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The Potential Industrial Application of *Bacillus Amyloliquefaciens* EGY3 Keratinolytic Protease: Optimization, Characterization and Application as Eco-Friendly Sustainable Alternative in Leather and Detergent Industry

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Abstract

Keratinolytic proteases are proteolytic enzymes specifically catalyse keratin hydrolysis that have been seen as efficient eco-friendly bio-catalysts for various industrial processes. In this study, an isolated keratinolytic bacterial strain namely *Bacillus amyloliquefaciens* EGY3 (Genbank accession number PP038117) was used for the enzyme production under submerged fermentation of feather in which the addition of corn steep liquor possessed a positive impact on the enzyme productivity. Furtherly, the fermentation conditions was statistically optimized and the optimized enzyme activity (391.5 ± 3.50 U/ml) was increased by 5.5-fold. Moreover, the partially purified enzyme was estimated to be alkalophilic (optimum activity at pH 9), thermophilic (optimum activity at 70°C) and surfactant stable enzyme. By examining the generated enzyme's suitability for dehairing bovine hide, complete dehairing was achieved after 2 h with the production of smooth clean surface and without the estimation of any negative impact on the skin structure manifested by scanning electron microscopy. Finally, the enzyme's possible application in getting rid of a stain made of proteins (chocolate-flavored milk stain) from cotton fabrics was examined in which the enzyme addition to a thermally-inactivated commercial detergent restored the whiteness index of the treated fabric by 86.28 % in compare to 65.26% for the use of the commercial detergent.

Keywords: *Bacillus amyloliquefaciens*, feather fermentation, keratinolytic protease, leather industry, detergent-formulation.

1. Introduction

The worldwide concern for a sustainable future, coupled with the significant increase in the biotechnology engineering, has increased the use of green products in various industrial processes. Nowadays, green catalysts possess substantial signs of progress as safe alternatives for conventional chemicals in various industries [1, 2]. In general, bio-catalysts catalyse various biological reactions in all domains of life but for their industrial production, microbial sources are advantageous as they provide a number of benefits such as quick processing, low energy needs, affordability, non-toxicity and eco-friendly [3]. Keratinolytic proteases are protein-hydrolyzing enzymes specifically catalyse the hydrolysis of keratin, the fibrous structural protein present in hair, nails, hoofs, horns, wool, feather and the epithelial cells of the skin outermost layers, that resists most common proteolytic enzymes. These enzymes have been produced by a diversity of microorganisms including bacteria [4], fungi [5] and actinobacteria [6] that have been isolated from various ecosystems. Moreover, these enzymes have been reported as efficient bio-catalysts for the biological conversion of keratinous wastes into products with additional value [7] in addition to their potential application in leather [8], detergent [9] and wool industry [10]. The need to produce keratinolytic proteases economically was driven by the increasing industrial applications of these enzymes. The isolation of highly productive microorganisms as well as the use of keratinous wastes in their production attracted the research focus as efficient steps participate in their economic-production approaches. In addition, the improvement of the fermentation process in order to increase the yield has become a fundamental step with a positive impact on cost reduction [4, 9, 11, 12, 13, 14, 15, 16]. Among different leather processing steps, the pre-tanning processes releases the majority of pollution load, particularly during dehairing and liming. Sodium sulfide and lime are the conventional applied chemicals during the dehairing process. These chemicals are extremely toxic and hazardous to the human and the environment. Dehairing and liming effluents are rich in

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2. organic compounds including proteins and fats in addition to inorganic salts. The direct disposal of these effluents in water bodies negatively affects the aquatic ecosystems and disrupts their balance. In addition, there is a possibility for the remaining unused fraction of sodium sulfide in these effluents to react with acids released from other processes leading to the formation of hydrogen sulfide gas. The inhalation of this gas (> 1 ppm) can cause human death [8]. The negative environmental and health impacts of leather industry have come under scrutiny and criticism, and there is a growing need for more eco-friendly and sustainable practices. Therefore, the application of keratinolytic proteases in the dehairing process has attracted the global interest with a great scope on highly specific efficient enzymes that achieved the dehairing process without a negative effect on the collagen fibers of the skin. Any degree of collagen damage as a result of enzymatic activity would result in finished leather with undesirable physical attributes, lowering its quality [4, 17]. Nowadays, proteases in general are seen as essential ingredients in all of detergent-formulations (laundry, dishwashing and industrial cleaning) as they can improve their cleaning efficiency in the removing of protein-based stains. Enzymes that are compatible with detergents need to be both stable and active across a broad temperature range, especially in alkaline environments. They also need to continue to be active when added to additional detergent ingredients. Therefore, there is a great interest on proteases with high stability in detergent-formulations [9, 18, 19]. In this study, a keratinolytic bacteria isolated from leather industry by-products was identified and examined for the production of keratinolytic protease under submerged fermentation of feather. The fermentation conditions were statistically optimized followed by partial purification of the produced enzyme. In addition, the enzyme that was produced was characterized in which its optimum activity conditions was determined and its Stability when metal ions are present, reducing agent and surfactants was evaluated. In the end, the generated enzyme's suitability for leather dehairing as well as the formulation of detergents was examined.

2. Materials and methods

2.1. Microorganism and culture conditions

The strain under examination was previously identified from leather-byproducts at the department of chemistry of natural and microbial products, National research centre, Dokki, Giza, Egypt. It was chosen based on the preliminary keratinolytic analysis using keratinous-byproduct (feather) as described by Emran et al., [20]. It underwent identification by matrix-assisted laser desorption ionization-time-of-flight mass spectroscopy (MALDI-TOF MS) performed in Children's cancer hospital (57357), Cairo, Egypt and 16s rDNA sequencing. After that, the obtained sequencing data was uploaded to the GenBank database and received an accession number. Moreover, the phylogenetic tree was constructed using the software MEGA 11 [21].

For the production of the enzyme, submerged fermentation of 0.5 g/flask native feather was carried out in 50 mL production medium composed of (g/L): K_2HPO_4 (1.4), KH_2PO_4 (0.7), $MgSO_4.7H_2O$ (0.1), NaCl (0.5) and adjusted at pH 7.0. The media was inoculated with 3 ml pre-incubated bacterial suspension grown on (g/L): glucose (10.0), yeast extract (3.0), peptone (10.0), and $CaCl_2.2H_2O$ (2.0) that adjusted by using 0.1 N NaOH and 0.1 N HCl to pH 7.0 [20].

The fermentation conditions were preliminary adjusted via the evaluation of the capacity of the examined strain to produce keratin-hydrolyzing enzyme over a range of time periods (1-5 days). Additionally, the adjustment of the starting pH at different values (4, 5, 6, 7, 8, and 10) as well as the use of different feather concentrations (0.1-3 g/flask) and different additives (Molasses (M), corn steep liquor (CSL), wheat bran (WB), whey protein concentrate (Wh)) at a concentration of 1 % were examined. Furthermore, different concentrations (0.1, 0.5, 1, 2, 3, 5 %) of the additive that positively influence the productivity was examined.

2.2. Enzyme assay and protein content determination

Initially, soluble keratin was prepared from feather as stated by Wawrzkiewicz et al., [22]. Briefly, ten grams of white feather were cooked in a reflux condenser for two hours at 100 degrees Celsius using 500 milliliters of dimethyl sulfoxide followed by the addition of 1.0 L of cold acetone and maintained under cooling conditions for two hours at -70 ºC. The mixture was centrifuged for 15 minutes at 5000 rpm and the resultant precipitate was dried at 40 ºC in a hot-air oven after being twice washed with distilled water followed by grinding. After that, twenty milliliters of 0.05 M NaOH were used to dissolve one gram of the dried precipitate followed by adjusting the pH to 8 using 0.1 M HCl, and finally, 200 milliliters of 0.1 M Tris/HCl buffer (pH 8) were added.

The keratin-hydrolyzing activity was estimated on the base of Cai et al., [23] instructions using the prepared soluble keratin substrate in which the amount of protein in the translucent supernatant after centrifugation was estimated according to Lowry et al., [24]. Under the reaction conditions, each keratinolytic activity was quantified as a rise in the absorbance by 0.01/min relative to the blank.

2.3. Optimization of enzyme productivity

Statistical optimization was carried out to enhance the enzyme production in which two-step optimization approach was conducted. The highest influencing variables were identified by Plackett-Burman (PB) design followed by the optimization of these variables by central composite design (CCD).

2.3.1. Plackett-Burman design

Seven parameters were examined by applying PB design in eight trials at two different levels, high concentration (+1) and low concentration (-1) [25]. These factors included feather concentration, CSL concentration, pH, incubation period, inoculum size, K_2HPO_4 concentration, and KH_2PO_4 concentration. After estimating the keratin-hydrolyzing activity of each experimental run, equation (1) was used to determine the effect of each variable ($E_{(Xi)}$).

$$E_{(Xi)}=2(\Sigma Mi+-\Sigma Mi-)/N \quad (1)$$

where Mi− and Mi+ were the keratin-hydrolyzing activity in trials where the variable (Xi) was present at high and low concentrations, respectively and N was the number of attempts.

2.3.2. Central Composite Design

Using CCD, the variables that had the biggest effects on the productivity of the enzymes were chosen and optimized at five different levels (−1, +1, 0, −2, and +2) in a set of 30 experimental trials [26]. By utilizing Design Expert 11, after computing and analyzing the second-order polynomial coefficients, the predicted keratin-hydrolyzing activity was determined using equation (2) in which all interaction terms were included.

$$Y_{activity} = B_0 + \Sigma B_i X_i + \Sigma B_{ij} X_i X_j + \Sigma B_{ii} X_i^2 \quad (2)$$

where: $Y_{activity}$, predicted keratin-hydrolyzing activity (U/ml); B_0, intercept; X_i and X_j were the independent variables; B_i, linear coefficients; B_{ii}, squared coefficients; and B_{ij}, interaction coefficients.

2.4. Enzyme partial purification and biochemical assessment

2.4.1. Partial purification process

The crude enzyme was partially purified by fractional precipitation with ethanol and acetone in addition to salting out by ammonium sulfate at intervals of 10 % with concentration varying between 30 and 90 %. Following fractionation, each fraction was subjected to protein and enzyme activity determination as described in section 2.2. After that, the SDS-PAGE method was employed to evaluate the molecular weight and purity of the fraction with the highest specific activity. Initially, a 1:1 ratio of the sample under investigation with a solution composed of 4 % SDS, 20 % glycerol, 200 mM DTT, 0.01 % bromophenol blue, and 0.1 M Tris HCl (pH 6.8) was prepared. After that, the mixture was heated to 100 °C for five minutes and centrifuged for 10 minutes at 4 °C at 1000 rpm. After cleaning the gel with distilled water, the prepared sample was loaded and Coomassie brilliant blue R-250 staining was employed to view the gel's protein bands [27].

