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Research article

KERATINOLYTIC PROTEASE PRODUCTION AND CHARACTERIZATION FROM *BACILLUS* SP. ISOLATED FROM POULTRY WASTES

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ABSTRACT: Keratinases originating from microorganisms are used in many industrial fields such as the recycle of keratinous wastes, leather, textile, the detergent industry and medical applications. In this study, 42 *Bacillus* strain were isolated from Cukurova University Research and Application and Chicken Management Unit. 8 of these isolates showed proteolytic activity on skim-milk and keratinolytic activity with keratin-azure on the basal feather-meal medium. Strain H62 with the highest keratinase activity was determined as the keratinase producer and identified as *Bacillus licheniformis* with microscopic, biochemical (VITEK-2, 90%) and molecular analysis (16S rRNA, 99%, *B. licheniformis* 9945A). The highest enzyme production was carried out at 40°C for 45 hours by adding 0.1 g/l mannitol (as carbon source), 0.1 g/l ammonium nitrate (as nitrogen source) and 15 g/l feather-meal into the basal feather-meal medium. Although keratinase showed the activity at 20-90°C and pH 5.0-13.0, optimum activity was obtained at 40°C and pH 9.5. 100% of stability was determined at pH 8.0, whereas the loss of activity was observed at pH 7.0-9.0. After a pre-incubation at 20-100°C enzyme was 100% stable whereas activity was decreased at the other temperatures. At room temperature, a loss of activity was determined after the 24th hour. EDTA, SDS and Urea increased the enzyme activity; however, Tween-20 was decreased. The enzyme was seen to be a single band with a molecular weight of 26 kDa. As a result, keratinase *B. Licheniformis* H62 is an enzyme that can be used in mesophyll and alkaline conditions, particularly in medical applications and as feed supplements.

Key words: Keratinase, *Bacillus licheniformis* H-62, carbon and nitrogen source

INTRODUCTION

Microbial keratinase is an enzyme capable of degrading the insoluble structural protein found in feathers, hair and wool known as keratin. This protein is resistant to degradation by proteolytic enzymes such as trypsin, pepsin, papain due to the composition and molecular conformation of the amino acids found in keratin. Consequently, huge amount of keratinase waste accumulate in nature and impose a great concern for the environment (Sivakumar et al., 2012). Keratinases (E.C 3.4.21/24/99.11) are a particular class of proteolytic enzymes that display the capability of degrading insoluble keratin substrates. These enzymes have gained importance in recent years, as several potential applications have been associated with the hydrolysis of keratinous substrates (Rayudu et al., 2013). Most keratinases have some common characteristics despite their different origins. They belong mainly to the extracellular serine proteases, with the exception of keratinases from yeasts, which belong to the aspartic proteases (Gradisar et al., 2005). The keratinous substrate such as feather and wool can be degraded in basal medium by microorganisms which are capable of utilizing keratin as a sole carbon and nitrogen source. Keratinolytic proteases have broad substrate specificity where they have the ability to hydrolyze soluble protein such as casein, gelatin, and bovine serum albumin. In recent years, demands to keratinolytic proteases are increasing due to their multitude in industrial applications such as the feed, fertilizer, detergent, and textile industries (Hassan et al., 2013). Keratin waste is mainly composed of keratinous protein and classified into α, β and γ keratin since the diverse percentage of disulfide bonds (Fang et al., 2013). For mature chicken, feather accounts up to 5% to 7% of the live weight and is composed of over 90% crude protein, the main component being keratin, a fibrous and insoluble protein (Lo et al., 2012).

At present, feathers are converted to feather-meal, a digestible dietary protein for animals, using physical and chemical treatments. These physico-chemical conversion methods involve costly treatments under harsh temperature and pressure conditions that result in a loss of certain heat sensitive amino acids, e.g., methionine, lysine and tryptophan.

Heat treatment also adds to non-nutritive amino acids such as lysinoalanine and lanthionine. The microbial degradation of feather represents an alternative eco-friendly technology to improve the nutritional value of feather-meal. Nevertheless, feathers do not accumulate in nature, since structural keratin can be degraded by some microorganisms (Lo et al., 2012).
Cultivation conditions are essential in successful production of an enzyme and the optimization of parameters such as pH, temperature and media composition is important in developing the optimum fermentation conditions. Keratinase production is affected by various nitrogen sources. Besides these, several other physical factors such as aeration, inoculum's density and incubation time also affect the amount of keratinase production (Sivakumar et al., 2012). Biotechnological processing of keratinous waste may represent an important added value to the industry (Brandelli et al., 2010). In addition, Research on keratinolytic microorganisms has been focused mostly on biotechnological applications involving the hydrolysis of keratinous byproduct, although promising new applications related to drug delivery and hydrolysis of prion proteins have been described (Bach et al., 2011).
In the current study, we focused on the isolation of extracellular keratinase producing bacteria from the soil of Cukurova University Research and Application and Chicken Management Unit., and identified by biochemical, morphological and molecular methods. Optimization of keratinase production conditions and enzyme characterization was also aimed. The enzymes produced according to the results obtained were determined, which can be used in biotechnological applications.

MATERIALS AND METHODS

Isolation of Microorganisms:

Feather-degrading bacteria were isolated from soil samples from Cukurova University Research and Application and Chicken Management Unit. The soil samples were collected in dark polythene bags and stored at 4°C until further use. About 1 g of soil sample was dispersed in 9 ml of sterile distilled water and then treated for 20 min at 80°C, in order to enable the isolation of the spore-forming bacteria. The samples were serially diluted 10^{-9} and six fold dilutions were plated on nutrient agar and incubated for 24 hours at 37°C. The colonies with different morphology were picked and purified using streak plate method.

