Journal of Applied Biological Sciences
E-ISSN: 2146-0108 18(2): 143-161, 2024
DOI: https://doi.org/10.71336/jabs.1161
Research Article

STUDY OF OPTIMIZATION, CHARACTERIZATION AND APPLICATIONS OF KERATINASE PRODUCED BY A BACILLUS STRAIN

Arjuman Surti*, Mugdha Taral

Sophia College, Department of Microbiology, Mumbai, Maharashtra

**Corresponding Author:*
E-mail: arjumansurti@gmail.com

(Received 11th November 2022; accepted 18th March 2024; published: 12th June 2024)

ABSTRACT. The poultry industry contributes to immense amounts of feather waste, over 90% of which is keratin. Although these industrial byproducts can be used as animal feed due to their high nutritional value, they are often landfilled or composted due to their low decomposition. The present study was carried out to isolate and identify a potential keratinase-producing bacterium from soil and waste water samples and to optimize cultural and physicochemical conditions to maximize enzyme production. The bacterium with the highest enzyme activity among the 17 isolates obtained in our study was identified as *Bacillus* sp. AM251. Optimal enzyme production was observed in media with the following composition (in g/L); NaCl (0.5), KH_2PO_4 (0.7), K_2HPO_4 (1.4), $MgSO_4$ (0.01), $CaCl_2$ (0.5), ammonium sulphate (2.5) and feather (10); pH 7.0. The optimum physicochemical parameters were determined to be 7.5% inoculum size, 37°C incubation temperature and shaking conditions (200 rpm). Characterization studies showed that the enzyme is a 43kDa serine protease with optimum activity at 50°C and pH 8.0. As an application of the enzyme keratinase, we also studied its substrate specificity and depilatory effect on goat skin. Both studies showed good enzyme activity. However, another study conducted to determine the use of enzyme-treated feather meal as an alternative to yeast extract and peptone for the growth of microorganisms in the laboratory showed limited applications. Overall, the results of our study show that the strain *Bacillus* sp. AM251 has great potential as a keratinase producer that can be used for bioremediation of feathers in landfills, preparation of animal feed or as a depilatory agent.

Keywords: *Bacillus sp. AM251, feather, keratinase, keratin, poultry, optimization.*

INTRODUCTION

Keratins are structural proteins found abundantly in the epithelial cells of vertebrates, where they function as mechanical support system. They form most of the skin and its appendages, including nails, hair, feathers, and wool [1]. Keratins belong to sclero-peptides and can be divided into hard and soft keratin based on their sulfur content. The soft keratins are fibrous and stringy skin modifications that form the basic skin structure and callus. The hard keratins occur as horns, nails, claws, scales and hooves of animals [2]. They are extremely stable proteins that are insoluble in water and organic solvents. Due to their chemically non-reactive nature, they are also stable to enzymatic hydrolysis by common peptidases such as trypsin, pepsin, and papain. This is due to the abundance of cross-linking disulfide bonds, salt bridges, hydrogen bonds and hydrophobic interactions in keratins. Consequently, they have a very slow rate of biodegradation [3].

In addition to the natural death and decay of animals, slaughterhouses and the meat processing industry generate large amounts of keratin-containing byproducts such as horns, feathers, nails, hooves, scales, and wool. Feathers which make up 5-7% of the total weight of adult chickens result in over 8.5 billion tons of feather waste being generated annually in the poultry industry worldwide. India alone produces over 350 million tons of feather waste every year [4]. Disposal of this waste over time results in environmental pollution due to slow biodegradation associated with the release of sulfur-containing compounds [5].

There are several approaches to the disposal of feather waste, including landfills, incineration, feedstock for natural gas production, and low-cost protein sources for animals. Presently, most of the feather wastes are dumped in land-fills or incinerated. Both these methods are largely associated with major ecological disadvantages like energy loss and production of a large amount of carbon dioxide [6]. Feather dumping grounds have also been associated with outbreak of chlorosis, mycoplasmosis, fowl cholera and H5N1 infections in the past [6, 7, 8]. The use of feathers as dietary proteins is also limited since hydrothermal processes, used to convert raw material into feather meal, destroy the amino acids and increase the cost of production. The high protein content also reduces the digestibility; further limiting its use as animal feed [9].

As a simple remedy, the use of keratinase enzymes of microbial origin, to improve the nutritional value of feathers has been suggested by several researchers. Keratinases are 20-50 kDa serine or metalloproteases capable of degrading the structure forming keratinous proteins. They are produced by several genera of bacteria (*Streptomycetes*, *Bacillus*, *Pseudomonas*) and fungi (*Aspergillus*, *Onygena*, *Absidia*, *Mucor* and *Rhizopus*) [10]. To date, none of the purified keratinases completely solubilize native keratin. Hence, isolation of keratinase producing microorganisms and purification of keratinase enzyme with high enzyme activity can contribute to voluminous reduction in keratin containing wastes disposed in the environment. It can also be applied to converting keratin wastes into useful biomass or obtaining specific amino acid and protein concentrates through biotransformation [11]. In India, keratinases are expected to find extensive application in livestock industries in near future as depilatory agents due to its huge livestock market that produces over 51 million bovine skin and 128 million cattle skin annually [12].

Considering the following factors, the present study was carried out with an objective to screen and isolate a potential keratinase producing microorganisms, and optimize the cultural and physicochemical parameters for maximizing its production. We also studied the application of keratinase as a depilatory agent on goat skin, and the efficacy of treated feather meal as an alternative to meat extract, and peptone for growth of microorganisms in the laboratory.

MATERIALS AND METHODS

Collection of samples

Screening of keratinase producing bacterium was done from soil and sewage samples. The sampling sites for the collection of soil included Sophia College Garden, Crawford market Slaughter house and compost collected from a Nursery in Dapoli, Mumbai. A partially degraded feather was collected from dumping ground of a chicken shop. A domestic sewage sample and wastewater from slaughter house in Borivali, India were also collected for screening of keratinase producers. The collection of samples from multiple sites increases the probability of isolation of keratinase producers with high enzyme activity. All samples were collected in washed, cleaned and dried plastic containers using a clean spatula and were processed immediately.

Preparation of chicken feather powder as keratin source

Feathers were collected from a slaughterhouse at Crawford market for the preparation of feather meal. They were washed with mild detergent and water to remove all the traces of blood and residual skin. They were dried at room temperature and then in the oven at 50°C until all moisture was removed (~ 1 week). After drying, the feathers were cut into smaller pieces and ground to fine powder in a standard mixer grinder. This powder was used for the preparation of selective media for the isolation of keratinase producers [13].

Isolation of feather degrading organisms

Feather meal (FM) agar plates [Composition in g/L: NaCl (0.5), K_2HPO_4 (0.3), KH_2PO_4 (0.4), agar (20) and feather meal (20), pH 7.4] were used for the isolation of keratinolytic bacteria. The phosphate salts in the medium act as buffers whereas magnesium acts as an inducer of keratinase production for microorganisms. Feather meal was used as a sole source of carbon and nitrogen. The samples were mixed with saline to prepare 10^{-1} dilutions and a loopful of samples were plated on the isolation media. The plates were incubated at 37°C for 48 h. After incubation, well differentiated colonies were picked up using a sterile loop and streaked again on FM agar plates for the selection of a pure colony. The pure cultures were maintained on nutrient agar slants at 4°C.

Detection of proteolytic activity

All the isolates obtained in our study were spot inoculated on Skimmed Milk Agar (SMA) [Composition in g/L: Peptone (10), Meat extract (3), NaCl (5), skimmed milk powder (10) and agar (13), pH 7.4] plates to check for proteolytic activity. The plates were incubated at 37°C for 48 h. After incubation, the SMA plates were checked for zones of clearance around the inoculated culture. The isolates showing clear and large zones were selected for further studies [14].