2.4.2. Biochemical assessment

2.4.2.1. Effect of pH and temperature

Initially, the optimum pH for the enzyme activity was estimated by examining the enzyme activity at multiple pH values for 15 minutes within the range of 7.0-10.5; 0.1 M Tris/HCl buffer for pH 7-8.5 and carbonate buffer for pH 9-10.5. Moreover, by measuring the enzyme residual activity every 30 minutes for up to two hours, the long-term stability of the enzyme's activity was determined after a pre-incubation at different pHs in which the enzyme activity without prior incubation was regarded as 100 % activity. Furthermore, by maintaining the reaction mixture for 15 minutes at different temperatures between 40 and 90 °C at the optimal pH in accordance with the conventional measurement procedures, the optimal temperature for the enzyme activity was estimated. On the base of the obtained data, the enzyme activation energy (Ea) was determined using Arrhenius plot according to the following equation:

$$-Ea/R=slope \quad (3)$$

where R was the gas constant (8.3145 $Jmol^{-1}K^{-1}$).

In order to perform a thermal stability investigation, the enzyme residual activity was estimated at the conducted optimal circumstances at different pre-incubation periods up to 2 hours following its pre-incubation without substrate-addition at temperatures ranged from 50 to 70 °C in which the enzyme activity without pre-incubation was considered to be 100 %. On the base of the obtained data, the enzyme's half-lives and its decimal reduction time (*D*-value) were determined as follow:

$$\ln(2)/K_d= T_{1/2} \quad (4)$$

$$\ln(10)/K_d=D\text{-value} \quad (5)$$

Where K_d was the thermal deactivation rate constant.

2.4.2.2. Effect of chemical agents on the enzyme activity

The effect of the presence of different chemical agents including NaCl, $HgCl_2$, $MgSO_4.7H_2O$, $CaCl_2$, KCl, EDTA sodium salt and SDS at 5mM final concentration and Tween 80, Propanol, DMSO, 2-Mercaptoethanol and Triton x-100 at 1 % (v/v) on the enzyme keratinolytic activity was evaluated [28]. The keratin-hydrolyzing activity was determined after individual combining the enzyme with each chemical additive and standing at room temperature for half an hour. Remaining activities were measured at 70 °C and pH 9.0 in which the control was analyzed under the same reaction conditions but without the addition of any chemical.

2.4.2.3. Substrate specificity

The catalytic activity of the selected fraction in the hydrolysis of other protein substrates including casein, collagen and gelatin, was examined according to the assay mentioned in Section 2.2.

2.5. Homology modeling and molecular docking

SWISS-MODEL, available at https://swissmodel.expasy.org/interactive, was used to create the 3D structure. Ramachandran was created, and the resulting model was evaluated using the general stereochemical parameters by PROCHECK, Verfiy3D, and ERRAT of the SAVES server https://saves.mbi.ucla.edu/. Utilizing ProsA-web (https://prosa.services.came.sbg.ac.at/prosa.php), the overall model quality was measured.

The three-dimensional structure of the enzyme and the substrate was created using the PyMOL program. The keratin substrate was retrieved from UniProt (protein ID: O13152) and the casein substrate was obtained from the PubChem database (CID: 2882155). The generated model was made to use the YASARA Server (http://www.yasara.org/minimizationserver.htm) for energy minimization. The molecular docking and interactions between the produced enzyme fraction and their substrates (keratin and casein) were done by byhttp://cao.labshare.cn/cb-dock/ and gramm web https://gramm.compbio.ku.edu/ [29]. Analysis of the protein-protein interaction was carried out via the Discovery Studio 4.5 and Pymol programs.

2.6. Dehairing application

2.6.1. Biotreatment process

It was investigated if the examined enzyme fraction could be used in the dehairing of bovine hide in which each sample measuring 5*3 cm^2 were placed in a 250 ml flask with 1 ml (50U of keratinolytic activity) of the enzyme fraction and 49 ml of carbonate buffer (0.1mM) of pH 9.0 and incubated at 37°C and 150 rpm. In order to elucidate the suitable incubation period for complete dehairing, the hide was mechanically examined via the gentle finger scraping at different periodic intervals. Moreover, the impact of different enzyme activities was evaluated.

2.6.2. Na_2S/Lime conventional process

After 15 minutes of immersion in 10 % NaCl, the hide was twice cleaned using wetting agent (Egyptol PLM). After that, the hide was dehaired using saturated solutions of lime and Na_2S then the dehaired hides underwent deliming using NH_4Cl followed by three-times of water washing.

2.6.3. Scanning electron microscope analysis

A number of SEM (High resolution field emission gun, Quanta 250, HRFEG, Czech) images were captured to the dehaired air-dried samples to investigate the effect of the examined enzyme fraction in compare to the applied chemicals on the morphology of the hide surface and cross section views.

2.6.4. Examination of pollution load parameters

Throught the estimation of the effluent content in terms of total dissolved solids (TDS), chemical oxygen demand (COD), and bio-chemical oxygen demand (BOD), the pollution load of the examined treatment process was evaluated. Every analysis was completed in compliance with the Standard Procedures for Water and Wastewater Examination [30].

2.7. Detergent application

2.7.1. The enzyme stability and compatibility with a commercial detergent

The aim of this investigation was to evaluate the stability as well as the suitability of the use of the examined enzyme in the production of a liquid laundry detergent. Initially, the commercial detergent (Persil gel, Henkel, Egypt) was heat-inactivated for an hour at 100 °C to render any endogenous enzymes inactive. After that,

pre-incubating the enzyme preparation at 40 °C with the heat-inactivated detergent for an hour followed by determining the residual activity. As a reference, a control sample (100 % activity) that was incubated under the same circumstances and without any detergent was employed [31].

2.7.2. Washing Performance

To assess the generated enzyme fraction's performance in the detergent industry, chocolate-flavored milk stained 3.5 cm^2 cotton pieces were put through a washing treatment simulation [31]. Two pieces of the stained fabrics were placed in different treatment solutions in a final capacity volume of 50 ml as follow:

1. Tape water
2. Tape water and 1 % final concentration of commercial detergent
3. Tape water and 1 % thermal-inactivated Persil gel
4. Tape water and the produced enzyme fraction (50 U/mL)
5. Tape water, 1 % thermal-inactivated Persil gel and the produced enzyme fraction (50 U/mL)

for one hour, each flask was shaken (200 rpm) at 40 °C then the fabrics were taken out, given a water rinse, and allowed to dry.

The effectiveness of the produced enzyme fraction in removing the stain was initially verified visually. After that, the whiteness level of the cotton fabrics was determined using a spectrophotometer with pulsed xenon lamp as the light source (Ultra-Scan Pro, Hunter Lab, USA).

2.8. Statistical analysis

The data of triplicate runs of each experiment with duplicate measurements for each replicate was presented as the average ± standard deviation. Furthermore, central composite design was assessed using Design Expert 11 and regression was conducted through Excel data analysis.

3. Result

3.1. Microorganism

Of all the isolated bacterial strains, the examined strain (Isolate Ⅱ) possessed the highest efficiency in the hydrolysis of feather (a keratin-based biomass). Moreover, it was the one with the highest keratin-hydrolyzing activity (data not shown). The isolate was preliminary identified on the base of its cultural, morphological and MALDI-TOF MS analysis as *Bacillus* candidate. After that, 16S rDNA sequencing manifested this result and the BLAST was used to compare the obtained sequence with that present in the GenBank database. The results indicated significant degrees of similarities with the other species of that genus with identity percentage of 95.18 % with *Bacillus amyloliquefaciens* DSM 7 (Fig. 1). The obtained sequence had been uploaded to the GenBank database with the name *Bacillus amyloliquefaciens* EGY3 and obtained accession number PP038117.

3.2. Enzyme production

The isolated *Bacillus amyloliquefaciens* achieved the highest enzyme production (30.00 ± 1.584 U/ml) after 4 days fermentation of 0.5 g/flask feather that increased to 47.00 ± 2.045 U/ml by the use of 2 g/flask. In addition, the suitable pH for the growth of the examined strain and production of the enzyme was examined. The results indicated that the enzyme optimal productivity was estimated at slight acidic to neutral pH. Moreover, the addition of CSL at concentration of 0.1% as an additional nutritional source led to a slight increase in the productivity that increased to 71.7 ± 2.193 U/ml by increasing the concentration to 1 % (Table 1).

3.3. Optimization of the enzyme production

3.3.1. Plackett-Burman design

We assessed the relationship relevance of seven distinct variables using PB design in which the trial run was represented by each row, and the levels of the examined independent variable was represented by each column (refer to Table 2). The average keratin-hydrolyzing activity for each trial was determined and presented in Table 2 in which The results showed that the enzyme activity varied widely, varying from 5 ± 0.58 to 111.2 ± 1.69 U/mL. ANOVA analysis indicated that the significance of the F-value was $8.35E^{-14}$ and the regression indicated that the R square (coefficient of determination) was 0.999756. Moreover, the regression demonstrated the significance of each variable under study and the coefficient analysis indicated that the variables; feather concentration, pH, incubation period, and K_2HPO_4 concentration had positive impacts on the keratin-hydrolyzing activity while CSL concentration, inoculum size, and KH_2PO_4 concentration had negative impacts (Table 3). In fig. 2, we computed and visually depicted the primary influences of the examined variables on the enzyme

activity. The variables with the highest level of confidence (CSL concentration, inoculum size, K_2HPO_4 concentration and KH_2PO_4 concentration) were selected for further optimization.

3.3.2. Central composite design

By utilizing CCD, four variables (CSL concentration, inoculum size, K_2HPO_4 concentration and KH_2PO_4 concentration) were optimized in which Table 4 displayed the design matrix, associated findings from the experimental trials, mean predicted values, and the residual values. The experiment involved a 30-trial matrix for the four variables examined at five levels (-2, -1, 0, +1 and + 2) with six central points. The quadratic regression model's analysis of variance revealed its high significance with 27.43 F-value and 0.01 % chance that such a large F-value could occur due to noise (Table 5). Moreover, the adjusted determination coefficient "Adj R-Squared" of 0.9283 yielded a predictive R Squared value of 0.7863, that considered very high according to the F-test (Table 5, 6). In addition, the R-squared value that indicated the proportion of the observed response values' variability that can be explained by the experimental factors and their interactions was 0.9629. The *P*-value less than 0.0500 indicated the significance of the model term, therefore, B, AB, AD, CD, A^2, B^2, C^2 were estimated as the significant model terms in the current applied design.

