM	**0.5 mM**
Oxidizing agent and surfactants	control	100^{f}	100^{f}
	Tween 80	100 ± 0.00^{f}	100^{f}
	SDS	91 ± 0.40^{d}	65 ± 0.90^{b}
	H_2O_2	80 ± 0.45^{c}	42 ± 0.68^{a}

Residual activity = (Activity of treated sample / activity of control samples) x 100
Residual activity: activity measured relative to that obtained at pH 9 and 60°C in control without metal ions, which is considered as 100%
Values with same superscripts did not vary significantly according to Tuky test at 5% level.
**Control without metal ions or inhibitor.*
***Phenylmethylsulphonyl fluoride (PMSF).*
The values are mean of three replicates ± standard deviation

(Fig. 1). The enzyme was able to hydrolyze gelatin in X-ray films better at higher temperature (50 and 60ºC) which was reflected in a shorter time for action at these temperatures. Maximum gelatin hydrolysis was observed in the initial 15 min and duration of 25 min was required for the complete stripping of gelatin from the X-ray films. With the increase in the incubation temperature from 30ºC to 60ºC, a corresponding increase in the rate of hydrolysis of gelatin was noted. In a similar study, Shankar et al. [16] have reported an increase in gelatin hydrolysis at 40ºC. Also, there was however no major difference in activity at 50 and 60ºC in gelatin hydrolysis. Therefore, 50ºC was chosen as the optimal temperature for hydrolysis due to lesser heating requirements. Moreover, Nakiboglu et al. [35] selected 50ºC for 15 min. as the optimum temperature for the complete hydrolysis of gelatin by proteases from *B. amyloliquefaciens* ATCC 6633. In another study, the proteases obtained from *B. amyloliquefaciens* 35s took 25 min for the complete removal of gelatin, whereas the proteases obtained from a fungal isolate *Conidiobolus coronatus* ATCC PTA-4132 [16] took only 6 min. Masui et al. [60] reported that the alkaline proteases from the *Bacillus* sp. B21-2 mutant took 30 min for the complete hydrolysis of gelatin layer at 50ºC.

Hydrolysis of gelatin occurred best when Tris-Cl buffer of pH 9 was used; indicating that it was the most ideal pH for hydrolysis of gelatin. The optimum pH for stripping of the waste film by the enzymatic extract of *B. amyloliquefaciens* 35s was found to be 8. Similar results were found by Nakiboglu et al. [35] where the optimum pH to strip the waste film was 10 by *B. amyloliquefaciens* ATCC 6633. Other workers found pH 10 or above to be ideal for gelatin hydrolysis. The ability of the proteases used in this study, to work in different pH environments (tap water, distilled water and buffer) is highly advantageous from the industrial point of view [16].

Table 4. Effect of different organic solvents on activity and stability of partially purified alkaline proteases produced by *B. amyloliquefaciens* 35s

Organic solvents	Relative activity (%)	Residual activity (stability %)
Control*	100±0.00^{f}	100±0.00^{f}
Benzene	72.54±0.17^{e}	69.7±0.20^{e}
Xylene	49.6±0.31^{d}	54.4±0.16^{d}
Toluene	47.8±0.14^{c}	45.5±0.55^{c}
Butanol	38.03±0.70^{b}	30±0.10^{b}
Pyridine	22.61±0.09^{a}	0±0.00^{a}

Relative activity: activity measured relative to that obtained at pH 9 and 60°C in control without any solvent, which is considered as 100%.
Relative activity = (Activity of treated sample / activity of control samples) x 100.
Residual activity = (Activity of treated sample / activity of samples at 0 time) x 100.
Values with same superscripts did not vary significantly according to Tuky test at 5% level.
**Control without organic solvents.*
The values are mean of three replicates ± standard deviation

Table 5. Comparison of *B. amyloliquefaciens* 35s alkaline proteases with the commercial alkaline proteases SAVINASE® (P3111)

Enzyme	Specific activity (u/mg)
Alkaline proteases from *B. amyloliquefaciens* 35s	5143
Savinase®	220

Specific activity (umg^{-1}) of the sample was calculated by dividing the enzyme units (u) on the total protein content