Strains Isolation

Luria-Bertani (LB) agar medium with 1% (w/v) skim milk was used for cultivation. Skim milk and LB agar made separately. LB was autoclaved at 121°C for 15 min, while 1% (w / v) skim milk solution was autoclaved at 115°C for 10 min. The milk solution was mixed with LB agar while still hot. Plates were incubated for 72 hours at 37°C after which time any clone with a clear halo was picked and stored for subsequent analysis.

Inoculum Preparation and Enzyme Production

Eight selected strains isolated according to the diameter of clear zone were cultured on LB-medium for 24 hours at 37°C in an orbital shaker. 5 mL of this culture (at OD_{660} ~1.0) was added to the feather-meal broth medium containing, per liter: 0.5 g of NH_4Cl, 0.5 g of NaCl, 0.3 g of K_2HPO_4, 0.4 g of KH_2PO_4, 0.1 g of $MgCl_2{\cdot}6H_2O$, 0.1 g of yeast extract, and 10 g of feather-meal. The pH was adjusted to 7.5 and incubated for 72 hours at 37°C under shaking condition (150 rpm). The feather-meal was used as the sole carbon and nitrogen source for detecting potent strains that have the ability to degrade the feather-meal completely. The culture was harvested by centrifugation at 5000 rpm for 20 min at 4°C. The cell-free supernatant was used as crude preparation of enzyme for keratinolytic activity.

Assay of Keratinolytic Activity

Keratinase activity was determined by the method of Suntornsuk et al. (2003). Keratin-azure (Sigma Chemical, St. Louis, MO, USA) was used as a substrate after first freezing at -20°C and then grinded into a fine powder. The keratin-azure powder was suspended in 0.01 M Tris-HCl buffer, pH 7.5 at a concentration of 4 mg/ml. The reaction mixture contained 1 ml of enzyme solution and 1 ml of keratin-azure suspension. The reaction was carried out at 50°C in a water bath with an agitation rate of 300 rpm for 60 min. The mixture was boiled for 5 min after incubation and followed by centrifuging at 5000 rpm for 20 min to remove the substrate. The supernatant was spectrophotometrically measured for the release of the azo dye at 595 nm. A mixture of enzyme and substrate was boiled before being carried out as a control. All assays were done in triplex. After determining the existence of the activity, *Bacillus* sp. H62 was selected and preserved for further investigation.

Bacterial Identification

Although the phenotypic characteristics and isolation method of the isolate indicate that it is related to *Bacillus* group, further identification was also conducted. The strains identification are included the spore morphology, Gram stain, and motility.

Bacillus sp. H62 that showed the highest keratinase activity was determined with the VITEK-2 (bioMerieux) bacteria identification system. Selected strain was inoculated in tryptic soy broth for 18 hours at 37°C for 18 hours at 150 rpm. Following the inoculation in tryptic soy broth, the mixture was incubated at 37°C for 24 hours. Fresh bacteria was homogenized with 0.45% sterile NaCl to achieve a turbidity equivalent to that of a McFarland 2.0 standard (range, 1.80 to 2.20). The VITEK 2 instrument automatically filled, sealed, and incubated the individual test cards with the prepared culture suspension. Cards were held at 35.5°C with optical readings taken automatically every 15 min. The results were obtained in 18 hours after loading.
Bacillus sp. H62 which was identified by morphological, microscopic, biochemical diagnostics, was sent to Karadeniz Technical University for diagnostic purposes of 16S rRNA gene sequences. The resulting sequence was assessed in national center for biotechnology information (NCBL GenBank).

Optimization of Cultural Conditions for Growth and Enzyme Production

To detect the influence of medium pH on growth and production of keratinolytic enzyme, *B. licheniformis* H62 was grown in the feather-meal broth at different initial pHs (4.0-9.0). The fermentation was carried out at 37°C for 72 hours under shaking at 150 rpm. To investigate the effect of the temperature on growth and keratinolytic enzyme production, fermentation was performed with the feather-meal broth medium at different temperatures (20, 30, 40, 50 and 60°C) for 72 hours at 150 rpm. The growth and enzyme activity was quantified as described above.

Effect of Different Carbon Sources on Growth and Enzyme Production

The growth and production of keratinolytic enzyme were investigated by adding 1% (w/v) various carbon sources (lactose, maltose, mannitol, starch, glucose) to feather-meal broth medium initial pH 7.0. The growth and enzyme activity were monitored after fermentation at 40°C for 72 hours under shaking at 150 rpm.

Effect of Different Nitrogen Sources on Growth and Enzyme Production

The growth and production of keratinolytic enzyme were investigated by adding 1% (w/v) various nitrogen sources (peptone, tryptone, casein, urea, ammonium nitrate and yeast (1 and 2%, w/v)) to feather-meal broth medium, initial pH 7.0. The growth and enzyme activity were monitored after fermentation at 40°C for 72 hours under shaking at 150 rpm.

The Effect of Different Feather-Meal Concentrations on Growth and Enzyme Production

The amount of keratinolytic enzyme production depended on feather concentrations; therefore, *B. licheniformis* H62 was cultivated at different concentration of feather-meal (5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20 g/l) in the feather-meal broth medium at initial pH 7.0, 0.1 g/l mannitol and 0.1 g/l ammonium nitrate incubated at 40°C for 72 hours under shaking condition (150 rpm) to compare the keratinase activity.

Effect of Incubation Period

B. licheniformis H62 was carried out individually at various incubation times ranging from 6 to 78 hours in the feather-meal broth medium at initial pH 7.0, 0.1 g/l mannitol, 0.1 g/l ammonium nitrate and 15 g/l feather-meal, incubated at 40°C under shaking condition (150 rpm) to compare the keratinase activity.

Enzyme Purification

The crude enzymatic extract was subjected to precipitation with commercial Chilled ethanol (-20°C) (96%) in the gradient concentration of 20 to 80%, for proteins to precipitate. After this solution was left to rest for 24 h at -20°C. The visible fractions were centrifuged at 5000 rpm for 20 min at 4°C, air dried and dissolved in minimal amount of 50 mM Tris HCl (pH 8.6) and obtain the concentrated protein.