The second-order polynomial equation obtained by the multiple regression analysis of the observed results was as follow:

$$Y_{Activity} = 111.30 + 50.30X_1 - 23.56X_2 - 24.45X_3 + 29.29X_4 - 49.64X_1X_2 - 10.53X_1X_3 - 0.7371X_1X_4 + 6.72X_2X_3 + 26.64X_2X_4 - 41.21X_3X_4 + 15.83X_1^2 - 4.99X_2^2 + 59.90X_3^2 + 48.58X_4^2 \quad (6)$$

where the response ($Y_{Activity}$) was the predicted keratin-hydrolyzing activity in which X_1, X_2, X_3 and X_4 were the codes of the examined variables; CSL concentration, inoculum size, K_2HPO_4 concentration and KH_2PO_4 concentration, respectively.

The results showed that 391.5 ± 3.50 U/ml of the enzyme activity could be produced when 0.5 % of CSL, 2 % inoculum size, K_2HPO_4 concentration of 1.4 g/L and KH_2PO_4 concentration of 0.6 g/L was applied. This result was equal to the estimated predicted value. Moreover, the actual and the predicted values were very close. The response surface plots (3D and the contour plots) that utilized to study the interactive effects of the examined variables on the enzyme productivity was shown in Fig. 3.

3.4. The enzyme partial purification and biochemical assessment

3.4.1. Partial purification process

By examining the precipitation of the crude enzyme with ethanol and acetone in addition to ammonium sulfate-salting out. The fraction precipitated at ammonium sulfate saturation levels of 40-50 % displayed the highest keratin-hydrolyzing activity with 42.06 % total recovered activity. Additionally, this fraction exhibited a substantial increase in the specific activity (40.63 U/mg protein) with 8.63-fold of increase. The SDS-PAGE for the 40–50 % fraction demonstrated the appearance of two bands; one approximately around 25 kDa molecular weight and the other was slightly higher (Fig. 4). As a result, this particular partially purified fraction was chosen for further investigation in the subsequent phases of the study.

Fig. 5A depicted the impact of pH on the enzyme activity in which the optimum activity was observed at pH 9.0. However, the activity of the enzyme at pH 10.5 decreased by approximately 25.9%. Moreover, the enzyme's stability at different pHs was evaluated and the results presented in figure (5B) indicated that the enzyme was more stable in alkaline conditions (pH 8-10) retaining 100 % of its activity up to 2 h.

Fig. 5C illustrated the temperature dependence of the enzyme activity at pH 9 and temperature between 40°C and 90°C. Based on our results, it was estimated that the optimal temperature for the enzyme activity was 70°C. Moreover, the energy needed for the activation of the enzyme activity was calculated using Arrhenius plot.

A linear relationship, depicting an enzyme's first-order kinetic reaction, was seen by charting the natural logarithm of its residual activity versus the reciprocal of the temperature (Fig. 5D) and consequently the calculated Ea was 33.70 $kJmol^{-1}$.

Thermal stability study indicated that the enzyme retained 100 % of its activity for more than 120 min at temperatures up to 45 °C while at 50, 55, 60, 65 and 70 °C, it retained 86.78, 70.38, 65.27, 60.38 and 58.99 %, respectively after pre-incubation for the same period (Fig. 5E). Based on the obtained data, the natural logarithmic value of the residual activity was plotted versus the pre-incubation period as shown in Fig. 5F.

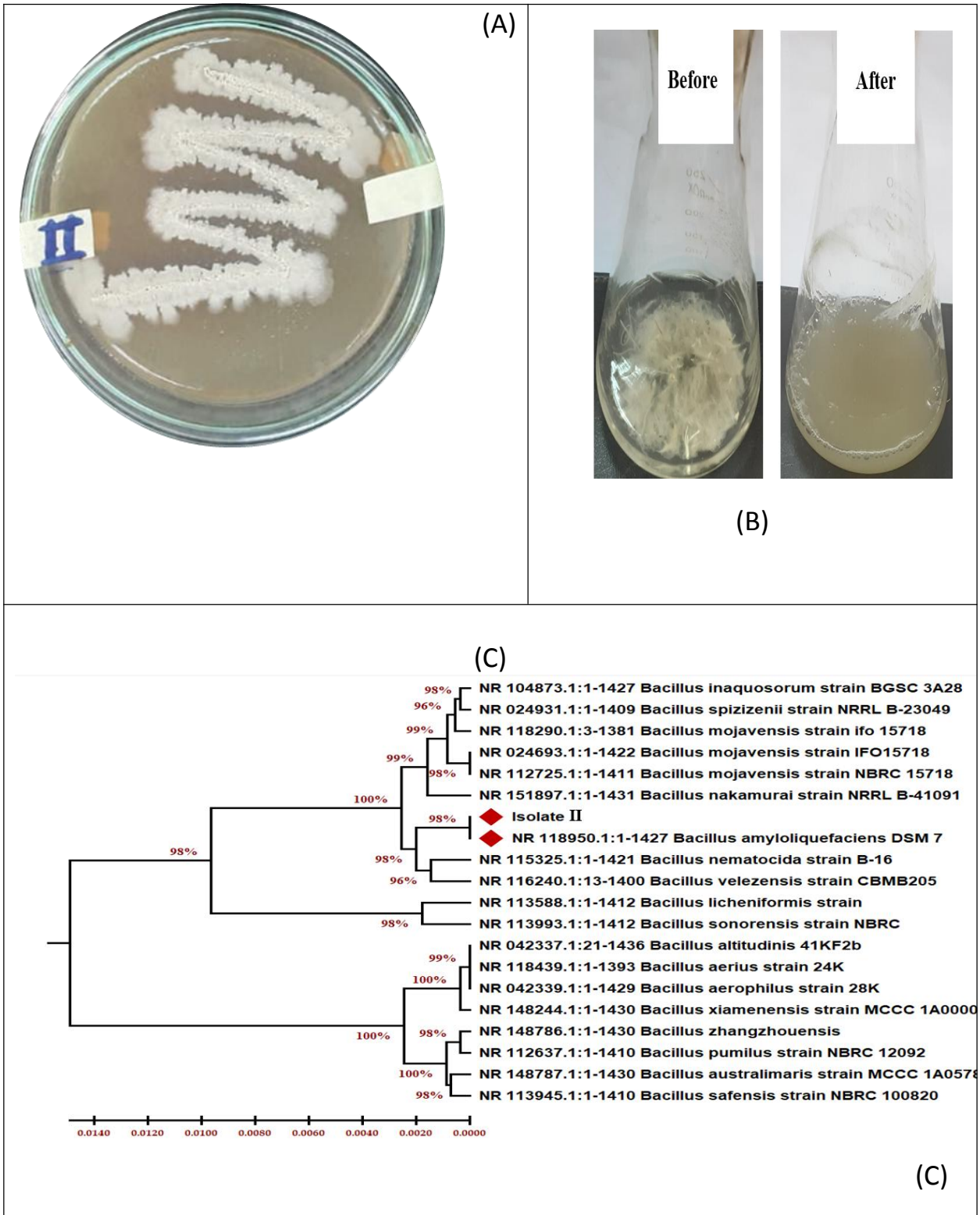


Figure (1): The cultural feature of the isolated strain on tryptic soy agar medium (A), feather before and after fermentation (B) in addition to the Phylogenetic tree based on partial 16S rDNA sequences, showing the relationship between *Bacillus amyloliquefaciens* and other species belong to the genus *Bacillus* (C). The tree was constructed using the MEGA 11 and neighbor-joining method.

Table (1): Effect of some cultural and nutritional parameters on *Bacillus amyloliquefaciens* keratinolytic protease production

Incubation period (days)	**1**	**2***	**3**	**4**	**5**
Enzyme activity (U/mL)	16.00 ± 1.067	16.6 ± 1.262	16.57 ± 1.256	30.00 ± 1.584	20.5 ± 0.582
Feather concentration (g/flask)	**0.1**	**0.5***	**1**	**2**	**3**
Enzyme activity (U/mL)	13.9 ± 0.673	30.00 ± 1.082	37.53 ± 1.56	47.00 ± 2.045	45.00 ± 2.036
pH	**4**	**5**	**6**	**7***	**8**
Enzyme activity (U/mL)	43.2 ± 2.153	47.00 ± 2.129	50.00 ± 2.354	47.00 ± 1.267	37.6 ± 1.075
Additive source at concentration of 0.1 %	**Feather***	**Corn steep liquor**	**Wheat bran**	**Whey protein concentrate**	**Molasses**
Enzyme activity (U/mL)	50.00 ± 2.821	50.75 ± 2.345	43.5 ± 2.436	37.00 ± 1.154	44 ± 1.421
CSL concentration (%)	**0.1***	**0.5**	**1**	**2**	**3**
Enzyme activity (U/mL)	50.75 ± 2.045	60.00 ± 2.513	71.7 ± 2.193	61.8 ± 2.283	53.75 ± 2.432

*control

Table (2): Coded levels and real values for Plackett- Burman design

Trial	**Feather concentration (g/flask)**	**Corn steep liquor concentration (%)**	**pH**	**Incubation period (Days)**	**Inoculum size (mL/flask)**	**K_2HPO_4 concentration (g/L)**	**KH_2PO_4 concentration (g/L)**	**Enzyme activity (U/mL)**
1	1(-)	0.5(-)	5(-)	6(+)	4(+)	1.8(+)	0.6(-)	99.2 ± 2.41
2	3(+)	0.5(-)	5(-)	2(-)	2(-)	1.8(+)	0.8(+)	111.2 ±1.69
3	1(-)	1.5(+)	5(-)	2(-)	4(+)	1(-)	0.8(+)	5 ± 0.58
4	3(+)	1.5(+)	5(-)	6(+)	2(-)	1(-)	0.6(-)	90.1 ± 2.13
5	1(-)	0.5(-)	7(+)	6(+)	2(-)	1(-)	0.8(+)	101.3 ±1.69
6	3(+)	0.5(-)	7(+)	2(-)	4(+)	1(-)	0.6(-)	90.4 ± 2.40
7	1(-)	1.5(+)	7(+)	2(-)	2(-)	1.8(+)	0.6(-)	101.7 ± 2.39
8	3(+)	1.5(+)	7(+)	6(+)	4(+)	1.8(+)	0.8(+)	67.9 ± 2.42