The conditions pH 9 and 50ºC were found to be most ideal for the alkaline proteases of *B. amyloliquefaciens* 35s for carrying out the hydrolysis of gelatin and the release of silver from X-ray films. The ability of an enzyme to retain its activity for repeated use makes it suitable for commercial application. It was found that the proteases of *B. amyloliquefaciens* 35s could be reused for 4 runs. The efficiency was found to drop after the 3rd run resulting in longer treatment time for achieving complete hydrolysis in the 4th run. Shankar et al. [16] noted that the proteases from *C. coronatus* could be reused 4 times and that, lowering the enzyme concentration resulted in longer reaction time for complete gelatin removal and also reduced the number of recycles. Masui et al. [60] observed that the treatment time increased after every reuse of enzyme and the first decomposition was complete in 60 min while the 2nd use required more than 2 h.

When compared to the proteases reported by Masui et al. [60], the alkaline proteases of *B. amyloliquefaciens* 35s used in this study seem to be more efficient as it was capable of withstanding reuse to a greater extent. Reuse of alkaline proteases of *B. amyloliquefaciens* 35s was carried out at 50ºC and pH 9. The proteases could be used up to 4 runs (Table 6). More than 30 min of exposure time was required to achieve completely clean sheets after the 3rd run. Thus, the produced alkaline proteases may exhibit an important trait for good commercial application.

3.3.2 Application of proteases as detergent additive

3.3.2.1 Stain removal

Wash performance revealed that the partially purified alkaline proteases (PPAP) of *B. amyloliquefaciens* 35s was very effective in removing blood and egg yolk stains from cotton fabric when used in combination with a detergent (Fig. 2). In case of egg yolk stain removal, the enzyme even in the absence of detergent was found to be quite effective. The proteases were capable of removing stains both at 50ºC and room temperature. Wash performance of partially purified alkaline proteases and/or detergent solution were evaluated by subjecting it to blood and egg yolk stain removal test from cotton fabric at 50ºC. The combination of detergent and

Table 6. Reuse of alkaline proteases for gelatin hydrolysis of X-ray film waste

Run	Reduction of weight (%) after 30 min of treatment based on the initial weight	Observation
1	4%	100% Clean polyester sheets
2	3%	100% Clean polyester sheets
3	2%	80% Clean polyester sheets
4	3%	100% Clean sheets were obtained when duration of exposure was prolonged up to 50 min

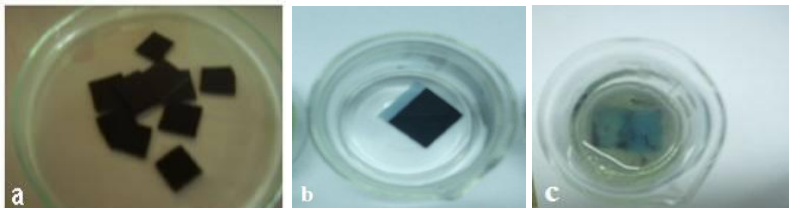

Fig. 1. Decomposition of gelatin and release of silver. (a) Cuttings of the untreated X-ray film (control). (b) Edges of film loss color after few minutes of exposure. (c) Clean bluish polyester sheets obtained after enzyme treatment of X-ray sheets

proteases solution was most effective in the removal of blood stain indicating that the enzyme aided the detergent in its cleaning action or improving the wash performance of the detergent. Moreover, the alkaline proteases of this study were capable of removing stains that had been made really stubborn by oven drying.

With drying and ageing proteinaceous stains strongly resist action of most low cost and high volume detergents [11]. These stubborn stains can be easily removed only by detergents incorporated with proteases that attack hydrolytically, the peptide bonds in the proteins. The ability of alkaline proteases to act on different types of stains satisfies the primary criteria of broad substrate specificity for its application as laundry detergent ingredient. The alkaline proteases from *B. amyloliquefaciens* 35s was capable of removing stain even at room temperature. This could be advantageous for detergent industry, as this industry is now looking for enzymes that work under lower wash temperatures. However, the washing efficiency of the enzyme increased with the increase in the temperature of incubation. The use of alkaline proteases for the removal of protein stains from cotton fabric has been reported by Abou-Elela et al. [12].