Characterization of Keratinase

Optimum pH and Temperature

The effect of pH on keratinase activity was studied in the pH range of 5.0-13.0 with a buffer system of citrate-phosphate buffer (pH 5.0-6.0), phosphate buffer (pH 6.5-7.5), Tris-HCl buffer (pH 8.0-9.0), carbonate-bicarbonate buffer (pH 9.5-105) and borax-NaOH buffer (pH 11.0-13.0), with an incubation of 60 min at 40ºC. The pH value that showed the highest activity was selected as the optimum pH for the enzyme.
The influence of temperature on keratinolytic activity was studied by incubation at 20-100°C at 10 degree intervals. The temperature exhibiting the highest keratinase activity was defined as the optimum temperature for this enzyme.

Effect of pH

For stability studies, the enzyme was pre-incubated for 60 min at different pH values (7.0-13.0) using different pH buffer systems, phosphate buffer (pH 7.0-7.5), Tris-HCl buffer (pH 8.0-9.0), carbonate-bicarbonate buffer (pH 9.5-105) and borax-NaOH buffer (pH 11.0-13.0) and the residual activity was assayed.

Effect of Temperature

Thermo-stability of the keratinase was examined by pre-incubation of the enzyme preparation at different temperatures ranging from 20 to 100°C at 10 degree intervals for 60 min, and then by determining the residual activity.

Effect of Inhibitors

The inhibitors, sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA), Urea and Tween20 were added at the concentration of 1 mM and 5mM, which contained the produced enzyme and were incubated at ambient temperature for 30 min. The activity of the enzyme preparation in the absence of chemicals was defined as the 100% level and the activity of the enzyme pre-incubated with the chemicals were estimated according to this level.

Effects of Storage Time at Room Temperature on Enzyme Stability

To determine the enzyme stability at room temperature, the enzyme stored at room temperature was measured by the standard method with samples taken at 12 hour intervals. The activity of the enzyme preparation taken before standing at room temperature was defined as the 100% level.

Determination Molecular Weight

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed with 12% polyacrylamide gels as described by Bressollier et al. (1999). Molecular weight markers (molecular weights, 20 kDa to 120 kDa; Thermo Scientific, Made in Lithuania) were included. Electrophoresis was done at a constant current of 12 mA. The gel was stained with Coomassie brilliant blue R-250 for 2 hours.

Zymogram

Zymogram was carried out on vertical slab gel, proteinase samples were mixed with the electrophoresis sample buffer without heat denaturation prior to electrophoresis. SDS-PAGE was carried out at 4°C by using a 12% polyacrylamide gel. After electrophoresis, the gel was washed with 2.5% (v/v) Triton X-100 for 30 min and then with 50mM phosphate buffer (pH 7.0) for 30 min. The gelatin (2%, w/v) in 50mM phosphate buffer (pH 7.0) was then poured onto the gel slab containing proteases. After 2 hours of incubation at 40°C, the gel was stained with Coomassie brilliant blue R-250 and then destained. Protease bands appeared as clear zones on a blue background.

RESULTS AND DISCUSSION

Strain Isolation

Microbial keratinolytic protease has been described for various biotechnological applications in food, detergent, textiles, removal of corns and calluses, acne treatment, leather dehairing, cosmetics, pearl bleaching, removal of earwax, and Bio-safety against infectious prions demand because these enzymes necessitate the screening for novel keratinolytic microorganisms with potential applications. Keratinolytic protease has been described for several species of *Bacillus* due to the broad distribution of keratinase among these genera. A total of 42 pure cultures of spore-forming bacteria were isolated and purified which obtained from different samples collected from Cukurova University Research and Application and Chicken Management Unit. All isolates were screened using the selective method for *Bacillus* isolation. The proteolysis activities of all the isolates were detected using the plate test method containing LB agar medium with 1% (w/v) skim milk (Figure 1). Among the isolates analyzed, 8 isolates exhibited proteolytic activity in which they had a halo diameter longer than the colony diameter. All isolates have the proteolytic activity but do not have the ability to degrade feather-meal. Eight selected isolates were grown using a feather-meal broth medium which contained, per liter: 0.5 g of NH_4Cl, 0.5 g of NaCl, 0.3 g of K_2HPO_4, 0.4 g of KH_2PO_4, 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.1 g of yeast extract, and 10 g of feather-meal, and the pH was adjusted to pH 7.5 using 2N NaOH and HCl and incubated for 72 hours at 37°C under shaking condition (150 rpm). For assay of keratinolytic activity, keratin-azure was used as a substrate and supernatant was spectrophotometrically measured for the release of the azo dye at 595 nm. The *Bacillus* sp.H62 showed the highest amount among the other isolates and was therefore selected for further experimental studies.

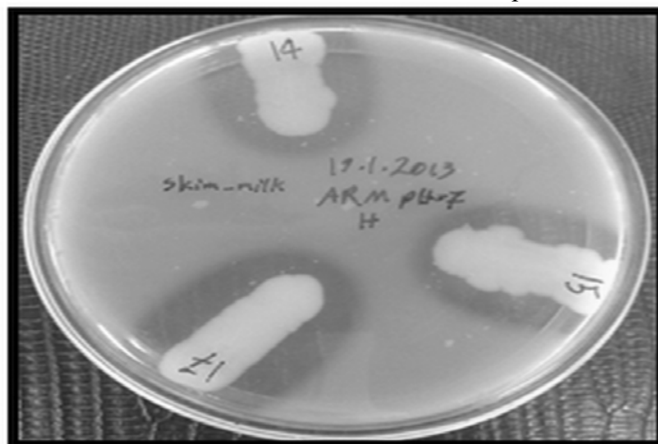

Figure 1. Isolated with activity zone at pH 7.0, 37°C on skim milk agar after a 24 h of incubation

Strains Identification

Phenotypic characteristics (spore-forming, Gram-positive and motility) and isolation method of the isolate indicate that it is related to the *Bacillus* group and further identification was conducted as 90% of *B. licheniformis* by VITEK2 bacteria identification system (Table 1).