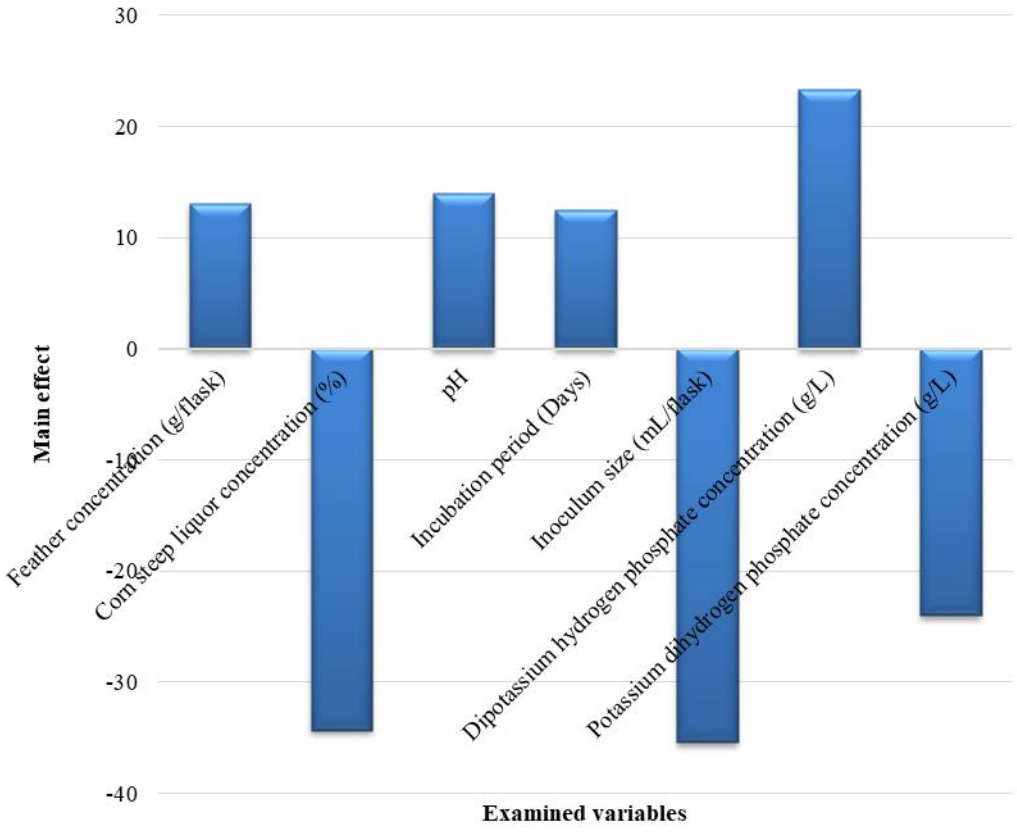


Figure (2): Main effects of the independent variables on the keratin-hydrolyzing activity according to the results of Plackett-Burman design.

Table (3): Multiple regression analysis results

	Coefficients	*Standard error*	*t Stat*	*P-value*
Intercept	575.15	7.683017	74.85992	1.13E-12
Feather concentration (g/flask)	27.45	0.703562	39.01573	2.05E-10
Corn steep liquor concentration (%)	-135.8	1.407125	-96.5089	1.48E-13
pH	28.35	0.703562	40.29494	1.58E-10
Incubation period (Days)	12.55	0.351781	35.67559	4.17E-10
Inoculum Size (mL/flask)	-70.85	0.703562	-100.702	1.06E-13
K_2HPO_4 concentration (g/L)	116.25	1.758906	66.09222	3.06E-12
KH_2PO_4 concentration (g/L)	-472	7.035624	-67.0872	2.71E-12
Multiple R	0.999878			
R Square	0.999756			
Adjusted R Square	0.999542			
Standard Error	2.814249			
Significance F	$8.35E^{-14}$			

Consequently, the K_d, $T_{1/2}$ and *D*-values of the enzyme at temperatures ranged from 50 to 70 °C were illustrated in Table 7.

3.4.2.2. Effect of chemical agents

It was investigated how different chemical agents affected the enzyme activity and the results shown in Fig. 6 indicated that DMSO, Propanol, NaEDTA, and $HgCl_2$ significantly inhibited the activity of *B. amylopeliquefaciens* keratinolytic protease while the other examined agents activated the enzyme activity in which magnesium sulfate heptahydrate was the most promising activator that enhanced the activity by about 66%. Furthermore, there was no discernible impact on the enzyme activity when NaCl was present. Moreover, the enzyme exhibited remarkable stability and improved activity in the presence of several surfactants and reducing agents, including Tween 80, 2-Mercaptoethanol, SDS and Triton X-100.

3.4.2.3. Enzyme's substrate specificity

The catalytic activity of the produced fraction in the hydrolysis of other protein substrates including casein, gelatin and collagen was examined at its optimum conditions in compare to that of its keratinolytic activity. The results revealed that the enzyme exhibited moderate caseinolytic activity (145.33 U/ml) in comparison to its keratinolytic activity (391.5U/ml) without the estimation of any gelatin or collagen-hydrolyzing activity.

3.5. Homology modeling and molecular docking

The sequence of *B. amyloliquefaciens* keratinolytic protease was taken from the NCBI database (accession no AKR05134.1) and the Swiss Model tool was used for searching and generating the best template model. The selected template model, proteolytic enzyme of *B. subtilis* (P35835.1), was shown in Fig. 7A. Moreover, the stereochemical parameters of the SAVES server were used to evaluate the created model: ERRAT, VERIFY3D, and PROCHECK. After that the Ramachandran plot of the keratinolytic protease model was created. The low-energy zone, the allowed region, the typically allowed in region, and the prohibited region that compromised the four sections that made up the x- axis of the Ramachandran plot were shown in fig. 7B.

The keratinolytic protease-generated model was analyzed, and the results indicated eighty-four percent of the residues were located in the most preferred regions and 12.9% in the additional authorized regions, 0.9% in the generously allowed regions and 2.2% residues found in the disallowed regions, indicating the good quality of the generated model. Moreover, the overall quality factor and the 3D model's compliance with the amino acid sequence (3D-1D) for the keratinolytic protease model were assessed using ERRAT and VERIFY 3D, the results were 95.54 and 82.46%, respectively. The overall model quality was estimated by Z-Score, -9.92 (Figure 8). The overall outcomes verify the validity of the produced keratinolytic protease model.

The molecular docking interaction between the generated model of keratinolytic protease *B. amyloliquefaciens* Pp038117 and keratin, casein substrates separately were performed. Many positions of protein-protein interactions for keratin and casein were generated in which the 3D and surface interactions were shown in Fig. 9.

3.6. Dehairing application

Currently, the use of the examined enzyme fraction in the dehairing of bovine hide was investigated. The results indicated that the use of 50 U/mL keratinolytic activity dispersed in carbonate buffer (0.1 mM) of pH 9.0 led to the complete removal of the hair after 4 h (Fig. 10). Furthermore, by examining the effect of different enzyme activities, the results estimated that the use of 100 U/mL completely removed the hair after 2 h without the appearing of any negative impact on the skin morphology, manifested by examining the surface as well as the cross-section appearance of the dehaired hide under scanning electron microscope. Moreover, the enzymatically treated hide was smooth and clean on contrast to the Na_2S/Lime conventional treated one that appeared with a rough grain deteriorated surface (Fig. 11).

To assess the environmental impact of the applied enzymatically dehairing process, various pollution control parameters including TDS, BOD and COD were evaluated and compared with that resulted from the conventional applied method. According to the data presented in Table 8, the enzymatically dehairing process resulted in significant reduction in TDS and COD values of the effluent by 5.7 and 51.87%, respectively. Moreover, the BOD/COD ratio for the enzymatically dehairing process was 0.47 that was 3.4-fold higher than that estimated for Na_2S/Lime treatment process.

Table (4): Central composite design

Run	Factor 1 A: Corn steep liquor concentration (%)	Factor 2 B: Inoculum size (mL/flask)	Factor 3 C: K_2HPO_4 concentration (g/L)	Factor 4 D: KH_2PO_4 concentration (g/L)	Observed enzyme activity (U/ml)	Predicted enzyme activity (U/ml)
1	0.3(1-)	1.5(1-)	2(1+)	0.8(1+)	78.74 ± 2.68	95.76
2	0.5(0)	2(0)	1.4(2-)	0.6(0)	391.5 ± 3.50	399.80
3	0.3(1-)	1.5(1-)	2(1+)	0.4(1-)	166.3 ± 2.26	171.42
4	0.7(1+)	2.5(1+)	1.6(1-)	0.8(1+)	345.35 ± 2.58	332.39
5	0.3(1-)	2.5(1+)	2(1+)	0.8(1+)	215 ± 1.62	214.66
6	0.5(0)	2(0)	1.8(0)	1(2+)	364 ± 2.82	364.18
7	0.5(0)	2(0)	1.8(0)	0.6(0)	111.3 ± 2.75	111.30
8	0.7(1+)	1.5(1-)	1.6(1-)	0.8(1+)	390.45 ± 2.51	438.94
9	0.7(1+)	1.5(1-)	2(1+)	0.8(1+)	301.083 ± 1.35	273.11
10	0.1(2-)	2(0)	1.8(0)	0.6(0)	46.88 ± 1.85	74.03
11	0.3(1-)	1.5(1-)	1.6(1-)	0.8(1+)	235.4 ± 2.82	219.48
12	0.7(1+)	1.5(1-)	2(1+)	0.4(1-)	302.5 ± 2.96	351.72
13	0.3(1-)	2.5(1+)	1.6(1-)	0.4(1-)	95.6 ± 1.37	115.73
14	0.7(1+)	2.5(1+)	1.6(1-)	0.4(1-)	135.45 ± 2.89	139.57
15	0.9(2+)	2(0)	1.8(0)	0.6(0)	315.66 ± 1.30	275.22
16	0.7(1+)	2.5(1+)	2(1+)	0.8(1+)	160.718 ± 3.36	193.43
17	0.5(0)	2(0)	1.8(0)	0.6(0)	111.3 ± 2.75	111.30
18	0.3(1-)	2.5	2(1+)	0.4(1-)	211.1 ± 2.30	183.75
19	0.3(1-)	1.5(1-)	1.6(1-)	0.4(1-)	141.86 ± 0.98	130.28
20	0.5(0)	2(0)	1.8(0)	0.6(0)	111.3 ± 2.75	111.30
21	0.7(1+)	1.5(1-)	1.6(1-)	0.4(1-)	360.19 ± 2.07	352.69
22	0.5(0)	2(0)	1.8(0)	0.6(0)	111.3 ± 2.75	111.30
23	0.5(0)	2(0)	1.8(0)	0.6(0)	111.3 ± 2.75	111.30
24	0.5(0)	2(0)	1.8(0)	0.6(0)	111.3 ± 2.75	111.30
25	0.7(1+)	2.5(1+)	2(1+)	0.4(1-)	157.4 ± 1.35	165.48
26	0.5(0)	2(0)	2.2(2+)	0.6(0)	323.58 ± 0.47	301.99
27	0.3(1-)	2.5(1+)	1.6(1-)	0.8(1+)	339.575 ± 3.31	311.50
28	0.5(0)	2(0)	1.8(0)	0.2(2-)	260.5 ± 2.84	247.03
29	0.5(0)	1(2-)	1.8(0)	0.6(0)	160.25 ± 2.12	138.46
30	0.5(0)	3(2+)	1.8(0)	0.6(0)	35.75 ± 1.92	44.24