From the characterization study, it is clear that the alkaline proteases of *B. amyloliquefaciens* 35s is a promising detergent additive as it is active at alkaline conditions and also over a broad temperature range which would facilitate washing even at room temperature. Apart from these properties, good detergent proteases are expected to be stable in the presence of commercial detergents as well. The compatibility of alkaline proteases varied with different detergent brands, however more than 42% residual activity was observed with all the commercial detergents after 60 min of incubation at 40ºC. A similar observation was made by Rai and Mukherjee [61] in which they found alkaline proteases from *Bacillus* sp. had a residual activity of 43% when incubated with different detergents. When compared to proteases of *V. fluvialis* [62], the proteases from *Bacillus sp.* exhibited greater compatibility with the laundry detergents Ariel (77.45%), Sunlight (63.15%) and Surf excel (78.83%). The enzyme produced by *B. cereus* retained more than 80% of its activity in the presence of detergents Surf excel, Ariel and Henko [39]. When weighed against the proteases from *Conidiobolus coronatus* (NCL 86.8.2) [63], *Bacillus* sp. SSR1 [64], and *Basidiobolus* [65] the proteases from *Bacillus sp.* was found to be more compatible with commercial detergents.

3.3.3 Amino acid production from chicken feather waste

Feather are a significant source of protein for livestock because of their high protein content (>85% CP) [66]. Raw chicken feather, however, are very poorly digested to be used as a feed ingredient for non-ruminant animals. Although keratin can be completely dissolved by reducing agents like copper sulphate, mercapto-acetate, iodoacetic acid, sodium sulphite, sodium tetrathionate [67], these methods are not suitable for the large scale application. In order to overcome these limitations, the use of microbial enzymes which improves the nutritional value of feather wastes have been implemented in recent years. In the current work, *B. amyloliquefaciens* 35s was grown in mineral medium containing different concentrations of raw feather as the sole carbon and nitrogen source. The strain exhibited the highest enzymes production (645 u/ml) after 72 h of culturing in medium containing 30 g/l of chicken feathers (Fig. 3). At this feather concentration, the highest levels of amino acid production (25.8 g/l) was reached after 120 h of cultivation (Fig. 4) and the maximum productivity of soluble proteins and peptides (6 g/l) was after 96 h of cultivation (Fig. 5). Fig. 6a illustrates feather hydrolysis during 144 h of cultivation by *B. amyloliquefaciens* 35s at 37ºC. Feather was

completely hydrolysed after 144 h of incubation. The direct treatment of feather with the partial purified alkaline proteases (PPAP) showed extensive hydrolysis after 48 h of incubation at 37°C (Fig. 6b).