Bacillus sp. H62 which was sent to Karadeniz Technical University for diagnostic purposes of 16S rRNA gene sequences, could grow on the minimal medium containing the feather-meal as the substrate for carbon and nitrogen source. The strain H62 exhibiting the highest feather degradation was chosen for taxonomical investigation. The phylogenetic analysis of the strain H62 based on the 16s rRNA showed high levels of sequence similarity to the species *B. licheniformis* (99%). Phylogenetic analysis was assessed at the national center for biotechnology information (NCBI GenBank) showed that isolate is closely related to *B. licheniformis* 9945A with the reference sequence No. NC_021362.1.

Table 1. Identification of *Bacillus licheniformis* H-62 by VITEK-2 syatem

Bionumber: 1373361557077671			
Selected organism: Bacillus licheniformis			
Identification information	Card: BCL	Lot Number: 239267720	Expires: Apr 13, 2014 13.00
	Completed: May 27, 2013 04:11 CDT	Status: Final	Analysis Time: 14.25 hours
Selected Organism	90 % Probability Bionumber: 1373361557077670	Bacillus licheniformis	
		Confidence: good identification	

Optimization of Cultural Conditions for Growth and Enzyme Production

The growth and keratinolytic enzyme synthesis with *B. licheniformis* H62 was greatly influenced by the initial medium pH showing maximum production at pH 7.0 (Figure 2). Temperature is an important environmental factor affecting the growth and production of metabolites by microorganisms (Hossain et al., 2007). The optimal temperature for growth and production of keratinolytic protease by *B. licheniformis* H62 was 40°C (Figure 2). This temperature optima for maximum keratinolytic enzyme production by this organism is almost similar to that of other *Bacillus* species (Hossain et al., 2007; Cheng et al., 1995), but dissimilar to keratinolytic *Vibrio* sp.kr2 (Sangali and Brandelli, 2000).

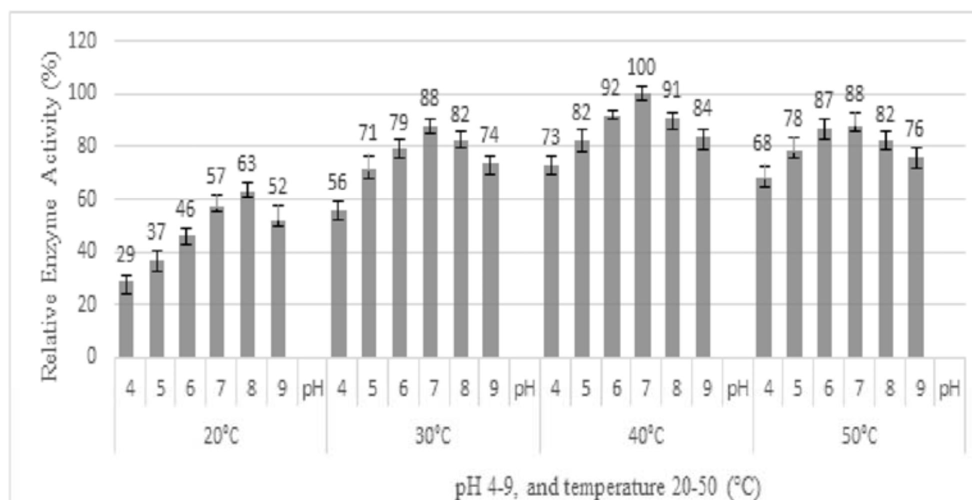

Figure 2. pH and temperature optimization of culture

Effect of Different Carbon Sources on Growth and Enzyme Production

During the growth of microorganisms, the carbon source in the culture medium plays an important role for the growth of cells, the production of metabolites and the availability of energy to drive endergonic reactions. Of all the carbon sources tested, the highest reduction activity was obtained with mannitol. The other carbon sources gave substantially lower reduction activities and were judged unsuitable for producing highly active biocatalyst (Figure 3).

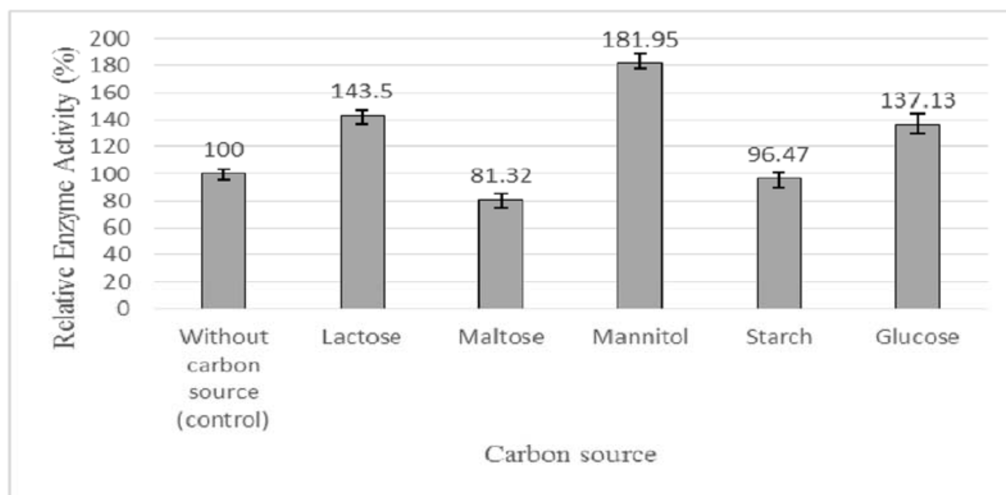

Figure 3. Keratinase production in different carbon sources

Effect of Different Nitrogen Sources on Growth and Enzyme Production

To optimize the keratinase production 1% (w/v) various nitrogen sources (peptone, tryptone, casein, urea, ammonium nitrate and yeast (1 and 2%, w/v)) were supplemented to feather-meal broth medium. Ammonium nitrate was the best nitrogen source for optimum keratinase and selected as the sole nitrogen source for the keratinase production by *B. licheniformis* H62 showed maximum production. The lowest keratinase production was observed in peptone and casein (Figure 4).