Table (5): Coefficients in terms of coded factors

Factor	Coefficient	df	Standard error	95% CI Low	95% CI High	VIF
Intercept	111.30	1	12.12	85.47	137.13	
A	50.30	1	6.06	37.38	63.21	1.0000
B	-23.56	1	6.06	-36.47	-10.64	1.0000
C	-24.45	1	6.06	-37.37	-11.54	1.0000
D	29.29	1	6.06	16.37	42.20	1.0000
AB	-49.64	1	7.42	-65.46	-33.83	1.0000
AC	-10.53	1	7.42	-26.34	5.29	1.0000
AD	-0.7371	1	7.42	-16.55	15.08	1.0000
BC	6.72	1	7.42	-9.10	22.54	1.0000
BD	26.64	1	7.42	10.83	42.46	1.0000
CD	-41.21	1	7.42	-57.03	-25.40	1.0000
A^2	15.83	1	5.67	3.75	27.91	1.05
B^2	-4.99	1	5.67	-17.07	7.09	1.05
C^2	59.90	1	5.67	47.82	71.98	1.05
D^2	48.58	1	5.67	36.50	60.66	1.05

A: Corn steep liquor concentration (%), B: Inoculum size (mL/flask), C: K_2HPO_4 concentration (g/L) and D: KH_2PO_4 concentration (g/L).

3.7. Detergent application

Proteases are the main enzymatic component in detergent-formulations. Therefore, the applicability of the generated keratinolytic protease in detergent-formulation was examined.

Table (6): ANOVA for quadratic model

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.430E+05	14	24501.00	27.81	< 0.0001	significant
A	60714.72	1	60714.72	68.92	< 0.0001	
B	13316.58	1	13316.58	15.12	0.0015	
C	14350.68	1	14350.68	16.29	0.0011	
D	20587.12	1	20587.12	23.37	0.0002	
AB	39430.04	1	39430.04	44.76	< 0.0001	
AC	1773.34	1	1773.34	2.01	0.1764	
AD	8.69	1	8.69	0.0099	0.9222	
BC	722.53	1	722.53	0.8202	0.3795	
BD	11356.63	1	11356.63	12.89	0.0027	
CD	27178.33	1	27178.33	30.85	< 0.0001	
A^2	6873.95	1	6873.95	7.80	0.0136	
B^2	682.08	1	682.08	0.7742	0.3928	
C^2	98409.06	1	98409.06	111.71	< 0.0001	
D^2	64720.55	1	64720.55	73.47	< 0.0001	
Residual	13214.56	15	880.97			
Lack of Fit	13214.56	10	1321.46			
Pure Error	0.0000	5	0.0000			
Cor Total	3.562E+05	29				
Std. Dev.	29.68		**R^2**	0.9629		
Mean	206.75		**Adjusted R^2**	0.9283		
C.V. %	14.36		**Predicted R^2**	0.7863		
			Adeq Precision	18.8060		

A: Corn steep liquor concentration (%), B: Inoculum size (mL/flask), C: K_2HPO_4 concentration (g/L) and D: KH_2PO_4 concentration (g/L).

3.7.1. The enzyme stability and compatibility with a commercial detergent

The stability of the examined enzyme in detergent-formulation was evaluated via its addition to a thermally-inactivated Persil gel and incubated for 1h. The results indicated that the produced enzyme retained 98.5 ± 0.19 % of its initial activity after its incubation at 40 °C.

3.7.2. Washing performance

The applicability of the produced enzyme in the removing of chocolate-flavored milk stain was examined. The visual appearance shown in figure (12A) indicated its efficiency in the removal of the stain by its addition to a thermally-inactivated detergent. Moreover, the total protein content of the detergency solution was estimated by Lowry method and the results shown in figure (12B) indicated the increase in the intensity of the produced color by the use of the thermal-inactivated detergent with the addition of the produced enzyme. Finally, the whiteness index of the treated samples was estimated and the results indicated that the whiteness index was restored by 86.28 % by the use of the thermal-inactivated detergent with the addition of the produced enzyme in compare to 65.26 % for the commercial detergent treated fabric (Table 9).

3. Discussion

The growing industrial applications of keratinolytic proteases increased the research focus for the isolation of efficient keratinolytic microorganisms from various sources [7]. In the current study, an isolate furtherly identified as *Bacillus amyloliquefaciens* was isolated from industrial leather-byproducts and it had been estimated as an efficient keratinolytic strain. The genus *Bacillus* had been estimated as being rich in keratinolytic strains in which various species including *B. amyloliquefaciens* [32], *B. licheniforms* [10], *B. cereus* [33], *B. tropicus* [18], *B. subtilis* [11], *B. halotolerans*[4], *B. mycoides*, *B. altitudinis* [34], and *B. pumilus* [35] had been stated.

The productivity of keratin-hydrolyzing enzyme using the isolated *Bacillus amyloliquefaciens* under submerged fermentation of feather was initially quantified and the results indicated the production of keratinolytic activity of 30.00 ± 1.584 U/ml by the fermentation of 1 % feather for 4 days. Feather is a keratinous biomass in which keratin compromises 90 % of its dry weight [36]. The use of feather in the production of industrial keratinolytic enzymes is an attracting approach as it provides combined environmental and circular economy benefits [4,9,11,12, 14, 16, 18].

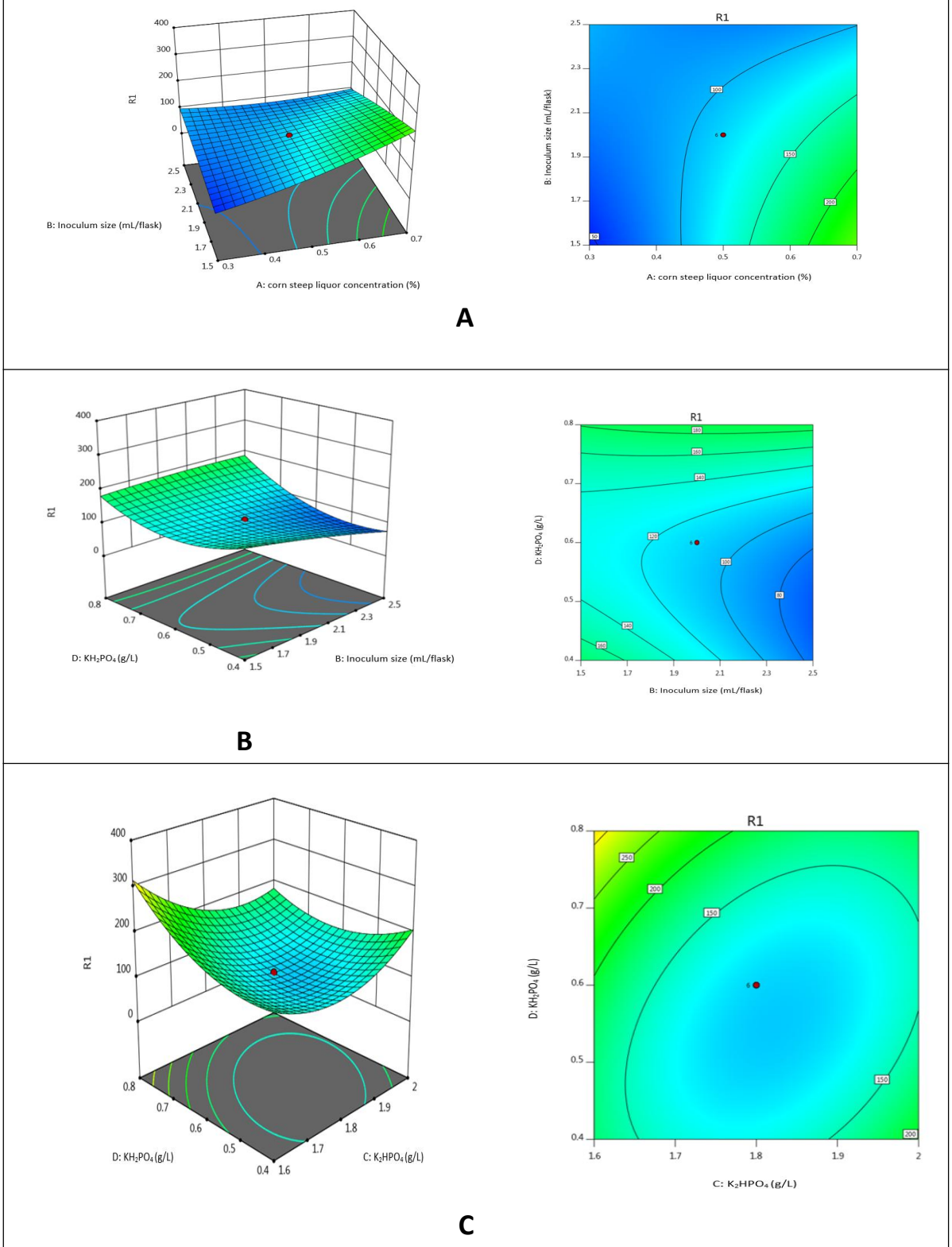


Figure (3): Three-dimensional response surface and contour plots showing the effect of different variables on *B. amyloliquefaciens* keratinolytic protease activity (R1): corn steep liquor concentration & inoculum size (A), Inoculum size & KH_2PO_4 concentration (B), K_2HPO_4 & KH_2PO_4 concentration (C). Green, yellow, and red colors showed low, medium, and high keratin-hydrolyzing activity (U/mL), respectively.