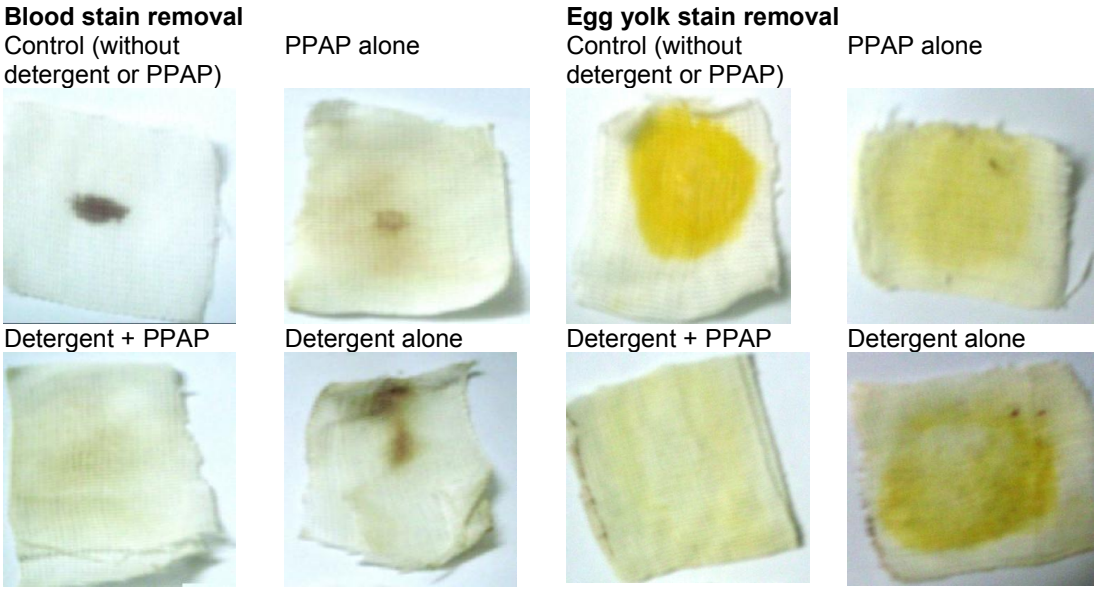

Fig. 2. Washing treatments of stained cotton fabric by the partially purified alkaline proteases (PPAP) of *B. amyloliquefaciens* 35s

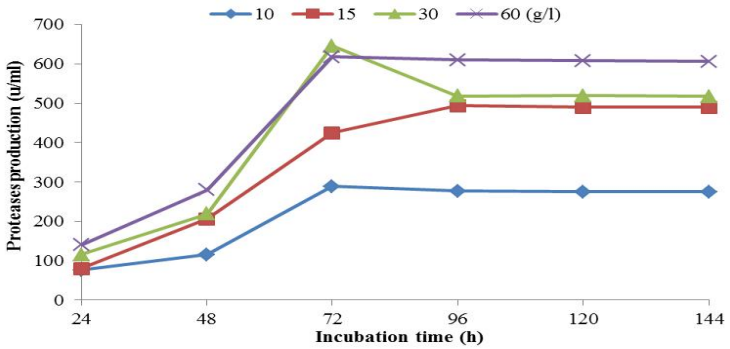

Fig. 3. Effect of feather concentration on proteases production in cultures of *B. amyloliquefaciens* 35s

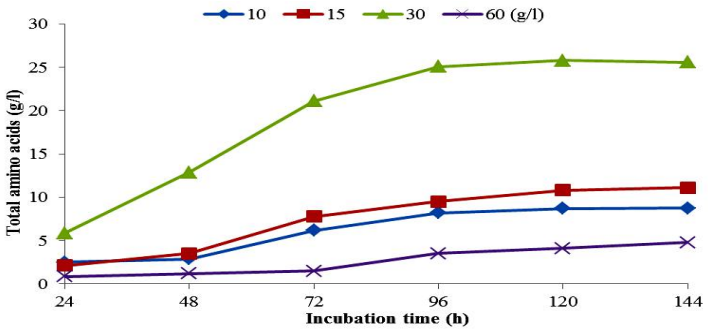

Fig. 4. Effect of feather concentration on amino acid production in cultures of *B. amyloliquefaciens* 35s

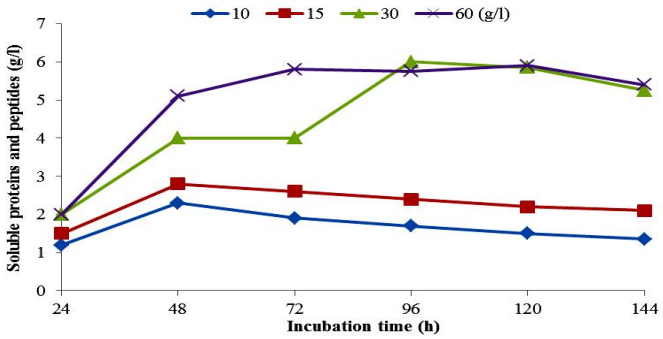

Fig. 5. Effect of feather concentration on soluble proteins and peptides in cultures of *B. amyloliquefaciens* 35s

Fig. 6. (A) Feather hydrolysis during 144 h of cultivation by *B. amyloliquefaciens* 35s at 37°C (B) The direct effect of the partial purified alkaline proteases (PPAP) on one feather hydrolysis (0.1 ml of PPAP was added to test tube containing one feather in 10 ml tap water and incubated at 37°C for 144 h of incubation

Similar results were obtained by Fakhfakh et al. [41] who used *B. pumilus* A1 to produce high levels of amino acids and peptides (34±2.0 g/l) and soluble proteins and peptides (5.9±2 g/l) in medium containing 30 g/l of feathers. However, at 60 g/l feather, proteases, amino acids, peptides and soluble proteins production were reduced. Moreover, Borah et al. [43] used the strain MPTK6 to degrade raw feather to feather protein hydrolysate (FPH) under optimal conditions (30 g/l chicken feather, pH 10.0 and 72 h of fermentation). While, Mariana et al. [68] used *Bacillus* sp. to degrade FPH under optimal conditions (945 g/l chicken feather, pH 9 and 96 h of fermentation).