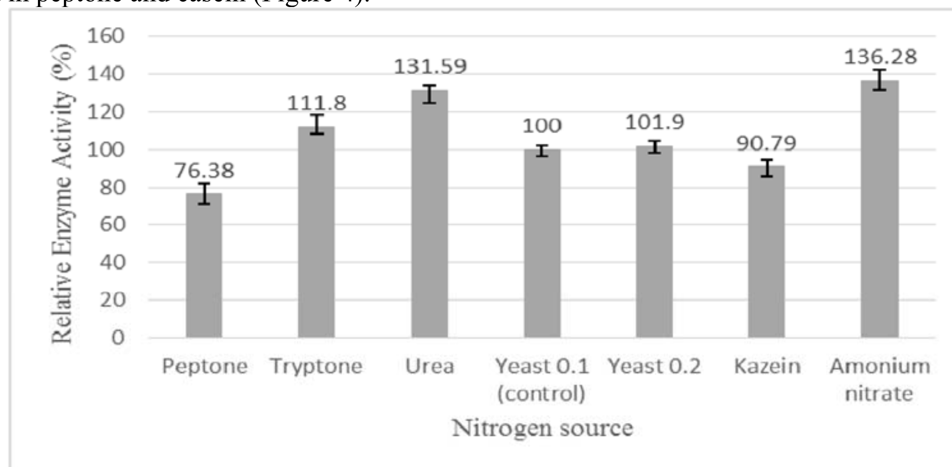

Figure 4. Keratinase production on different nitrogen sources. Cultivation was maintained at 40 °C and 150 rpm for 72 h

The Effect of Different Feather-Meal Concentrations on Enzyme Production

The effect of different feather-meal concentrations on keratinolytic enzyme production is shown in Figure.5. The amount of keratinolytic enzyme production depended on feather concentrations, confirming the presumption of Chen et al. (2002) that keratinase was induced to breakdown the feather into soluble protein to support cell growth. Keratinolytic enzyme production increased as the amount of feather increased; but when the concentration was raised to 17.5 g/l, enzyme production slightly decreased. The highest enzyme production was obtained at 15 g/l feather-meal (Figure 5). Cheng et al. (1995) reported that 1% (w/v) feather-meal gave the highest keratinase activity for *B. licheniformis* PWD-1.

Similar result was also reported for *Bacillus* sp. FK46 (Suntornsuk and Suntornsuk, 2003). The latter authors reported that higher concentrations (3% and 5%, w/v) may cause substrate inhibition or repression of keratinase production. On the other hand, high feather concentration increased medium viscosity which possibly resulted in oxygen limitation for bacterial growth.

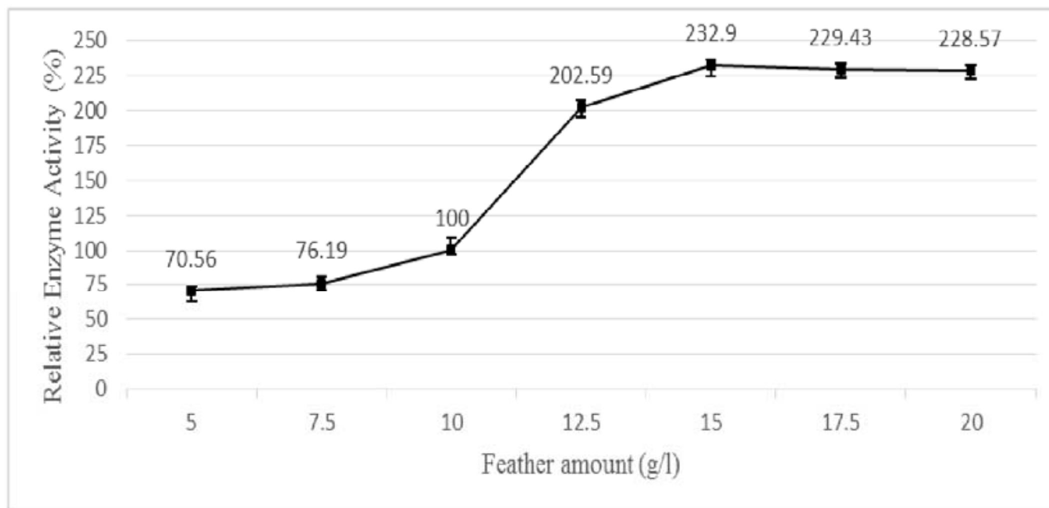

Figure 5. Effect of feather concentration on keratinolytic enzyme production

Effect of Incubation Period

The commercial production of the enzymes always depends on the maximum enzyme yield. The keratinase activity of the *B. licheniformis* H62 strain was at its maximum at 45 hours of incubation time (Figure 6). The minimum production of Keratinase was obtained at 9 hours of incubation. Sivakumar et al. (2012) reported that maximum keratinase production was at 96 hours and minimum keratinase production was recorded at 12 hours.