Keratinase enzyme assay

The isolates showing good proteolytic activity were further selected for determination of their keratinase activity. For this purpose, the isolates were grown in sterile nutrient broth for 20 h, washed with saline and inoculated in the FM basal medium (with same composition indicated above, without agar). The cultures were allowed to grow at 37°C for 48 h under shaker conditions. The keratinase assay was carried out by using standard protocol described by Cai et al. [15]. The enzyme activity was assayed using keratin-azure (Sigma Aldrich) as a substrate (0.2%-0.4%) and Tris-HCl buffer (pH 8.5) as a diluent. The crude enzyme was obtained by centrifuging the culture medium (cell free supernatant) at 3000 rpm for 30 min. The enzyme was stored in chilled condition. These tubes were incubated at different temperatures like 37°C, 45°C, 55°C for varied incubation period of 2, 4, 6, 24, 48, 72 and 96 h to determine the optimum activity of enzyme and standardize the optimization studies. Blank tubes were prepared by replacing keratin azure/ enzyme with buffer.

Keratin azure is an insoluble substrate made up of sheep wool coated with azo dye. On the reaction of enzyme with keratin azure, the azo dye is released and its concentration is proportional to the enzyme activity. The resulting color change was detected using a UV-Visible spectrophotometer (Systronics) at 595 nm. The assay was carried out in triplicates. The basic assay system was 1 ml enzyme with 1 ml Tris-HCl buffer (pH 8.5). One enzyme unit (EU) of keratinase activity was defined as the amount of enzyme leading to an increase in absorbance by 0.01 at 595 nm in 1 h under standard conditions.

Determination of whole feather degradation capacity

The isolates showing potential keratinase activity was selected and their ability to degrade whole feather (~10-12cm) was determined. For this purpose, the isolates were inoculated in basal medium containing 1 medium sized feather and incubated on shaker conditions at 37°C. The flask was monitored every 24 h for visible degradation of feather.

Protein estimation of culture supernatant

The concentration of proteins in the culture supernatant (also used as crude enzyme) was determined by Folin Lowry Ciocalteau method [16].

Specific activity of enzyme

The specific activity of the enzyme was calculated by using the formula [15]

$$Specific\ activity = \frac{Enzyme\ units\ in\ media}{'mg'protein\ in\ media}$$

Identification of isolate

The isolate with maximum specific activity was identified based on cultural, morphological and biochemical tests and comparison of observations with Bergey's manual [17]. The strain identification was done by 16s rRNA analysis, which was outsourced to Codon Life Sciences, Goa, India.

Optimization of culture conditions

Optimization of culture conditions for keratinase production was studied in 50 mL FM basal medium. The standard incubation conditions were 37°C for 48h. During all optimization steps, both enzyme activity and protein content were determined by varying one parameter at a time while keeping the others constant. The varying physical parameters included the inoculum size (2.5%, 5%, 7.5%, 10%, 12.5% and 15%), pH (4, 5, 6, 7, 7.5, 8, 9 and 10), temperature (room temperature- 28°C and 37°C) and aeration condition i.e., static or shaker (150 rpm and 200rpm). The varying nutritional parameters included the addition of 5% carbon sources such as glucose, sucrose, mannitol, starch, wheat flour, corn cob and coconut shell, and 5% nitrogen sources such as casein, gelatin, soy flour, peptone, ammonium sulfate, urea, ammonium chloride and potassium nitrate. The effect of calcium and magnesium salts was studied during optimization by adding varied concentrations of $CaCl_2$ (0.1 mM - 10 mM) and $MgSO_4$ (0.004 g/l - 0.24 g/l). After studying the effect of different parameters, the estimation of keratinase production was done under optimized conditions [15, 18, 19, 20].

Partial purification and characterization of enzyme

Optimum enzyme yield obtained in our study was partially purified by ammonium sulphate precipitation (achieved at 80% saturation) and dialysis (using cellophane bag with cut off 10kDa). The specific activity, fold of purification and % yield were calculated after the purification of the enzyme. The enzyme was characterized by SDS-PAGE. Zymogram analysis helped in determination of proteolytic ability of enzyme eluted on the electrophoresis gel. Both SDS-PAGE and Zymogram analysis was performed at Millipore Ltd. Andheri, India.

Characterization of keratinase activity

The enzyme activity and stability were studied under different conditions. During standard assay, the tubes containing 1 ml of enzyme and 4mg/ml keratin azure substrate were placed on shaker at 55°C at 200 rpm for 4 h to measure enzyme activity. The activity was also checked after 2, 4, 6, 8, 24, 28, 32 and 48 h to determine its stability. The varying parameters for the study were pH (4, 5, 6, 7, 7.5, 8, 9 and 10), temperature (30°C, 40°C, 50°C and 60°C), substrate concentration (1-7mg/ml keratin azure), metal ions (3mM of $MgCl_2$, $CaCl_2$, $CuCl_2$, $MnCl_2$, $ZnCl_2$, $BaCl_2$, $PbCl_2$, $FeCl_3$ and $HgCl_2$), inhibitors (1% PMSF, DMSO), organic solvents (1% isopropanol, 0.1mM mercaptoethanol) and detergents (0.5% of SDS and Triton X 100, 5Mm EDTA) [15, 18].

Application of keratinase

Substrate specificity and de-hairing ability of keratinase

The substrate specificity of keratinase was determined by incubating it with various substrates like nail, hair, silk, cotton, feather, wool and goat skin for 24 h. To determine the de-hairing ability, goat skin was washed and over laid with 2 ml of enzyme in a petridish. It was incubated at room temperature for 24 h. Macro and microscopic observations were noted [15].

Conversion of feather meal into microbial nutrient for laboratory cultures

Keratinase enzyme produces peptides and soluble proteins (amino acids) on the degradation of feathers. These degradation products can be inexpensive alternatives to peptone, tryptone or meat extract in bacteriological media for the growth of lab cultures. For testing this application, the optimum inoculum of potential keratinase producer was inoculated in sterile feather basal medium and incubated for 24 h at 37°C. After incubation, the feather basal medium was centrifuged at 3000 rpm for 30 min. The supernatant (cell free extract) was stored as enzyme source, in chilled conditions. The precipitate was washed three times to remove dead cells with sterile distilled water. Both supernatant and precipitate were autoclaved and incorporated in different media containing (i) NaCl + supernatant, (ii) NaCl + feather meal, (iii) NaCl + Meat extract (ME), (iv) NaCl + ME + supernatant, (v) NaCl + ME + feather meal and (vi) Nutrient agar (Control). The lab cultures were spot inoculated on these plates and checked for growth after 24 h incubation.

Statistical analysis

All studies were carried out in triplicates and results are reported as mean ± standard deviations.

RESULTS AND DISCUSSION

Screening and isolation of potential keratinase producer

In the present study, seventeen keratinase producing microorganisms were isolated from different sites on FM agar plates. The sources and growth characteristics of all isolates are represented in Table 1. Among the isolates obtained, 8 (N_1, N_2, N_3, N_5, N_6, N_{10}, S_3 and S_4) showed clear and large zones on SMA indicating better protease activity compared to other isolates. These isolates were grown in basal medium with feathers as the sole source of carbon and nitrogen to determine and confirm their keratinase activity. We observed visible degradation of native feathers into small particle size within 48h by 2 isolates (N_1, N_{10}) and within 24h by another 2 isolates (N_2 and S_4). The maximum specific activity was observed for the enzyme produced by isolate N_2 at 24 h, 48 h as well as 72 h intervals (Fig. 1).