In this study, results of characterization of the proteases produced by *B. amyloliquefaciens* 35s showed that the enzyme functions well at relatively high temperature (60ºC) and at pH 9, indication thermal and alkalinity classification of the enzyme. Stability of the enzyme in the presence of surfactants and oxidizing agents, stain removing capabilities along with its detergent compatibility indicated a promising suitability for application in laundry detergent formulations. The enzyme showed to have great potency to be used in recovery of silver from used X-ray films, and hydrolysis of chicken feather for the production of amino acids.

4. CONCLUSION

Purification with ammonium sulfate at 70% achieved specific activity of 5143 (u/mg), which is 2.6 fold the activity of the crude enzyme (1408.5 u/ml). Optimum pH of 9 indicates alkalinity class and optimum temperature of 60ºC indicates

thermal classification. The enzyme retained nearly 100% and 65% activity in presence of 0.1% and 0.5% of SDS respectively. Tween 80 at 0.5% and 1% enhanced the activity of proteases, and increasing concentration above that did not cause significant difference in activity. The produced protease exhibited significant stability towards almost all the tested organic solvents. The purified proteases produced by *Bacillus amyloliquefaciens* was 23 fold more active than the commercial Savinase® under the standard assay conditions. The above data suggest great reliability of applicability of the produced proteases in diverse fields, such as detergent industry and environmental fields.

Testing proteases produced by *Bacillus amyloliquefaciens* isolate 35s in various applications showed it to have great potency to be used in detergent formula, recovery of silver from used X-ray films, and hydrolysis of chicken feather for the production of amino acids from hydrolysate which can be used in further various applications, such as foliar fertilizers.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Walsh G. Proteins Biochemistry and Biotechnology. 2nd Ed. England. John Wiley & Sons. 2014;91–140.
2. Gupta R, Beg QK, Khan S, Chauhan B. An overview on fermentation, downstream processing and properties of alkaline protease. Appl Microbiol Biotechnol. 2002;60:381–395.
3. Kumar CG, Takagi H. Microbial alkaline proteases: From a bio industrial view point. Biotechnol Adv. 1999;17:561–594.
4. Kumar CG, Tiwari MP, Jany KD. Novel alkaline serine proteases from alkalophilic *Bacillus* spp. purification and some properties. Process Biochem. 1999;34: 441–449.
5. Shankar S, Rao M, Laxman RS. Purification and characterization of an alkaline protease by a new strain of *Beauveria* sp. Process Biochem. 2011;46:579–585.
6. Patel RK, Dodia MS, Joshi RH, Singh SP. Purification and characterization of alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp. Process Biochem. 2006;41:2002–2009.
7. Arulmani M, Aparanjini K, Vasanthi K, Arumugam P, Arivuchelvi M, Kalaichelvan PT. Purification and partial characterization of serine protease from thermostable alkalophilic *Bacillus laterosporus* –AK1. World J Microbiol Biotechnol. 2007;23: 475–481.
8. Moradian F, Khajeh K, Naderi–Manesh H, Sadeghizadeh M. Isolation, purification and characterization of a surfactants, laundry detergents and organic solvents resistant alkaline protease from Bacillus sp. HR–08. Appl Biochem Biotechnol. 2009;159:33–45.
9. Sigma DS, Moser G. Chemical studies of enzyme active sites. Ann Rev Biochem. 1975;44:889–931.
10. Karan R, Khare SK. Purification and characterization of a solvent stable protease from *Geomicrobium* sp. EMB2. Environ Technol. 2010;31:1061–1072.
11. Saxena RK, Davidson WS, Gupta R. Microbial enzymes for detergent application. In: Advances in microbial biotechnology, Eds. Tewari JP, Lakhanpal TN, Singh J, Gupta R, Chamola BP. New Delhi, A.P.H. Publishing Corporation. 1999;77–91.
12. Abou–Elela GM, Ibrahim HAH, Hanan SWH, El–Toukhy NMK. Alkaline protease production by alkaliphilic marine bacteria isolated from Marsa Matrouh (Egypt) with special emphasis on *Bacillus cereus* purified protease. Afr J Biotechnol. 2011;10:4631–4642.
13. Johnvesly B, Naik GR. Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus* sp. JB–99 in a chemically defined medium. Process Biochem. 2001;37:139–144.
14. Saeki K, Okuda M, Hatada Y, Kobayashi T, Ito S, Takami H, Horikoshi K. Novel oxidatively stable subtilisin–like serine proteases from aklaiphilic *Bacillus* spp.: enzymatic properties, sequences and evolutionary relationships. Biochem Biophys Res Commun. 2000;3:313–319.
15. Swati J, Satyanarayana T. Characteristics and applications of a recombinant alkaline serine protease from a novel bacterium *Bacillus lehensis*. Bioresour Technol. 2013;131:76–85.
16. Shankar S, More SV, Laxman RS. Recovery of silver from waste X–ray film