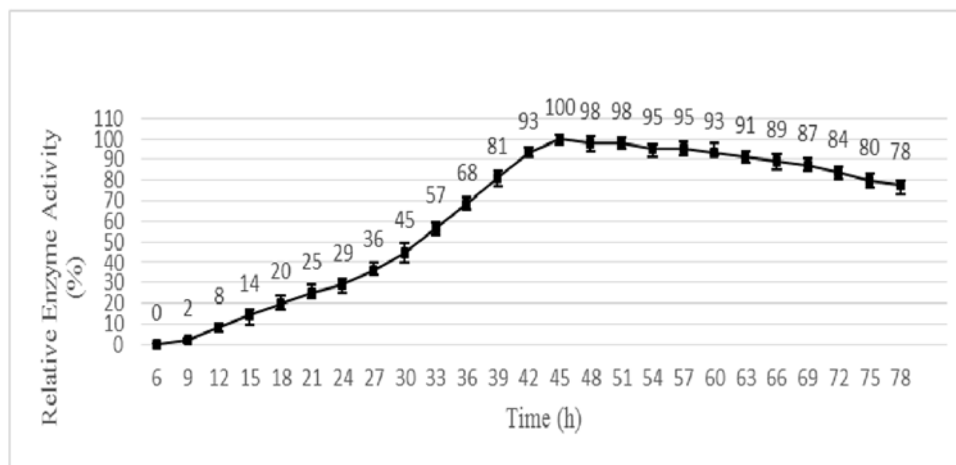

Figure 6. Effect of incubation period on keratinolytic enzyme production

Characterization of Keratinase

Optimum pH and Temperature

The effects of pH on the enzyme activity was examined over a pH range of 5.0-13.0 at 40°C. The maximum activity obtained at pH 9.5 was considered 100%. The *B. licheniformis* H62 strain showed more than 50% activity over a broad pH range of 8.0-12.0 (Figure 7). Kainoor et al. (2010) reported that the optimum pH of *Bacillus* JB99 was 10.0. The optimum activity of the *B. licheniformis* enzyme was observed at pH 8.5 and showed activity in neutral pH and alkaline which was reported by Cai et al. (2008).

The optimum temperature of the *B. licheniformis* H62 strain was determined by assaying enzyme activity at various temperatures for 60 min at pH 7.0. The optimum activity of the enzyme was observed at 40°C. The *B. licheniformis* H62 strain retained, on average, 51% and 60% of its activity between 20°C and 60°C, respectively. However activity of the enzyme decreased to 32% at 70°C (Figure 8). Vigneshwaran et al. (2010) reported that the maximum activity of the newly isolated *B. licheniformis* strain was determined at 60°C.

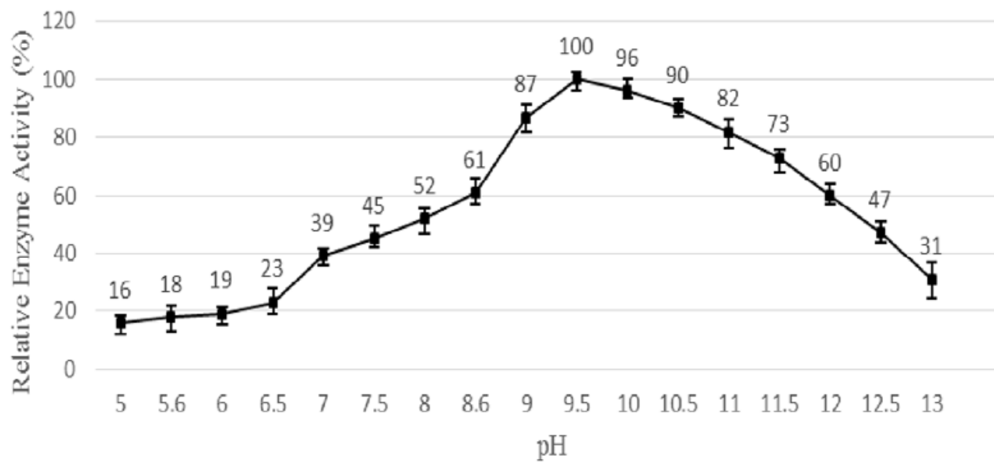

Figure 7. Effect of pH on the activity of keratinase

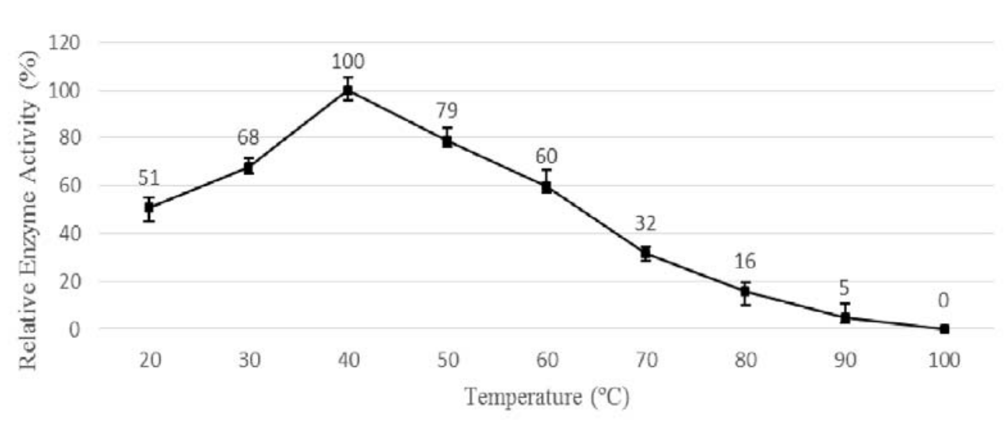

Figure 8. Effect of temperature on the activity of keratinase

Effect of pH

The keratinase enzyme was pre-incubated in buffers at various pH values (7.0-13.0) at 40°C for 60 min. The enzyme from *B. licheniformis* H62 was highly stable over a pH range of 7.0-11.0, averaging more than 49%. The enzyme retained 86% of its original activity at pH 9.0. However, the activity decreased rapidly to 25% at pH 13.0 (Figure 9). Rajput et al. (2010) reported that the enzyme from *B. pumilus* KS12 was active in neutral to alkaline pH, ranging from pH 7.0-12.0 with maximum activity corresponding at pH 10.0 and showed completely stable in the pH range of pH 6.0-10.0 with no loss in activity and retained up to >50% activity at extreme acidic (pH 4.0-5.0) and alkaline pH (pH 11.0-12.0) after 2 hours of incubation at room temperature.