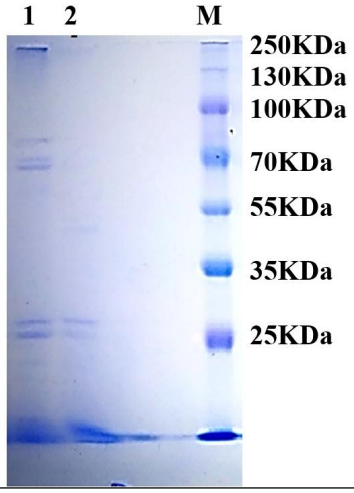


Figure (4): SDS-PAGE analysis of (1) the culture supernatant, (2) 40-50% ammonium sulfate-precipitated fraction in which M was the marker.

The adjustment of the fermentation condition for the maximum production of the enzyme has been turned to be inevitable part for its industrial application since the cost is a major obstacle and the production medium accounts for about 40 % of the enzyme's total production costs [37]. Herein, the impact of feather concentration as well as the impact of additional nutritional compounds had been preliminary evaluated. The findings estimated that the increase in the feather concentration increased the enzyme's production up to the use of 4 % concentration. Furtherly, the addition of CSL at concentration of 1 % increased the enzyme's production to 71.7 ± 2.193 U/mL. The concentration of feather that influence the microbe's growth and consequently the optimum production of the enzyme varied according to the used strain; 0.367 % reported for *B. halotolerans* [4], 1 % reported for *B. cereus* L10 [33], 2 % reported for *B. subtilis* ES5 [9]. Moreover, CLS is a corn wet-milling by-product rich in amino acids, minerals and vitamins that can be used as a feedstock for the growth of varies microorganisms for the producing of products with additional value [38]. It had participated in the formulation of production media for various enzymes; fibrinolytic enzyme [39], lipase [40] and protease [41].

Furtherly, There was statistical optimization done in two-stage strategy for optimization wherein the impact of seven variables on the enzyme productivity was screened by applying PB design and the highest significant variables were optimized by applying CCD. In PB design, the ANOVA analysis indicated the overall significance of the design (significance of the F-value was less than 0.05) and the regression indicated that the R square value was 0.999756, confirming the model accuracy as it was greater than 0.9 [42]. Moreover, the regression demonstrated the importance of each variable under study in which they were adjusted according to their coefficient values in the second optimization step. The variables with the highest level of confidence; CSL concentration, inoculum size, K_2HPO_4 and KH_2PO_4 concentration, were selected and optimization by CCD in which their zero level were the positive value for K_2HPO_4 concentration and the negative value for the other three variables. In CCD, the significance of the F-value was < 0.0001 and the R square value was 0.9629, manifesting its significance and accuracy. The optimized keratinolytic activity (391.5 ± 3.50 U/ml) was accomplished by fermenting 6% feather and 0.5 % of CSL in a salt solution composed of (g/L); K_2HPO_4 (1.4), KH_2PO_4 (0.6), $MgSO_4.7H_2O$ (0.1), NaCl (0.5) and adjusted at pH 7.0 that inoculated by 4 % inoculum and fermented for 6 days, in which the activity was increased by 5.5-fold. The optimized activity was higher than 92.9 U/mL reported for *B. haynesii* [20], 140.83 U/mL reported for *B. halotolerans* [4] and 195.05 U/mL reported for *Bacillus* sp. CN2 [43]. Through the process of salting out ammonium sulphate, an enzyme was partially purified and the fraction precipitated at 40-50 % possessed purification fold of 8.63 with specific activity of 40.63 U/mg protein. Furtherly, the partially purified enzyme was characterized in which it possessed optimum activity and stability in alkaline condition (pH 9). In case of *Bacillus* keratinolytic proteases, alkaline condition was mostly reported as the optimal for their activities [4, 9, 17, 34, 44].

In general, the change in pH alters the process of certain groups of chemicals across protein structures being protonated or deprotonated that significantly affects the durability and the function of enzymes [45], reflecting the importance for the estimation of the appropriate pH for the enzyme activity. In addition, the change in the temperature not only affects the enzyme activity but it affects the pH of the buffer solution as the temperature changes the dissociation constants for weak acids or base in the buffer solution which in turn influences the enzyme activity [34].

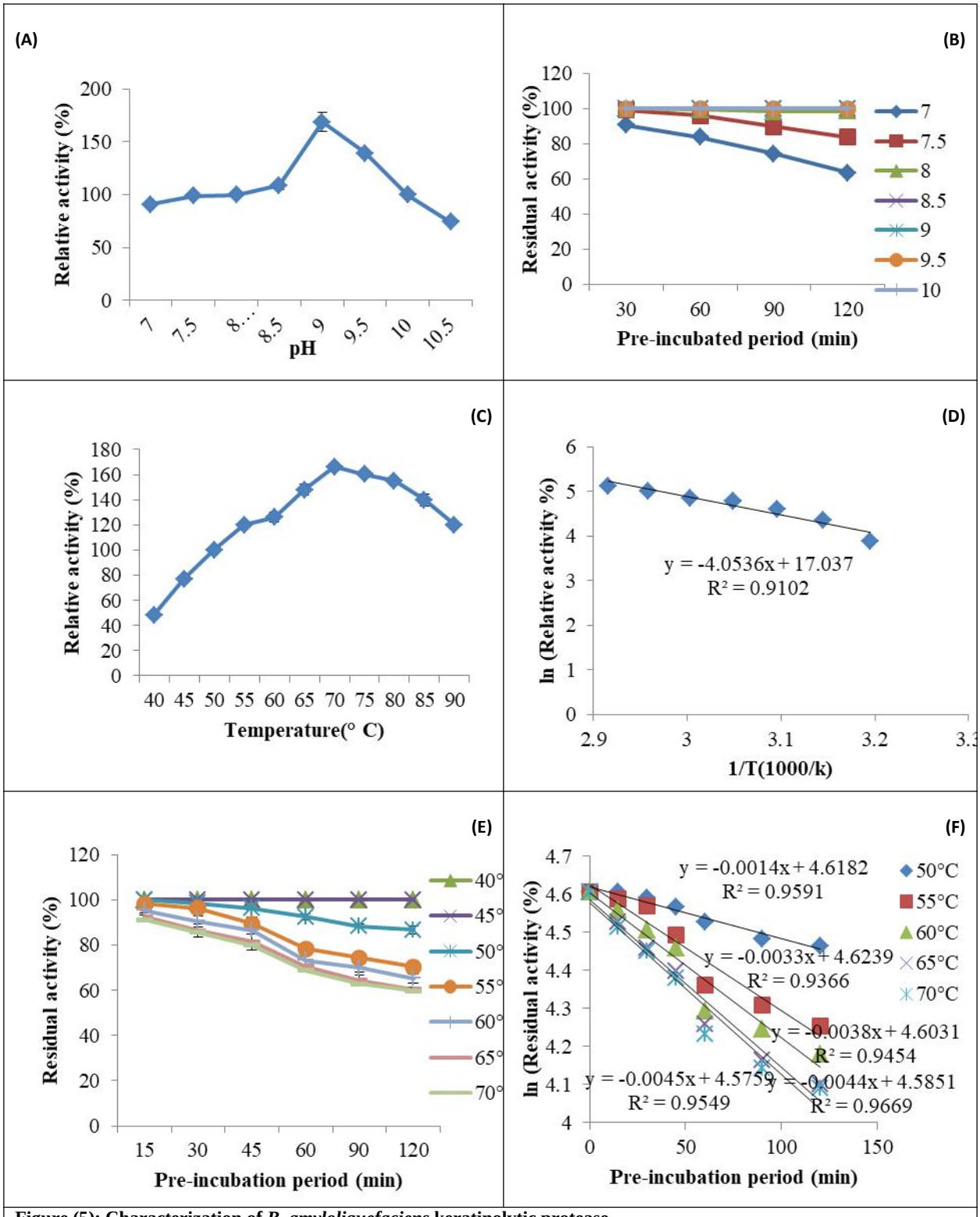


Figure (5): Characterization of *B. amyloliquefaciens* keratinolytic protease

(A)The effect of the reaction mixture's pH on the enzyme activity, (B) pH stability, (C) Effect of temperature of the reaction on the enzyme activity, (D) Arrhenius plot, (E) Temperature-stability profile, (F) First order of thermal deactivation of *B. amyloliquefaciens* keratinolytic protease.

Table (7): Thermo-kinetic parameters of the keratin-hydrolyzing activity

Temperature (°C)	K_d (min^{-1})	$T_{1/2}$ (min)	*D*-value (min)
50	0.0014	495.1051	1644.704
55	0.0033	210.0446	697.7531
60	0.0038	182.4072	605.9434
65	0.0044	157.5335	523.3148
70	0.0045	154.0327	511.6856

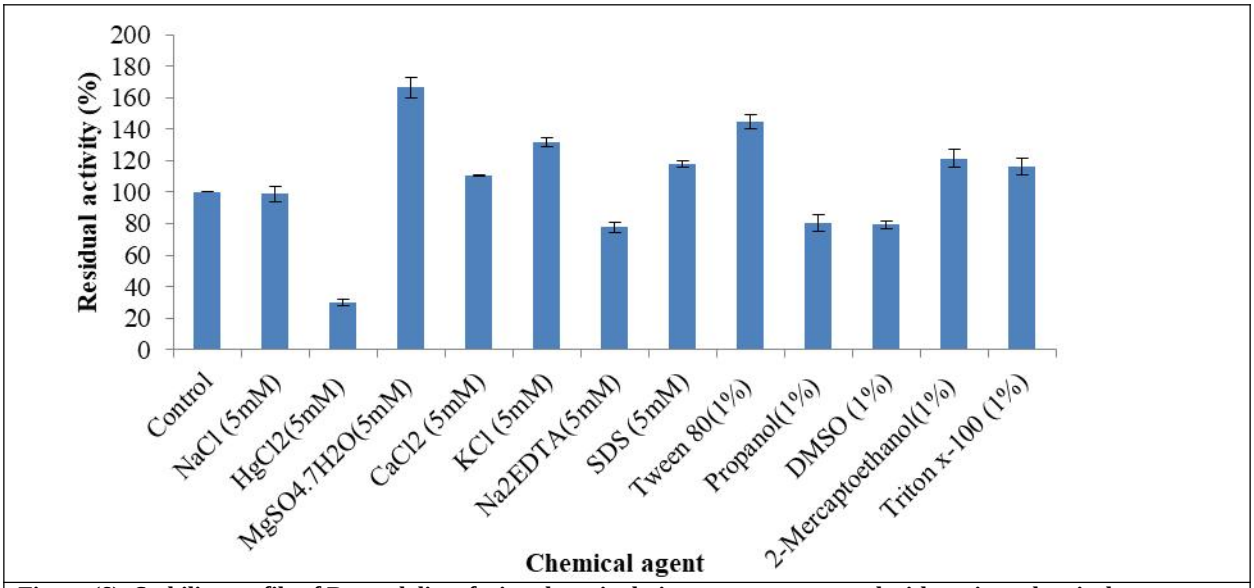


Figure (6): Stability profile of B. amyloliquefaciens keratinolytic protease pretreated with various chemical agents.