Table 1. *Sources of Sampling and Growth Characteristics of Isolates*

Isolate	Source	Gram nature	Colony characteristics	Zone size on SMA(in mm)
N1	Soil	Gram +ve	3 mm, irregular, opaque, orange pigment, flat surface, butyrous consistency	1.3 ± 0.2
N2	Soil	Gram +ve	2 mm, irregular, translucent, orange pigment, flat surface, butyrous consistency	1.45 ± 0.25
N3	Soil	Gram +ve	2.5 mm, irregular, opaque, cream coloured, rough surface, butyrous consistency	1.05 ± 0.55
N4	Soil	Gram +ve	4 mm, irregular, opaque, white coloured, flat surface, butyrous consistency	0.85 ± 0.15
N5	Soil	Gram +ve	0.5 mm, circular, entire margin, cream, translucent, flat surface, butyrous consistency	1.2 ± 0
N6	Soil	Gram +ve	0.5mm, circular, white, opaque, entire margin, raised surface, butyrous consistency	1.35 ± 0.05
N7	Soil	Gram +ve	Spreading, mycelia producing organism, cream in color, butyrous consistency	0.6 ± 0.1
N8	Soil	Gram +ve	Spreading, mycelia producing organisms, cream in color, butyrous consistency	0.55 ± 0.05
N9	Soil	Gram +ve	Spreading, mycelia producing organisms, cream in color, butyrous consistency	0.5 ± 0.1
N10	Soil	Gram +ve	1mm, circular, yellow colored, translucent, entire margin, slightly raised, sticky consistency	1.4 ± 0.2
N11	Waste water	Gram +ve	2mm, circular, cream colored, entire margin, translucent, slightly raised, butyrous consistency	0.4 ± 0.2

Table 1. *Continued.*

Isolate	Source	Gram nature	Colony characteristics	Zone size on SMA(in mm)
N12	Waste water	Gram +ve	3mm, irregular, opaque, mycelia producer, cream colored, rough surface	0.75 ± 0.05
N13	Waste water	Gram +ve	2mm, irregular, cream, opaque, rough surface, slightly raised	1.65 ± 0.75 (opaque zone)
S1	Waste water	Gram +ve	2mm, irregular, cream colored, opaque, flat surface, butyrous consistency	0.25 ± 0.05
S2	Poultry wastes	Gram +ve	1mm, circular, cream colored, translucent, entire margin, butyrous consistency	0.35 ± 0.05
S3	Poultry wastes	Gram +ve	3mm, circular, opaque, white colored, flat surface, butyrous consistency	0.7 ± 0.1
S4	Compost	Gram +ve	2mm, irregular, cream colored, opaque, rough surface, flat colony	0.75 ± 0.15

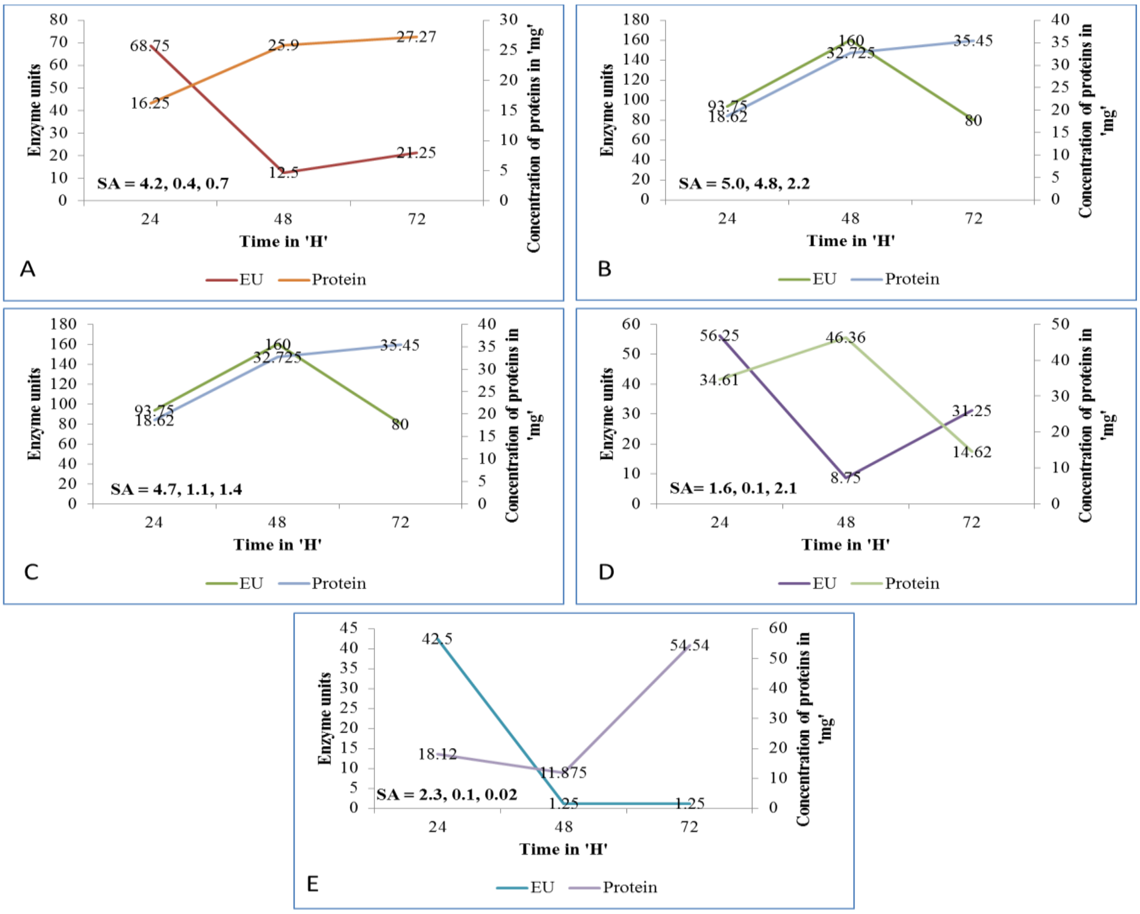

Fig. 1. *Enzyme Activity of Potential Keratinase Producers*
**A to D: represent isolates N1, N2, N10 and S4 respectively; E: represents un-inoculated sample used as control; SA indicates specific activity of enzyme in 24, 48 and 72 h respectively*

Most of the published studies report steps for enrichment of keratinase producers from soil samples before its isolation to ensure presence of significant bacterial load [21, 22, 23]. In the present study, we skipped this step since the selected sampling sites were assumed to be naturally enriched with keratinase producers. A similar strategy was followed by Prasad et al. [24] where 8 potential keratinase producers were obtained in their study. Another study from Aurangabad reported screening of protease producing bacteria from 6 soil samples on SMA [25]. Among the 17 proteolytic enzyme producing bacteria isolated, they selected the isolate with highest enzyme activity, and tested it for keratinase production. This study was also based on the assumption that since all samples were collected from feather dumping grounds, the strains showing proteolytic activity may be keratinase producers.

Identification of potential isolates

The isolate with maximum specific activity (Isolate N_2) was identified as *Bacillus* sp. based on cultural, morphological, biochemical (Table 2) as well as 16s rRNA sequencing (with 97% similarity profile). The sequences were submitted to GenBank (accession no. OP882018) as *Bacillus* sp. AM251. The microscopic characteristics (including gram staining and endospore staining) of *Bacillus* sp. AM251 and visible degradation of the whole feather within 24 h is represented in Fig. 2.

Bacillus species are among the most commonly reported keratinase producers in literature. Raju and Divakar, [26] isolated 6 keratinase producing bacteria from soil samples, out of which 4 were identified as Bacillus spp. A novel strain *Bacillus tropicus* Gxun-17 was recently reported from a marine duck farm in China. The analysis of metabolic end products of this strain further suggested that feather degradation may be a result of synergistic activity between the keratinase enzyme and sulphites [27]. Maximum keratinase activity has also been reported in Bacillus spp. isolated from poultry farm (*Bacillus* sp. MK3), feather dumping grounds (*B. subtilis*) and puffer fish tannery waste (*B. firmus* BRAW_PI) [28, 29, 30].