by alkaline protease from Conidibolus coronatus. J Sci Eng Technol. 2010;6:60–69.
17. Singh J, Vohra RM, Sahoo DK. Alkaline protease from a new obligate alkalophilic isolate of *Bacillus sphaericus*. Biotechnol Lett. 1999;21:921–924.
18. Ramakrishna DPN, Gopi Reddy N, Rajagopal SV. Purification and properties of an extra cellular alkaline protease produced by *Bacillus subtilis* (MTTC N0–10110). Int J Biotechnol Biochem. 2010;6:493–504.
19. Manczinger L, Rozs M, Vagvolgyi Cs, Kevei F. Isolation and characterization of a new keratinolytic *Bacillus licheniformis* strain. World J Microbiol Biotechnol. 2003; 19:35–39.
20. Dalev PG. Utilization of waste feathers from poultry slaughter for production of a protein concentrate. Bioresour Technol. 1994;48:265–267.
21. Papadopoulos MC. Amino acid content and protein solubility of feather meal as affected by different processing conditions. Neth J Agric Sci. 1985;33:317–319.
22. Kumar DJM, Priya P, Balasundari S, Nandhini-Devi GSD. Production and optimization of feather protein hydrolysate from *Bacillus* sp. MPTK6 and its Antioxidant Potential. Middle-East J Sci Res. 2012;11:900–907.
23. Srivastava A, Sharma A, Suneetha V. Feather Waste biodegradation as a source of amino acids. Eur J Exp Biol. 2011;1:56–63.
24. Nassar FR, Abdel-hafez AA, El-Tayeb TS, Abu-Hussein SH. Proteases production by a bacterial isolate *Bacillus amyloliquefaciens* 35s obtained from soil of the Nile Delta of Egypt. Brit Microbiol Res J; 2015. (accepted for publication).
25. Nassar FR, Abdel-hafez AA, El-Tayeb TS, Abu-Hussein SH. Response surface methodology for the optimization of proteases production by the novel Egyptian isolate *Bacillus amyloliquefaciens* 35s. Brit Microbiol Res J; 2015. (accepted for publication).
26. APHA (American Puplic Health Association). Compendium methods of food microbiology 8th ed. APHA, INC. New York; 1992.
27. Abu–Sayem SM, Alam MJ, Hoq MM. Effect of temperature, pH and metal ions on the activity and stability of alkaline proteases from novel Bacillus licheniformis MZK03. Proc. Pakistan Acad Sci. 2006;43:257–262.
28. Green AA, Hughes WL. Protein fractionation on the basis of solubility in aqueous solutions of salts and organic solvents. Methods Enzymol. 1955;1:67–90.
29. Anustrup K. Proteinases. In: Economic microbiology: Microbial enzymes and bioconversions (Ed.: A.H. Rose). Academic Press, New York, 1980;5:50–114.
30. Sadia AA, Shelly D, Mohan P. Purification and characterization of a novel protease from *Bacillus* strain Sal1. Afr J Biotechnol. 2009;8:3603–3609.
31. Anson ML. The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J Gen Physiol. 1938;22:79–89.
32. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193:265–75.
33. Jellouli KGB, Olfa BA, Hanen LM, Agrebiand R, Nasri M. Alkaline–protease from *Bacillus licheniformis* MP1: Purification, characterization and potential application as a detergent additive and for shrimp waste deproteinization. Process Biochem. 2006;41:2002–2009.
34. Barindra S, Debashish G, Malay S, Joydeep M. Purification and characterization of a salt, solvent, detergent and bleach tolerant protease from a new gamma *Proteobacterium* isolated from the marine environment of the Sundarbans. Process Biochem. 2006;41:20–215.
35. Nakiboglu N, Toscaliand, D, Yasa I. Silver recovery from waste photographic films by an enzymatic method. Turk J Chem. 2001;25:349–353.
36. Kanekar PP, Nilegaonkar SS, Sarnaik SS, Kelkar AS. Optimization of protease activity of alkaliphilic bacteria isolated from alkaline lake in India. Bioresour Technol. 2002;85:97–93.
37. Jyothi B, Yan S, Rojan PJ, Tyagi RD, Surampalli RY. Recovery of *Bacillus licheniformis* alkaline protease from supernatant of fermented waste water sludge using ultrafiltration and its characterization. Biotechnol Res Int. 2011;23:8549.
38. Kamoun SA, Haddar A, El–Hadj AN. Stability of thermostable alkaline protease from *Bacillus licheniformis* RP1 in