Currently, the activity of the enzyme at pH 9 was evaluated at various temperatures in which the optimum temperature for the enzyme activity was 70 °C with $T_{1/2}$ of 154 min at this temperature. Similarly, Ismail et al., [4] and Priyanka et al., [44] reported 70 °C as the optimum temperature for other Bacillus sp. Keratinolytic proteases. This optimum temperature was more than that mentioned for further Bacillus species. keratinolytic proteases; 45 °C for B. subtilis GH2 [11], 50 °C for B. paralicheniformis and 60 °C for B. cereus strain [17].

Moreover, metal ions play an essential role in keeping the enzyme structure intact, prevent collapse and maintaining the structure of the created transition products between the enzyme and its substrate [11]. In the current study, Mg^{+2}, Ca^{+2} and K^{+1} had an improvement effect on the enzyme activity. The positive impact of Mg^{+2} and Ca^{+2} on Bacillus keratinolytic proteases had been previously reported as they can function as ion bridges or salts capable to preserve the binding of the complex's substrate and enzyme that are crucial for protecting the enzyme from autolysis [11, 46, 47]. Contrary, Hg^{+2} had a negative impact on the enzyme activity that was in agreement with previous published results. This negative impact can be attributed to the attachment of the metal ions to catalytic residues in the enzyme active-sites, preventing the enzyme-substrate binding [11, 48]. In addition, EDTA had a negative impact on the enzyme activity. In general, metalloproteases has various catalytic sites in need for one or two divalent ions to functionlize; therefore, EDTA, as a chelating agent, is a typical inhibitor of metalloproteases. On contrast, the effect of reducing agents, that assist disulfide bonds breakage, on keratin-hydrolyzing activity is contradictory [16]. Currently, the enzyme exhibited improved activity in the presence of 2-Mercaptoethanol. Moreover, the stability of the produced enzyme in the presence of surfactants was examined. A positive impact on the enzyme activity was estimated by the presence of Tween 80, SDS and Triton X-100. All of the above properties suggested the generated enzyme's potential applications in the detergent and leather industries.

Among the enzymes that find application in bioprocessing of leather, keratinolytic proteases have essential signs of progress as they offer notable advantages for enhancing the quality of the end product in an eco-friendly manner [8]. In leather industry, the conventional dehairing and liming process is an environmentally deleterious

step with the highest emerging pollution load [49], reflecting the importance for finding sustainable and eco-friendly alternatives. Proteases had been reported as an eco-friendly alternative solution to the conventional dehairing process but their distructive effect to the collagen grain layer due to non-specificity and lack of efficacy in eliminating tiny hairs limited their commercial applicability [17]. In the current study, the produced keratinolytic protease did not possess any collagen or gelatine hydrolyzing activity and by examining its applicability in the dehairing of bovine hide, complete dehairing was acheived after 2 h without the estimation of any deleterious effect on the skin, manifisted by scanning electron microscopy. The skin surface was smooth and cleans with the complete removal of the hair from the follicles. On the other hand, the conventional applied method led to a rough grain deteriorated surface. This result could be attributed to the specificity of the produced enzyme in the breakdown of the structural protein of the hair and epidermis, facilitating their removal. Moreover, the time required for the complete dehairing of the hide was much lower than that spent in the conventional chemical dehairing and lower that that reported in the literature ; 16-48 h had been reported for other *Bacillus* keratinolytic proteases [12, 18, 33], reflecting the effectiveness of the enzyme produced in the present research. Furtherly, a significant reduction in the pollution load parameters was observed in the effluent produced from the examined enzymatic dehairing in compare to that resulted for the conventional chemical method. The high pollution load parameters estimated for chemical dehairing was mainly attributed to the dissolving of the hair while enzymes mainly act on the hair root, removing the hair intact [12, 17].

In the last few years, the application of enzymes in detergent-formulations become essential as they provided environmental and economic advantages, they could efficiently remove stains at low temperatures with a reduction in water consumption. Detergent-compatible enzymes are in need to be active in alkaline environments and over a wide temperature range in addition to maintaining activity when mixed with other detergent ingredients, like surfactants, bleaching agents and calcium-chelating agents [50]. In the present work, the produced keratinolytic protease possessed activity in alkaline conditions (up to 10) and over a wide temperature range (up to 90 °C) with an enhanced activity in the presence of surfactants. Moreover, the enzyme retained 98.5 ± 0.19 % of this initial activity following incubation with commercial detergent for 1 h at 40 °C. All these parameters suggested its application in detergent-formulations and by examining its efficiency in the removal of chocolate-flavored milk stain; it possessed an excellent activity manifested by the restoring of the whiteness index of the treated fabric by 86.28 % when the enzyme was added to a thermal-inactivated commercial detergent in compare to 65.26% for the use of the commercial detergent. Comparable results concerning the improved efficiency of detergents in the removal of protein-based stains by the addition of keratinolytic proteases had been reported [9, 19].

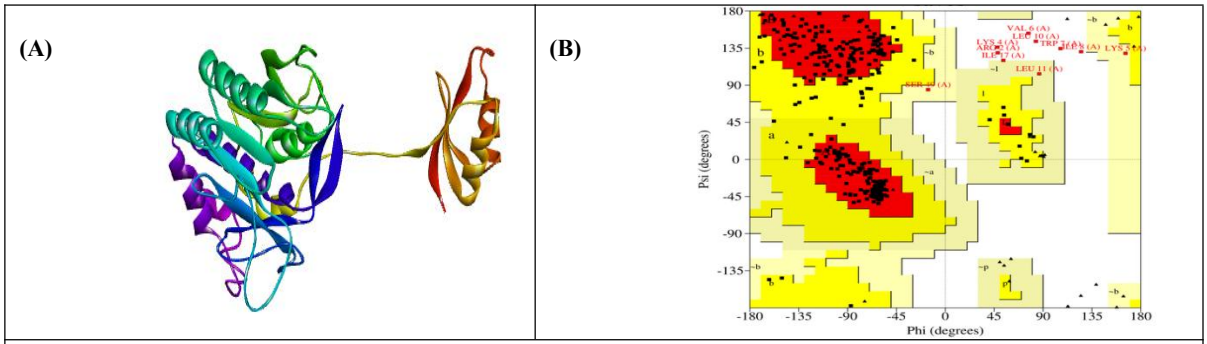


Figure (7): (A) 3D structure and (B) Ramachandran plot of keratinolytic protease *B. amyloliquefaciens* Pp038117.

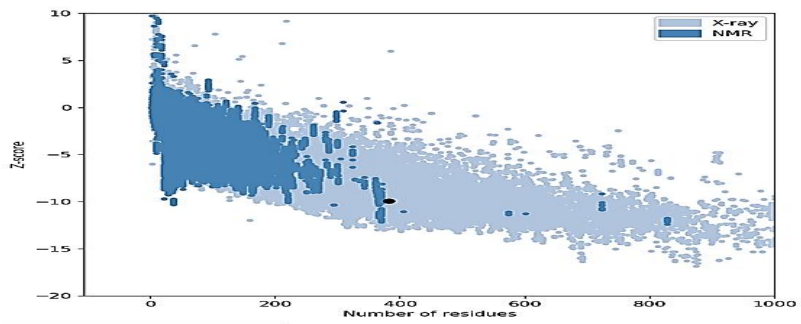


Figure (8): Overall model quality Z-Score, -9.92

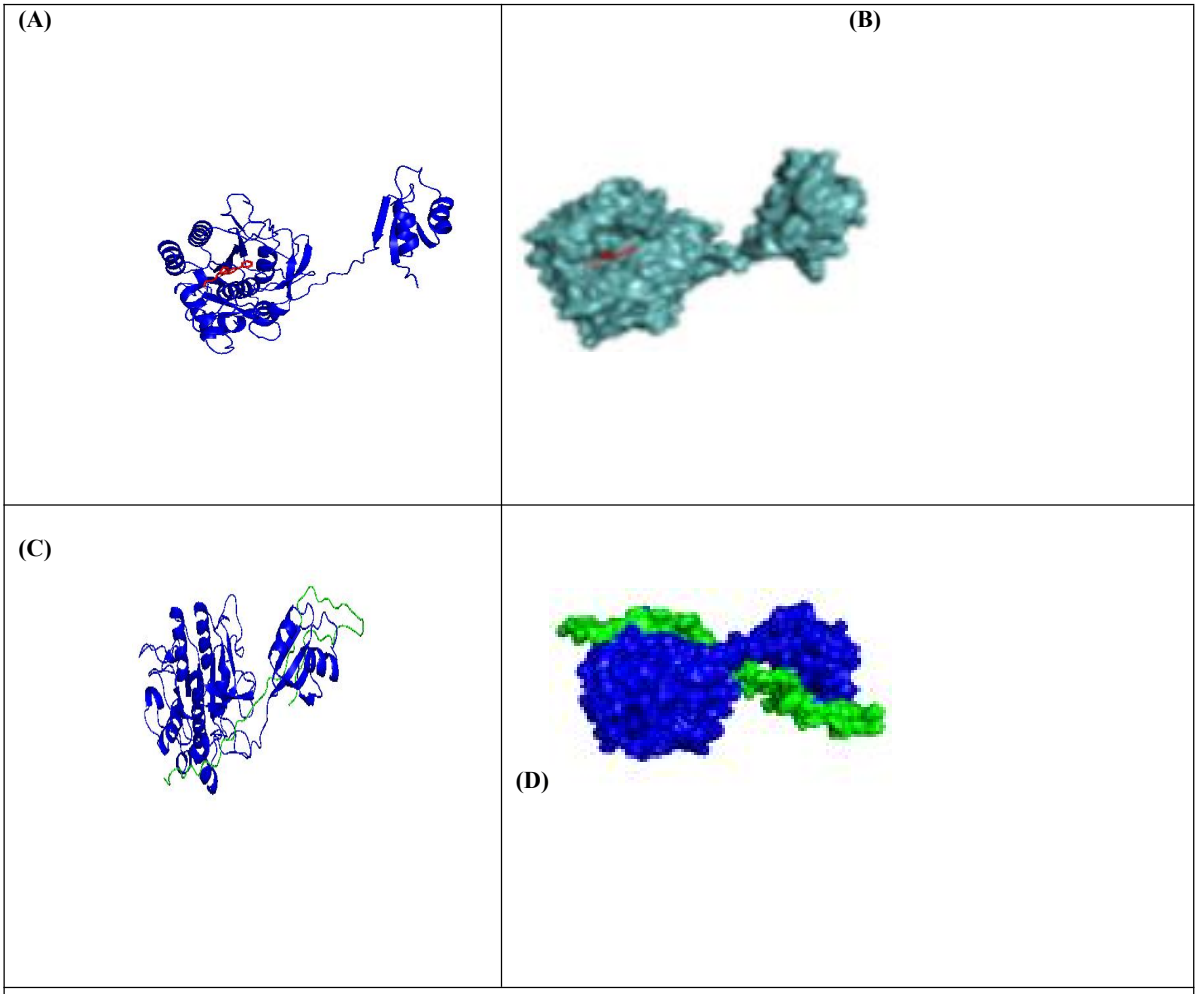


Figure (9): (A) 3D and (B) surface interaction between the keratinolytic protease *B. amyloliquefaciens* (Blue color) and casein (red color); (C) 3D and (D) surface interaction between the keratinolytic protease *B. amyloliquefaciens* (Blue color) and beta keratin, chicken (Green color).