Table 2. *Biochemical Characteristics of Keratinase Producer*

Biochemical tests	**Results**
Catalase test	+ ve
Lecithinase production	-ve
Nitrate reduction test	-ve
Indole test	-ve
Voges-proskauer test	-ve
Starch hydrolysis test	+ve
Citrate test	+ve
Gelatin hydrolysis test	-ve
Glucose	+ve
Maltose	+ve
Sucrose	+ve
Mannitol	-ve
Motility test	-ve

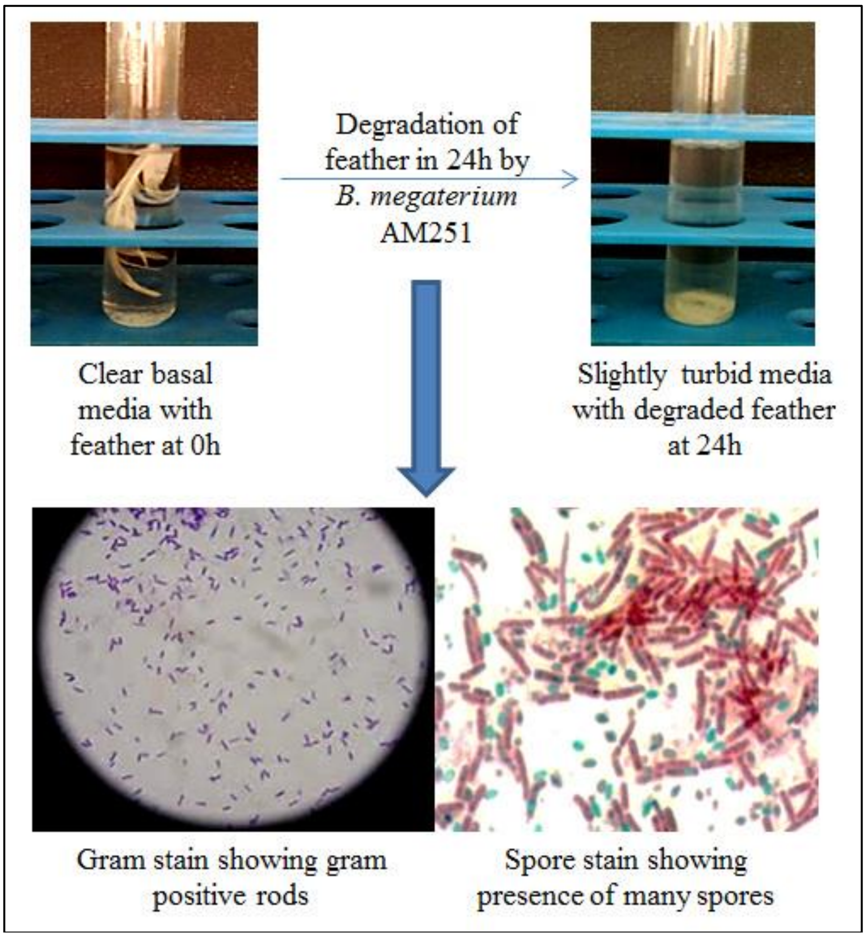

Fig. 2. *Morphological Characteristics and Feather Degrading Ability of Bacillus sp. AM251*
**Gram staining and spore staining was observed under 100x magnification of oil immersion lens*

Optimization of nutrient and incubation conditions

On standardization of Keratin azure assay, optimum enzyme activity was observed at 55˚C in 4 h using 0.4% substrate by all four isolates (N_1, N_2, N_{10} and S_4) when incubated at shaker conditions (200 rpm). Hence for optimization studies, 1 ml of crude enzyme (cell free supernatant) was incubated with 0.4% of keratin azure substrate at the above conditions for further studies. The parameters were optimized one at a time by keeping the others constant. The Fig. 3 represents the effect of nutritional and physicochemical parameters on enzyme production by *Bacillus* sp. AM251. Maximum enzyme production was observed in basal media (pH 7) containing ammonium sulfate (2.5g/L), $MgSO_4$ (0.002g/L), $CaCl_2$ (0.05g/L) and feather (10g/L) on the addition of 7.5% inoculum size. On the optimization of nutrient sources, the maximum specific activity was observed in 24h. Bacillus species are normal soil flora and show optimum growth under natural environmental conditions [31]. *Bacillus* sp. AM251 showed negligible growth at temperatures below 20°C and above 45°C. For this reason, keratinase production was determined only under room temperature (~28°C) and 37°C. The enzyme activity was lowest under static conditions (1.068 EU/mg) followed by incubation at 150rpm (1.346 EU/mg). Optimum incubation parameters were observed to be 37°C (1.723 EU/mg) and shaker conditions (200 rpm; 3.33 EU/mg) for keratinase production by *Bacillus* sp. AM251. Overall, on optimization, the specific activity of the enzyme was improved by 3.973 folds. Among other *Bacillus* sp. maximum enzyme yield from strain IIB-B5 was obtained at pH 7.0 and 40°C in 72 h. In the same study, another thermotolerant strain IIB-B9 was isolated and showed maximum yield at pH 8.0 in 96 h, at 50°C [32]. In a recent study, a novel *B. tropicus* Gxun-17 strain showed optimum keratinase production in basal medium (pH 7.0) containing maltose (10 g/L), feather (15 g/L) and $MgSO_4$ (0.1 g/L) when it was incubated at 32.5°C. The enzyme yield was 3.18 fold higher under optimum conditions [27]. Compared to these strains, *Bacillus* sp. AM251 showed better activity in a shorter incubation period (of 24 h).

In our study, keratinase activity was enhanced by ammonium sulphate (nitrogen source) whereas it was repressed by the addition of carbon sources. Generally, the secretion of microbial proteases is regulated by nutritional stress [10]. However, few studies have indicated that carbon and nitrogen sources may aid in the induction of keratinase enzyme activity. For instance, yeast extract, $MgSO_4$ and corn flour improved keratinase production by *Amycolatopsis* sp. strain MBRL 40 isolated from a limestone habitat [33]. Similarly, fructose and peptone in 40:1 ratio, in the growth medium of *Pseudomonas* sp. LM19 showed optimum yield [34]. One study reported optimum keratinase production by *Pseudomonas aeruginosa* YK17 in a medium containing 2% chicken feathers, beef extract, ammonium nitrate and glucose [35]. However, *Bacillus* sp. AM251 preferred other carbon sources like glucose, maltose, sucrose and starch over feather (keratin source) when supplemented together in the medium (data not shown). The magnesium ions in the medium act as cofactors whereas calcium is required to improve the thermostability of enzymes [36, 37]. Hence, these sources were added to the basal medium. The addition of 0.5mM Mn^{2+}, Ca^{2+} and Mg^{2+} also led to 1.5 fold increase in keratinase production by *Bacillus subtilis* amr [38]. Similar to our study, repression of keratinase synthesis has been reported in *B. licheniformis* PWD-1 in the presence of glucose, sucrose, starch and mannitol [39]. Few studies have also reported enzyme repression in the presence of inorganic or organic nitrogen sources [18, 22, 39]. This observation was true for all nitrogen sources used in our study except ammonium sulphate. In many studies, the addition of yeast extract has shown enhanced keratinase yield [20, 33, 41, 42]. Overall, these observations suggest unique metabolic response of microorganisms to the neighboring environment which is not only specific to one species but also characterizes the biochemistry of individual strains. Hence, the isolation and optimization of enzyme producers from the environment remain the most crucial step in industrial biotechnology.