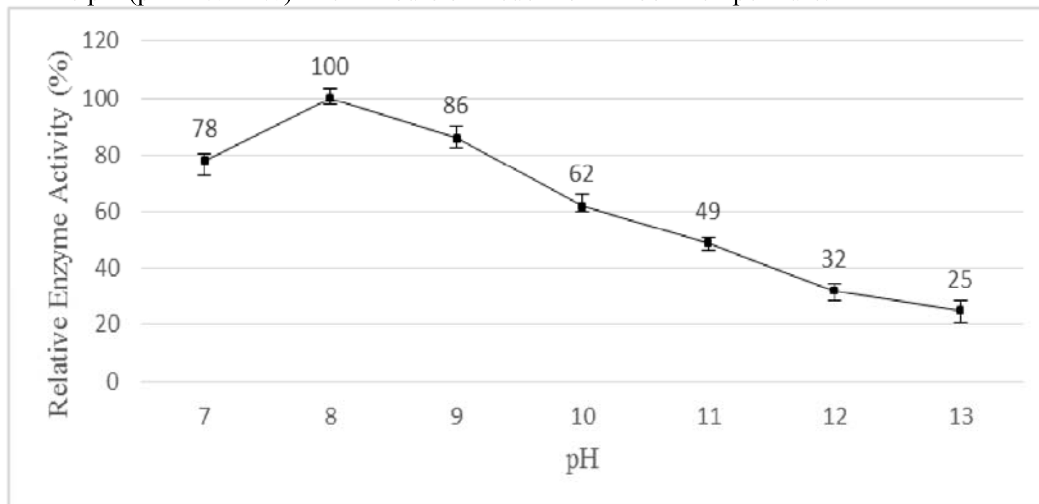

Figure 9. Effect of pH on the stability of keratinase from *B. licheniformis* H62

Effect of Temperature

Thermo-stability of the keratinase was examined by pre-incubation of the prepared enzyme at different temperatures ranging from 20 to 100°C at 10 degree intervals for 60 min, by determining the residual activity. The thermal stability analysis showed that the enzyme from *B. licheniformis* H62 was highly stable at temperatures below 60°C. It retained 87% and 60% of its initial activity at 50°C and 60°C, respectively (Figure 10). Rajput et al. (2010) reported that the enzyme from *B. pumilus* KS12 was active over a wide temperature range from 30 to 60°C with maximum activity at 60°C and stabled over temperatures ranging from 40-60°C with 97% residual activity at 40°C, followed by 89% activity at 50°C, while 82% residual activity at 60°C after 1 hour of incubation, at respective temperatures.

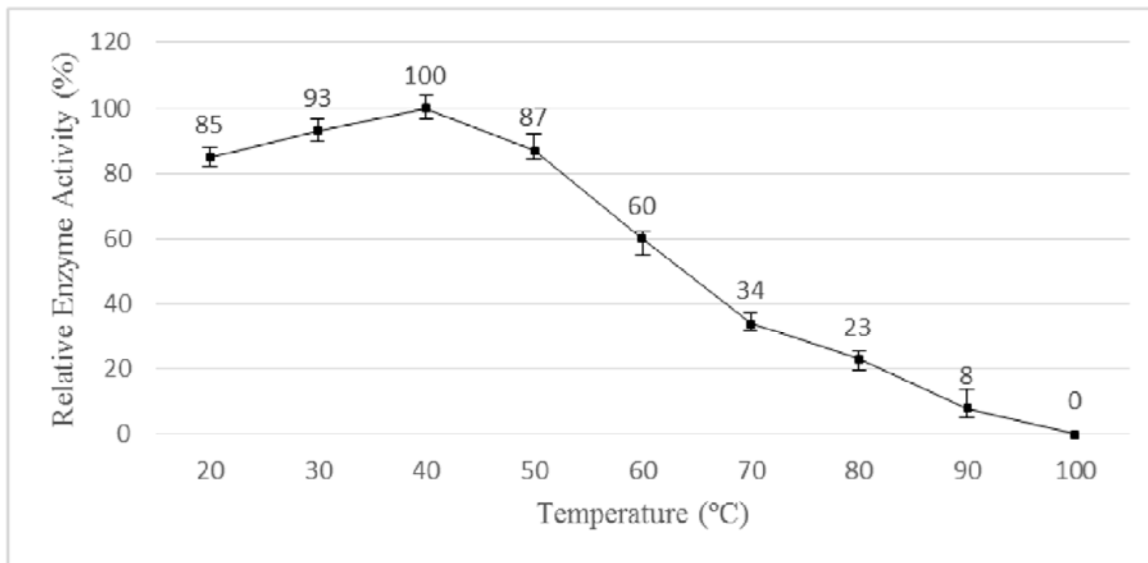

Figure 10. Effect of temperature on the stability of keratinase from *B. licheniformis* H62

Effect of Inhibitors

Among the various inhibitors, Tween20 partly inhibited keratinase activity. In the presence of EDTA, Urea and SDS, enzyme activity from *B. licheniformis* H62 was increased by 135%, 115% and 125%, respectively (Figure 11). Rajput et al. (2010) reported that the enzyme from *B. pumilus* KS12 was completely inhibited by PMSF. Cai et al. (2008) reported that the keratinase from *B. Subtilis* KD-N2 was partially inactivated by PMSF, SDS and EDTA had a positive effect on the keratinase activity.