commercial solid laundry detergent formulations. Microbiol Res. 2008;3:299–306.
39. Banik RM, Prakash M. Laundry detergent compatibility of the alkaline protease from *Bacillus cereus*. Microbiol Res. 2004;159:135–140.
40. El–Hadj–Ali N, Agrebi R, Ghorbel–Frikha B, Kanoun S, Nasri M. Biochemical and molecular characterization of a detergent stable alkaline serine–protease from a newly isolated *Bacillus licheniformis* NH1. Enz Microb Technol. 2007;40:515–523.
41. Fakhfakh N, Kammoun S, Manni L, Nasri M. Production and biochemical and molecular characterization of a keratinolytic serine protease from chicken feather degrading *Bacillus licheniformis* RPk. Can J Microbiol. 2009;55:427–436.
42. Padmapriya M, Williams BC. Purification and characterization of neutral protease enzyme from *Bacillus Subtilis*. J. Microbiol Biotechnol Res. 2012;2:612–618.
43. Borah D, Kumar T, Mishra V. Process optimization, partial purification and characterization of protease enzyme from *Bacillus altitudinis* (MCCB–0014). Int J Pharm Sci. 2012; 4: 483–89.
44. Tsujibo H, Miyamoto K, Hasegawa T, Inamori Y. Purification and characterization of two types of alkaline serine proteases produced by an alkalophilic actinomycete. J App Bacteriol. 1990;69:520–529.
45. Anwar A, Saleemuddin M. Alkaline pH–acting digestive enzymes of the polyphagous insect pest *Spilosoma oblique*: Stability as detergent additives. Biotechnol Appl Biochem. 1997;25:43–46.
46. Takami H, Akiba T, Horikoshi K. Production of extremely thermostable alkaline protease from *Bacillus* sp. Appl Microbiol Biotechnol. 1998;30:120–124.
47. El–Kastawy SFM. Application of enzyme inhibitor technique in regulating the activities of purified constitutive protease produced by *Staphylococcus aureus* 110 isolated from a clinical source. Egy J Biomed Sci. 1998;2:5366–5372.
48. Gerze A, Omay D, Guvenilir Y. Partial purification and characterization of protease enzyme from *Bacillus subtilis* and Bacillus megatherium. Appl Biochem Biotechnol. 2005;45:121–124.
49. Ammar MS, El–Louboudy SS, Abdulraouf UM. Protease (s) from *Bacillus anthracis* S–44 and B. cereus var. mycoids, S–98 isolated from a temple and slaughter house in Aswan city. Az J Microbiol. 1991;13:12–29.
50. Sookkheo B, Sinchaikul S, Phutrakul S, Chen ST. Purification and characterization of the highly thermostable proteases from *Bacillus stearothermophilus* TLS33. Protein Expr Purif. 2000;20:142–151.
51. Nadeem M, Qazi JI, Syed Q, Gulsher M. Purification and characterization of an alkaline protease from *Bacillus licheniformis* UV–9 for detergent formulations. Songklanakarin. J Sci Technol. 2013;35:187–195.
52. Tari C, Genckal H, Tokath F. Optimization of growth medium using a statistical approach for the production of an alkaline protease from a newly isolated *Bacillus* sp.L21. Process Biochem. 2006;41:659–665.
53. Mabrouk SS, Hashem AM, El–Shayeb NMA, Ismail AMS, Abdel–Fattah AF. Optimization of alkaline protease productivity by *Bacillus licheniformis* ATCC21415. Bioresour Technol. 1999;69: 155–159.
54. Reddy LVA, Wee YJ, Ryu HW. Purification and characterization of an organic solvent and detergent–tolerant novel protease produced by *Bacillus* sp. RKY3. J Chem Technol Biotechnol. 2008;83:1526–1533.
55. Sana B, Ghosh D, Saha M, Mukherjee J. Purification and characterization of a salt, solvent, detergent and bleach tolerant protease from a new gamma–*Proteobacterium* isolated from the marine environment of the Sundarbans. Process Biochem. 2006;41:208–215.
56. Frikha B, Kamoun A, Fakhfakh N, Haddar A, Manni L, Nasri M. Production and purification of a calcium–dependent protease from *Bacillus cereus* BG1. J Ind Microbiol Biotechnol. 2005;32:186–194.
57. Ogino H, Ishikawa H. Enzymes which are stable in the presence of organic solvents. J Biosci Bioeng. 2001;91:109–116.
58. Geok LP, Razak CNA, Abd Rahman RNZ, Basri M, Salleh AB. Isolation and screening of an extracellular organic solvent–tolerant protease producer. J Biochem Eng. 2003;13:73–77.
59. Abd–Rahman RNZR, Geok LP, Basri M, Salleh AB. An organic solvent tolerant protease from *Pseudomonas aeruginosa* strain K nutritional factors affecting protease production. Enz Microb Technol. 2005;36:749–757.