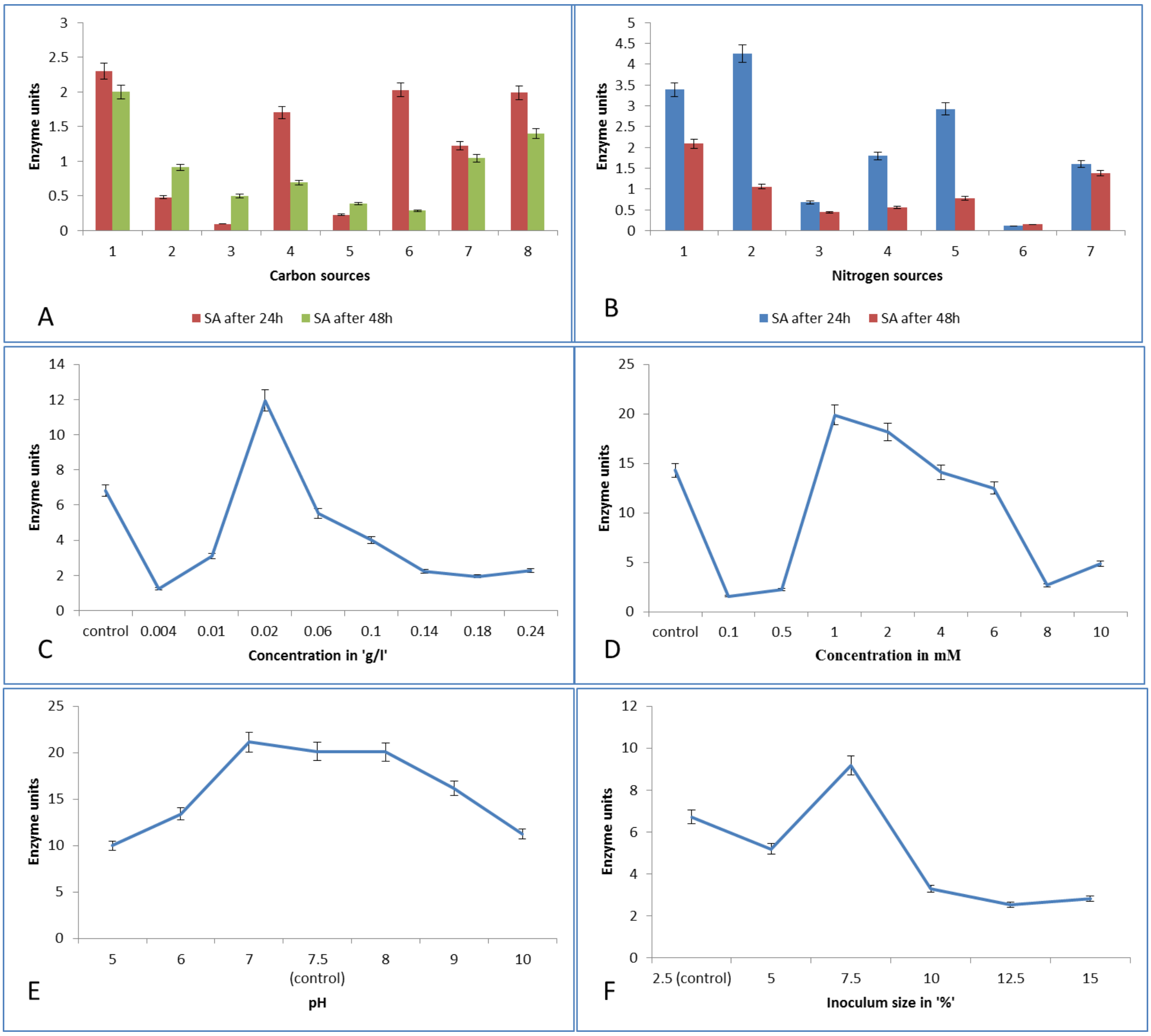

Fig. 3. *Effect of Nutritional and Incubation Conditions on Keratinase Production observed in 24h*
**A: bar 1 represents 'control' and bars 2-8 represent 5% glucose, sucrose, starch, mannitol, wheat flour, corn cob and coconut shell respectively; B: bar 1 represents 'control' and bars 2-7 represent 2.5% ammonium sulphate, urea, potassium nitrate, peptone, yeast extract and soy flour respectively; C: Effect of varying concentration of Magnesium; D: Effect of varying concentration of Calcium; E:Effect of varying pH; F: Effect of varying inoculum size); SA indicates specific activity of enzyme.*

Partial purification and characterization of enzyme

Precipitation and dialysis of enzyme resulted in 1.5 fold increase in purification and 9% increase in yield (Table 3). The Fig. 4 represents SDS-PAGE (A) and zymogram (B) analysis of keratinase enzyme. The Zymogram analysis confirmed the proteolytic activity of the partially purified enzyme. The analytical studies indicated that the keratinase enzyme produced by *Bacillus* sp. AM251 is a 43kDa serine protease. Fig. 5 represents the activity and stability of enzyme under different conditions. The optimum enzyme activity was observed at 50°C and pH 8.0. It was stable between pH 8 and 9.5 and 30°C for 2 h. It also showed stability in presence of metals like $MgSO_4$ and $CaCl_2$ and detergents like SDS and Triton, whereas $MnCl_2$, $HgCl_2$, $CuCl_2$ and $ZnCl_2$ decreased the enzyme activity. Inhibition of enzyme activity in presence of PMSF indicated that it is a serine protease.

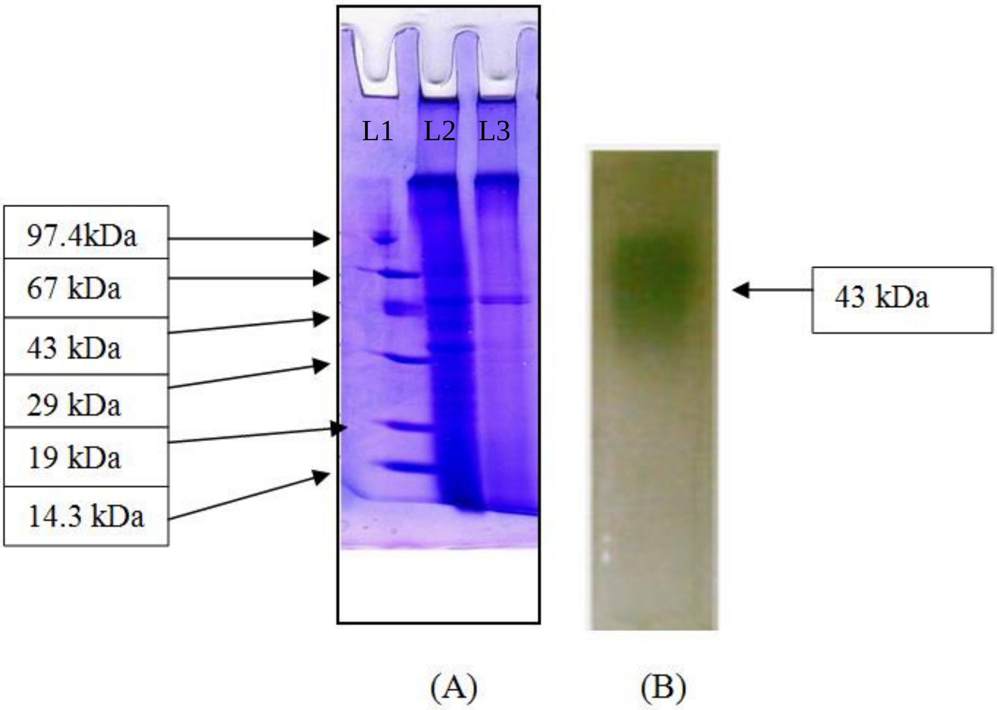

Fig. 4. *SDS-PAGE (A) and Zymogram (B) analysis of keratinase*
L1: Molecular weight marker; L2: Crude enzyme; L3: purified enzyme

Table 3. *Activity and Yield of Partially Purified keratinase*

Purification	Total protein (mg/ 50mL)	Enzyme units	Specific activity	Purification fold	Yield %
Before precipitation	6.55	80	12.2137	-	-
After precipitation	-	0.5	-	-	-
After dialysis	10.6	198.75	18.75	1.5351	9.0625