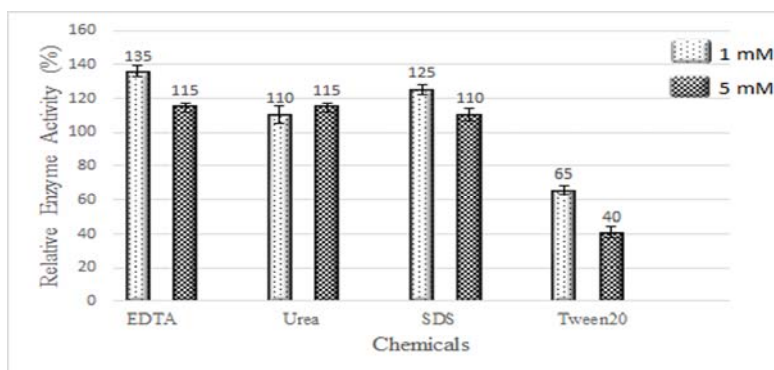

Figure 11. Effect of chemicals

Effects of Storage Time at Room Temperature on Enzyme Stability

The enzyme was stored at room temperature, measured by the standard method with samples taken at 12 hour intervals. The activity of the prepared enzyme showed a >54.3% increase at 84 hours of incubation at room temperature and remained 36.2% active at the end of 132 hours incubation in room temperature (Figure 12).

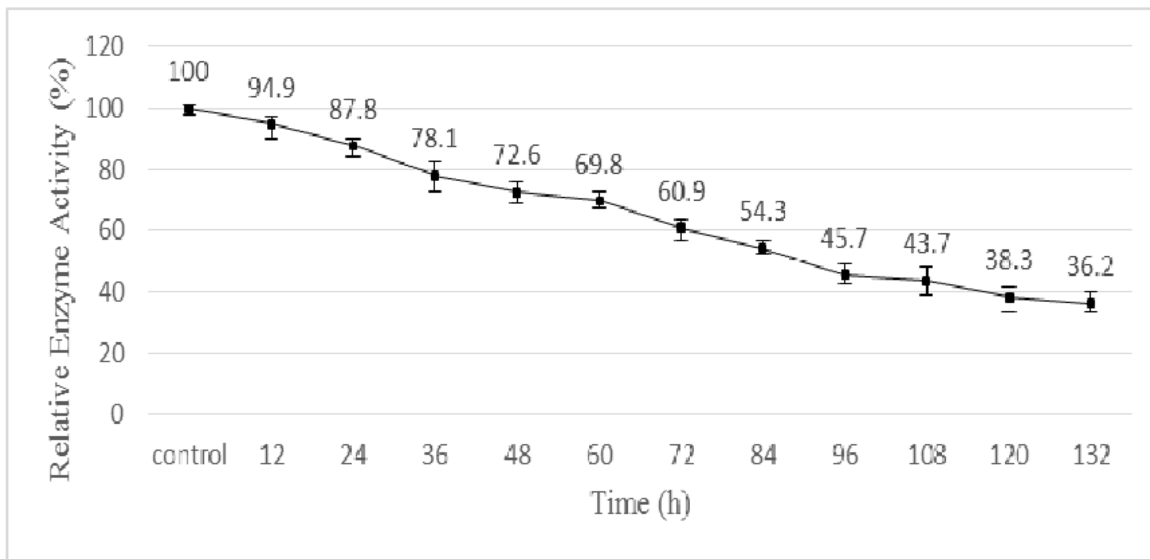

Figure-12: Effects of Storage Time at Room Temperature on Enzyme Stability

Determination Molecular Weight

The molecular mass of the keratinase was estimated by comparing the electrophoretic mobility of the enzyme with the electrophoretic mobilities of marker proteins. SDS-PAGE analysis of the sample revealed a single band at 26 kDa (Figure 13A). In zymogram, a clear zone of hydrolysis was observed against a white background coinciding with the protein band, at the same place where a single band was observed on SDS-PAGE gel (Figure 13B).

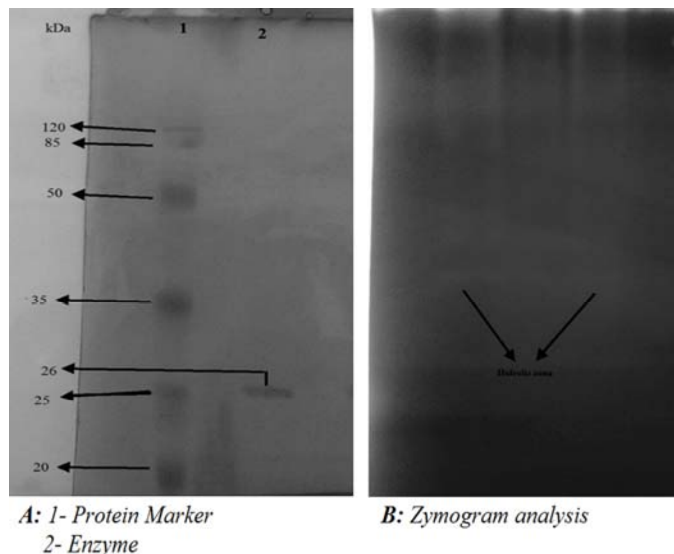

Figure 13. SDS-PAGE and Zymogram analysis

CONCLUSION

The newly isolated *B. licheniformis* H62 is a novel *Bacillus* strain showing the highest keratinase activity. The production of keratinase from *B. licheniformis* H62 is straightforward and easy to scale up; the organism grows on simple media with feather-meal as its sole carbon, nitrogen, and energy source. Hence, it is possible to culture an organism with great commercial potential using an inexpensive substrate, resulting in low production cost. At the same time, it transforms a kind of industrial waste into the required nutritional feed additives, thus, utilizing poultry feathers as a fermentation substrate in conjunction with keratin-degrading microorganism or enzymatic biodegradation may be a better alternative to improve nutritional value of poultry feathers and reduce environmental waste.

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