60. Masui A, Fujiwara N, Takagi M, Imanaka T. Feasibility study for decomposition of gelatin layers on X–ray films by thermostable alkaline protease from alkaliphilic *Bacillus* sp. Biotechnol Lett. 1999;13:813–815.
61. Rai SK, Mukherjee AK. Statistical optimization of production, purification and industrial application of a laundry detergent and organic solvent–stable subtilisin–like serine protease (*Alzwiprase*) from Bacillus subtilis DM–04. Biochem Eng J. 2010;48: 173–180.
62. Venugopal M, Saramma AV. Characterization of alkaline protease from *Vibrio fluvialis* strain VM 10 isolated from mangrove sediment sample and its application as a laundry detergent additive. Process Biochem. 2006;41:1239–1243.
63. Bhosale SH, Rao MB, Deshpande VV, Srinivasan MC. Thermostability of high–activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20). Enz Microb Technol. 1995;17:136–139.
64. Singh J, Batra N, Sobti RC. Serine alkaline protease from a newly isolated *Bacillus* sp. SSR1. Process Biochem. 2001;36:781–785.
65. Ingale SS, Rele MV, Srinivasan MC. Alkaline protease production by *Basidiolobus* (NCL 97.1.1), Effect of 'darmform' morphogenesis and cultural conditions on enzyme production and preliminary enzyme characterization. World J Microbiol Biotechnol. 2002;8:403–408.
66. MacAlpine R, Payne CG. Hydrolyzed feather protein as a source of amino acids for broilers. Br Poult Sci. 1977;18:265–273.
67. Krilova V, Popov V. A method for production of protein hydrolysate from a keratin source. SU Patent. 1983;1:161–164.
68. Mariana C, Contiero J, De Lima CJB, Lovaglio RB. Characterization of a feather degrading by *Bacillus amyloliquefaciens* Protease: World J Agric Sci. 2008;4:648–656.

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