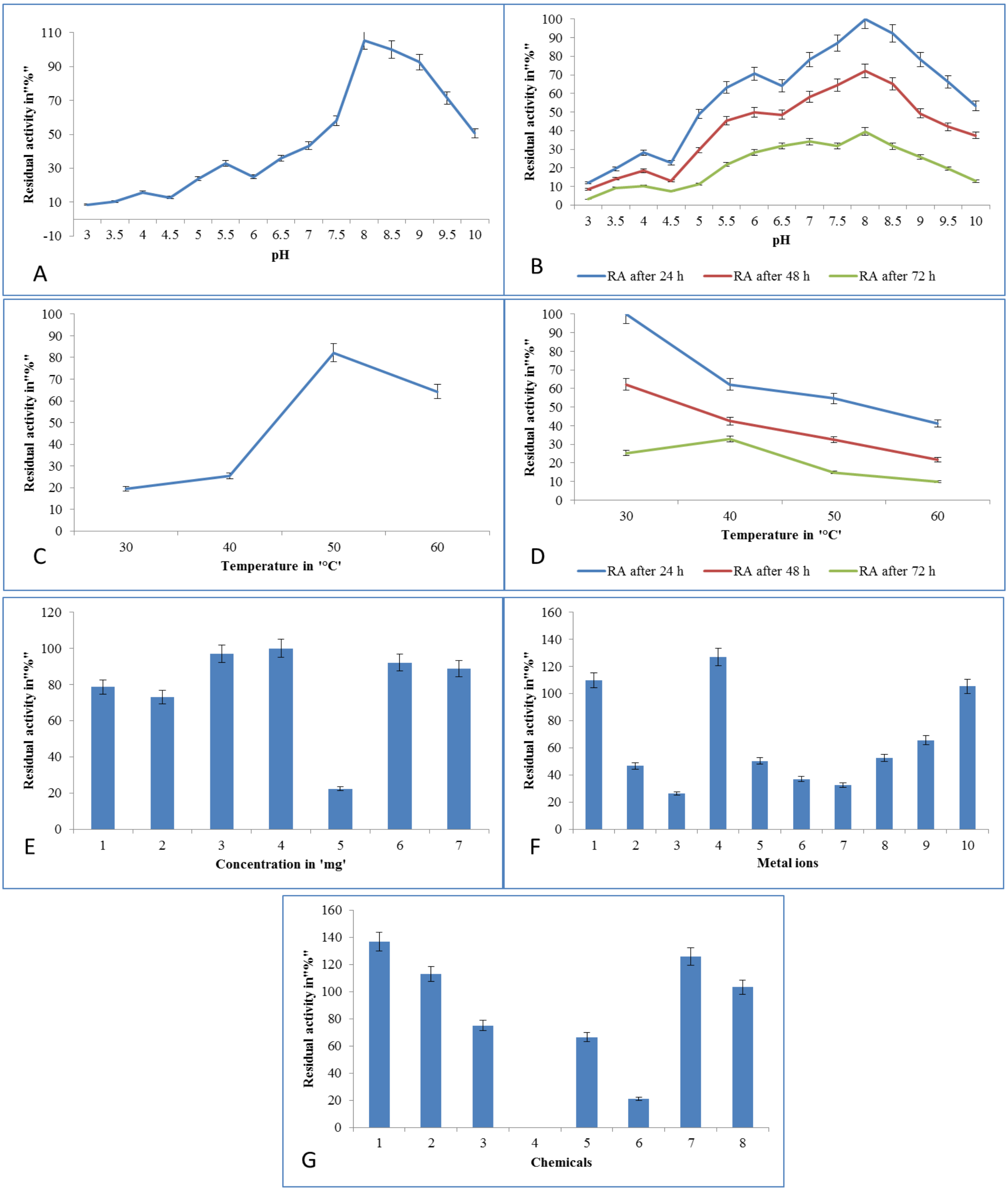

***Fig. 5.** Enzyme activity and stability under different conditions*
**A: Effect of varying pH; B: Stability at varying pH; C: Effect of Temperature; D: Stability at varying Temperature; E: Effect of varying substrate concentration; F: Effect of metal ions where bars 1-10 represents $MgCl_2$, $MnCl_2$, $ZnCl_2$, $CaCl_2$, $CuCl_2$, $HgCl_2$, $BaCl_2$, $FeCl_3$ and $PbCl_2$ respectively; G: Effect of inhibitor, solvents and detergents where bars 1-8 represents 0.5% SDS, 0.5% Triton X 100, 5Mm EDTA, 1% PMSF, 1% DMSO, 1% isopropanol, 0.1mM mercaptoethanol and control respectively.*

The proteolytic activity and de-amination reactions, occurring during feather degradation, lead to the accumulation of excess nitrogenous compounds like ammonium salt. In turn, it causes an increase in the pH of the medium and consequently reduces keratinase activity [43, 44]. Hence, keratinases which are stable at higher (alkaline) pH are preferred for industrial applications. As observed in Fig. 5, significant specific enzyme activity was observed up to pH 10 in this study. At the same time the enzyme activity of partially purified keratinase was not affected in the presence of detergents like SDS and Triton; indicating its potential in the detergent industry. The activity of serine protease obtained from *B. tropicus* Gxun-17 showed optimum activity at pH 7.0 and temperature of 60°C. The Km and Vmax of keratinase activity using casein substrate were reported to be 15.24 mg/mL and 0.01 mg/mL/min, respectively. Unlike our study, this enzyme was inhibited in the presence of Mg^{2+} and Ca^{2+} but showed increased activity in the presence of Mn^{2+} and β-mercaptoethanol. In addition, the enzyme activity was inhibited by K^{+}, Co^{2+} and Al^{3+} [27]. The enzyme activity of keratinase produced by *B. subtilis* KD-N2 was optimum over a much wider temperature range (40~70°C) compared to our study. Similar to the above study, and contrary to our observation, this enzyme was also inhibited by Mg^{2+} and Ca^{2+}. Additionally, many metal ions like Cu^{2+}, Mn^{2+}, Zn^{2+} and Al^{3+} also inhibited the enzyme produced by *B. subtilis* KD-N2 [15]. Similar to our study, serine protease produced by *Bacillus* sp. P7 isolated from an Amazonian environment was stimulated by Mg^{2+} and Ca^{2+} and inhibited by other metal ions [45]. Overall, keratinase activity over broad pH and temperature range and, in presence of common detergent like SDS is an important characteristic required in various industries, and primarily in the detergent industry. Besides, inhibition of enzyme activity in presence of heavy metals is a common feature [15, 46]. Hence, it can be stated that the keratinase obtained from *Bacillus* sp. AM251 showed comparable characteristics to those enzymes obtained from bacterial strains using sophisticated enrichment and optimization protocols.

Applications of keratinase

At present, the global market of enzymes accounts for approximately 1.19 billion USD and is expected to reach 9.2 billion USD by 2027 [47]. This tremendous demand is due to its application in various industries including tanning, poultry, detergent, construction (for removal of clogging in sewage systems), cosmetic and medical. For an enzyme to be practically applied in industries, its activity on a wide range of substrates is of foremost importance. Table 4 represents the residual activity of partially purified enzyme using different substrates. It showed maximum residual activity in the presence of chicken feathers and wool, and least activity in the presence of human hair. This observation suggests that *Bacillus* sp. AM251 strain may have adapted to the polluted environment of dumping grounds over time leading to production of enzymes with high substrate specificity. Our previous observations indicating that the test strain prefers other carbon sources over feather further supports the hypothesis of bacterial adaptation. However, an in-depth evolutionary analysis is required to confirm the same.

Another application tested in our study was the de-hairing ability of keratinase. The alkaline keratinases cause swelling of hair roots and weakening of hair follicles. Under these conditions, the protease activity of the enzyme enables easy removal of hair. As observed in Fig. 6, the partially purified keratinase used in our study selectively damaged the fibrous collagen dermis preventing any visual damage to the goat skin and leading to hair removal within 24 h. This indicates that the enzyme may be useful as a depilatory agent in tanning industries. Similarly, keratinases obtained from *B. subtilis* S14 and *B.*

subtilis KD-N2 have shown efficient dehairing potential without harming the collagen layer [15, 48].