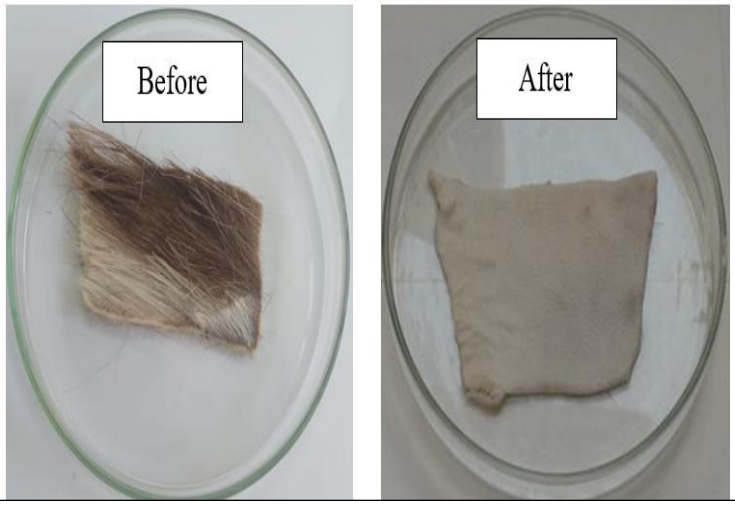
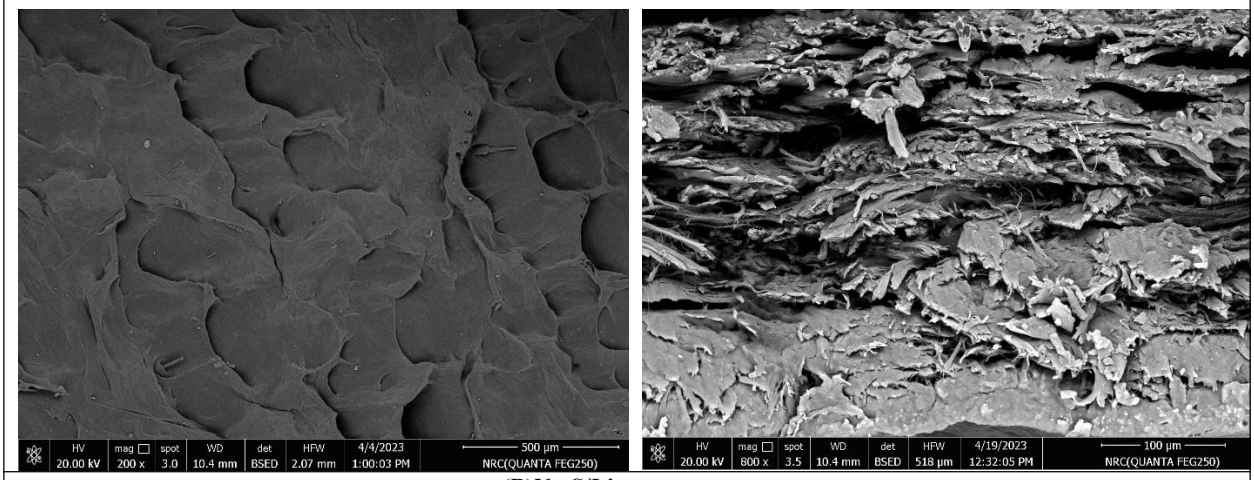


Figure (10): Visual appearance of the bio-treated dehaired bovine hide.

(A)Enzymatic treatment

Surface view **Cross-section view**



(B)Na_2S/Lime treatment

Surface view **Cross-section view**

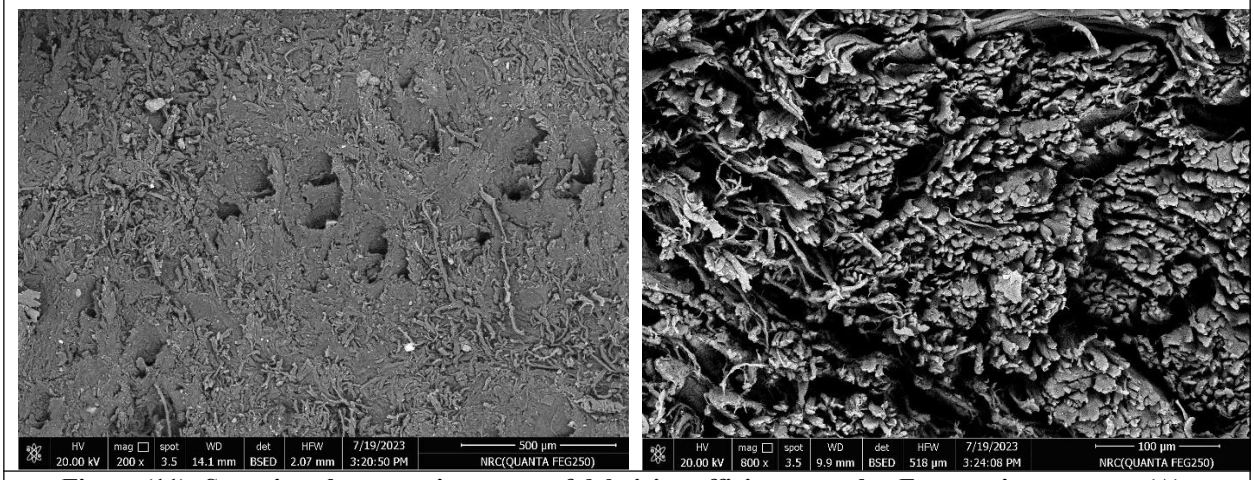
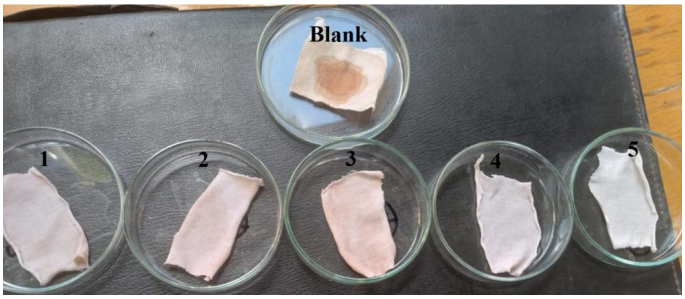


Figure (11): Scanning electron microscope of dehairing efficiency results, Enzymatic treatment (A); Na_2S/Lime

(A)



(B)

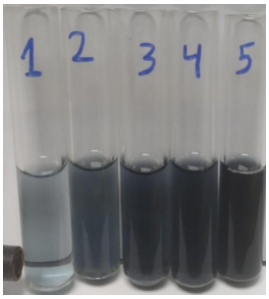


Figure (12): (A) Visual appearance for the removal of chocolate-flavored milk stain from cotton fabrics (Blank) under different treatment conditions in addition to (B) Lowry protein content determination in the detergency solutions; (1) tape water, (2) enzymatic, (3) thermal-inactivated Persil gel, (4) Persil gel and (5) added enzyme to thermal-inactivated Persil gel treatments.

Table (8): Pollution load parameters

Parameter	Enzymatic treatment	Na_2S/Lime treatment
TDS	3470	3680
COD	12200	25350
BOD	5770	3650

Table (9): Whiteness index determination

Treatment	L*a*b*Values			whiteness index (%)	Relative whiteness intensity to the control (%)
	L*	a*	b*		
Control (without any stain)	84.89	1.47	-4.87	89.99	100
Tape water	67.21	7.73	5.81	2.78	3.09
Persil gel	77.24	2.51	-1.25	58.73	65.26
Enzyme + thermal-inactivated Persil gel	78.5	3.67	-4.46	77.64	86.28

4. Conclusion

The current study concentrated on the economic production of a keratinolytic protease via the fermentation of feather. A keratinolytic bacteria was initially isolated and identified as *Bacillus amyloliquefaciens*. After that, it was used for the submerged fermentation of feather in which the fermentation conditions were statistically optimized by applying PB and CCD. The enzyme was partially purified and characterized as alkalophilic, thermophilic and surfactant-stable enzyme. The applicability of the produced enzyme as an eco-friendly contributive in leather and detergent industries was evaluated. The enzyme was efficiently applied in the dehairing of bovine hide in which the hide was completely dehaired after 2 h without the estimation of any negative impact on the skin surface as that observed for the chemically dehaired hide. Moreover, the pollution load parameters were significantly reduced via the enzymatically dehairing process. Finally, the suitability of the produced enzyme for use in detergent-formulation was evaluated in which its addition to a thermally-inactivated commercial detergent efficiently removed chocolate-flavored milk stains.

5. Contribution of Authors

Shaimaa A Nour: Conceptualization; Methodology ; Data curation; Formal analysis; and Writing - review & editing.
Shaymaa A Ismail: Conceptualization; Methodology ; Data curation; Formal analysis; and Writing - review & editing & Writing the orginal draft .
El-Shahat H A Nashy: Methodology ; Investigation and Data curation.
Azza M Abdel-Fattah: Project administration; Funding acquisition ; Conceptualization and Methodology.

6. Conflict of interest

"There was no conflict of interest to declare"

7. Funding

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