Lastly, we checked the application of keratinase to convert feathers into microbial feed that can be utilized as an alternative to peptone and meat extract. Table 5 represents the growth of laboratory isolates on media supplemented with keratinase treated feathers and control. As observed in the table, only *E. coli* could grow on agar plates supplemented with feather meal, while all other isolates were inhibited. However, this growth was observed after 5 days of incubation. It may be possible that other laboratory isolates also showed growth on further incubation, but long incubation time is not feasible for routine laboratory work. Hence, this application did not give the expected outcomes in this study. To the best of our knowledge, none of the published studies have studied the application of keratinase to transform feathers into microbial supplement for growth of laboratory isolates. This study was conducted to explore another possible application of keratinase enzyme to avail cheap sources of microbial nutrients.

Table 4. *Substrate specificity of Partially Purified Enzyme*

Substrate	Residual activity %
Casein	68
Gelatin	34
Feather (chicken)	85
Nail (human)	23
Hair (human)	2
Silk	57
Wool	80
Control	100

Table 5. *Application of Keratinase Enzyme to Convert Feather into microbial feed*

Media	A	B	C	D	E	F
NaCl + supernatant	-	-	-	-	-	-
NaCl + feather meal	+ *	-	-	-	-	-
NaCl + ME	+	+	+	+	+	+
NaCl + ME + supernatant	+	+	+	+	+	+
NaCl + ME + feather meal	+	+	+	+	+	+
Nutrient agar (Control)	+	+	+	+	+	+

**: + = growth; - = no growth; ME=Meat Extract;*

*A:Escherichia coli; B: Staphylococcus aureus; C: Streptococcus pyogenes; D: Bacillus subtilis; E: Corynebacterium diphtheria; F: Salmonella typhi *Growth observed after 5 days of incubation*

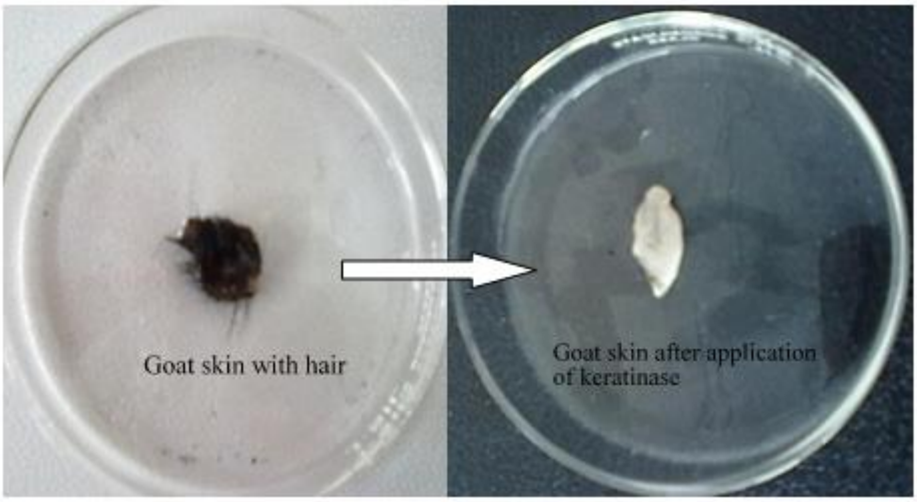

Fig. 6. *De-hairing ability of keratinase on goat hides*

CONCLUSION

The tolerance of partially purified enzyme to alkaline conditions and higher temperature is an advantageous characteristic that can be exploited in various industries. In addition, the high residual activity observed in the presence of SDS and triton suggests a significant application of enzyme obtained in this study in the detergent industries. Also, the selective action of enzyme on goat hair makes this enzyme a valuable candidate for use in tannery and food industries. Most interestingly, the *Bacillus* sp. AM251 strain isolated in this study showed maximum enzyme yield with good activity within 24 h. Thus, our study reports a valuable strain isolated from soil samples with minimum efforts that show higher keratin degrading capability compared to many isolates indicated in the literature.

Conflict of Interest. The authors declared that there is no conflict of interest.

Authorship Contributions. Concept: A.S., M.T., Design: A.S., M.T., Data Collection or Processing: A.S., M.T., Analysis or Interpretation: A.S., M.T., Literature Search: A.S., M.T., Writing: A.S., M.T.

Financial Disclosure. This research received no grant from any funding agency/sector.

REFERENCES

[1] Bragulla, H. H., Homberger, D. G. (2009): Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia. Journal of Anatomy 214(4): 516–559.

[2] Moll, R., Divo, M., Langbein, L. (2008): The human keratins: biology and pathology. Histochemistry and Cell Biology 129(6): 705–733.

[3] Qiu, J., Wilkens, C., Barrett, K., Meyer, A. S. (2020): Microbial enzymes catalyzing keratin degradation: Classification, structure, function. Biotechnology Advances 44: 107607.

[4] Paul, T., Mandal, A., Mondal, K. C. (2018): Waste to value aided fertilizer: an alternative cleaning technique for poultry feathers waste disposal. Annals of Microbiology and Immunology 1(2): 1006.
[5] Lange, L., Huang, Y., Busk, P. K. (2016): Microbial decomposition of keratin in nature- a new hypothesis of industrial relevance. Applied Microbiology and Biotechnology 100(5): 2083–2096.
[6] Franke- Whittle, I. H., Insam, H. (2013): Treatment alternatives of slaughterhouse wastes, and their effect on the inactivation of different pathogens: a review. Critical Reviews in Microbiology 39(2): 139–151.
[7] Woo, Y. K., Kim, J. H. (2006): Fowl cholera outbreak in domestic poultry and epidemiological properties of *Pasteurella multocida* isolate. Journal of Microbiology (Seoul Korea) 44(3): 344–353.
[8] Yamamoto, Y., Nakamura, K., Yamada, M., Mase, M. (2010): Persistence of avian influenza virus (H5N1) in feathers detached from bodies of infected domestic ducks. Applied and Environmental Microbiology 76(16): 5496–5499.
[9] Kumar, J. (2021): Microbial hydrolysed feather protein as a source of amino acids and protein in the diets of animals including poultry. In (Ed.) Advances in Poultry Nutrition Research. IntechOpen. https://doi.org/10.5772/intechopen.96925
[10] Vidmar, B., Vodovnik, M. (2018): Microbial Keratinases: Enzymes with promising biotechnological applications. Food Technology and Biotechnology 56(3): 312–328.
[11] Peng, Z., Mao, X., Zhang, J., Du, G., Chen, J. (2020): Biotransformation of keratin waste to amino acids and active peptides based on cell-free catalysis. Biotechnology for Biofuels 13: 61.
[12] Lagos, J. E., Intodia, V. (2016): Market development reports on livestock and products. Hides and Skins - 2016 Global Agricultural Information network. GAIN Report Number IN6078. Available at: https://agriexchange.apeda.gov.in/MarketReport/Reports/Hides_Skins%20-%202016_New%20Delhi_India_6-9-2016.pdf (Accessed 20th October 2020).
[13] Mokrejš, P., Huťťa, M., Pavlačková, J., Egner, P. (2017): Preparation of keratin hydrolysate from chicken feathers and its application in cosmetics. Journal of Visualized Experiments: JoVE (129): 56254.
[14] Hassan, M. A., Haroun, B. M., Amara, A. A., Serour, E. A. (2013): Production and characterization of keratinolytic protease from new wool degrading *Bacillus* species isolated from Egyptian ecosystem. BioMed Research International 2013: 175012.
[15] Cai, C. G., Chen, J. S., Qi, J. J., Yin, Y., Zheng, X. D. (2008): Purification and characterization of keratinase from a new *Bacillus subtilis* strain. Journal of Zhejiang University. Science B 9(9): 713–720.
[16] Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951): Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry 193(1): 265–275.
[17] Bergey's Manual of Determinative Bacteriology (7th ed.). (1964): American Journal of Public Health and the Nations Health 54(3): 544.
[18] Barman, N. C., Zohora, F. T., Das, K. C., Mowla, M. G., Banu, N. A., Salimullah, M., Hashem, A. (2017). Production, partial optimization and characterization of keratinase enzyme by Arthrobacter sp. NFH5 isolated from soil samples. AMB Express 7(1): 181.
[19] Ramnani, P., Gupta, R. (2004): Optimization of medium composition for keratinase production on feather by *Bacillus licheniformis* RG1 using statistical methods involving response surface methodology. Biotechnology and Applied Biochemistry 40(2): 191–196.
[20] Mazotto, A. M., Coelho, R. R., Cedrola, S. M., de Lima, M. F., Couri, S., Paraguai de Souza, E., Vermelho, A. B. (2011): Keratinase production by three *Bacillus* spp. using feather meal and whole feather as substrate in a submerged fermentation. Enzyme Research 2011: 523780.
[21] Suharti, D. R., Tyas., and Nilamsari, N. R. (2019): Isolation and characterization of a newly keratinase producing *Bacillus* sp. N1 from tofu liquid waste. IOP Conference Series: Earth

and Environmental Science, International Conference on Green Agro-industry and Bioeconomy 18–20 September 2018, Universitas Brawijaya, East Java Indonesia, 230:012088.

[22] Nnolim, N. E., Udenigwe, C. C., Okoh, A. I., Nwodo, U. U. (2020): Microbial keratinase: next generation green catalyst and prospective applications. Frontiers in Microbiology 11: 580164.

[23] Zhang, R. X.., Wu, Z. W., Cui, H. Y., Chai, Y. N., Hua, C. W., Wang, P., Li, L., Yang, T. Y. (2022): Production of surfactant-stable keratinase from *Bacillus cereus* YQ15 and its application as detergent additive. BMC Biotechnology 22: 26.

[24] Prasad, H. V., Kumar, G., Karthik, L., Rao, K. V. B. (2010): Screening of extracellular keratinase producing bacteria from feather processing areas in Vellore, Tamil Nadu, India. Journal of Scientific Research 2(3): 559-565.

[25] Subugade, S., Gupta, S. G., Mokashe, S. (2017): Isolation and screening of keratinase producing bacteria from chicken feather dumping site. International Journal of ChemTech Research 10(5): 900-905.

[26] Raju, E. V. N., Divakar, G. (2013): Screening and isolation of keratinase producing bacteria from poultry waste. International Journal of Pharmaceutical Research & Allied Sciences 2(1): 70-74.

[27] Shen, N., Yang, M., Xie, C., Pan, J., Pang, K., Zhang, H., Wang, Y., Jiang, M. (2022): Isolation and identification of a feather degrading *Bacillus tropicus* strain Gxun-17 from marine environment and its enzyme characteristics. BMC Biotechnology 22: 11.

[28] Karadaglibahri, M., Ozcan, D. (2022): Isolation of keratinase-producing *Bacillus* strains and enhanced enzyme production using in vitro mutagenesis. Cellular and molecular biology 94 (1): 2022.

[29] Sekar, V., Kannan, M., Ganesan, R. Dheeba, B., Sivakumar, N., Kannan, K. (2016): Isolation and screening of keratinolytic bacteria from feather dumping soil in and around Cuddalore and Villupuram, Tamil Nadu. Proceedings of the National Academy of Sciences, India Section B: Biological Sciences 86: 567–575.

[30] Martindro, R. L., Wibowo, S. A., Bachruddin, Z., Fitriyanto, N. A., Nakagawa, T., Hayakawa, T., Pertiwiningrum, A. (2017): Screening and characterization of keratinolytic bacteria from Puffer fish skin waste. Pakistan Journal of Nutrition 16: 488-496.

[31] Buchanan, R. E., Gibbons, N. E. (1974): Bergey's Manual of Determinative Bacteriology. 8th Edition, Williams and Wilkins, Baltimore, pp. 1268.

[32] Ahmed, M., Arshad, Y., Mukhtar, H. (2019): Isolation and screening of keratinase producing bacteria from soil. Biologia – Journal of Biological Society of Pakistan 65(2): 1-6.

[33] Ningthoujam, D. S., Jaya, L., Devi, P. J., Kshetri, P., Tamreihao, K., Mukherjee, S., Devi, S. S., Nongthombam, B. (2016): Optimization of keratinase production by *Amycolatopsis* sp. Strain MBRL 40 from a Limestone Habitat. Journal of Bioprocess and Biotechnology 6: 282.

[34] Nurliyana, M., Lai-yee, P., Suraini, A. A. (2017): Optimization of metallo-keratinase production by Pseudomonas sp. LM19 as a potential enzyme for feather waste conversion. Biocatalysis and Biotransformation 35(1): 41-50.

[35] Moonnee, Y. A., Foysal, M. J., Hashem, A., Miah, M. F. (2021): Keratinolytic protease from *Pseudomonas aeruginosa* for leather skin processing. Genetic Engineering & Biotechnology 19(1): 53.

[36] Razzaq, A., Shamsi, S., Ali, A., Ali, Q., Sajjad, M., Malik, A., Ashraf, M. (2019): Microbial Proteases Applications. Frontiers in Bioengineering and Biotechnology 7: 110.

[37] Sharma, M., Gat, Y., Arya, S., Kumar, V., Panghal, A., Kumar, A. (2019): A review on microbial alkaline protease: an essential tool for various industrial approaches. Industrial Biotechnology 15(2): 69-78.

[38] Mazotto, A. M., Cedrola, S. M. L., de Souza, E. P., Couri, S., Vermelho, A. B. (2022): Enhanced keratinase production by Bacillus subtilis amr using experimental optimization tools to obtain feather protein lysate for industrial applications. 3 Biotech 12(4): 90.

[39] Lin, X., Wong, S. L., Miller, E. S., Shih, J. C. (1997): Expression of the *Bacillus licheniformis* PWD-1 keratinase gene in *B. subtilis*. Journal of Industrial Microbiology and Biotechnology 19(2): 134-138.

[40] Gurav, R. G., Jadhav, J. P. (2013): Biodegradation of keratinous waste by Chryseobacterium sp. RBT isolated from soil contaminated with poultry waste. Journal of Basic Microbiology 53: 128–135.

[41] Fakhfakh, N., Kanoun, S., Manni, L., Nasri, M. (2009): Production and biochemical and molecular characterization of a keratinolytic serine protease from chicken feather-degrading *Bacillus licheniformis* RPk. Canadian Journal of Microbiology 55: 427–436.

[42] de Paiva, D. P., de Oliveira, S. S., Mazotto, A. M., Vermelho, A. B., de Oliveira, S. S. (2019): Keratinolytic activity of *Bacillus subtilis* LFB-FIOCRUZ 1266 enhanced by whole-cell mutagenesis. 3 Biotech 9(1): 2.

[43] Jeevana, L. P., Kumari, C. C., Lakshmi, V. V. (2013): Efficient degradation of feather by keratinase producing Bacillus sp. International Journal of Microbiology 2013: 608321.

[44] Nagal, S., & Jain, P. C. (2010): Feather degradation by strains of Bacillus isolated from decomposing feathers. Brazilian Journal of Microbiology 41(1): 196–200.

[45] Corrêa, A. P. F., Daroit, D. J., Brandelli, A. (2010): Characterization of a keratinase produced by *Bacillus* sp. P7 isolated from an Amazonian environment. International Biodeterioration & Biodegradation 64(1): 1-6.

[46] Thys, R. C. S., Lucas, F. S., Riffel, A., Heeb, P., Brandelli, A. (2004): Characterization of a protease of a feather-degrading Microbacterium species. Letters Applied Microbiology 39(2): 181–186.

[47] News article by Globe Newswire. (2021): Global Industrial Enzymes Market Size & Share Witnessing Consistent Growth—Projected to Reach worth USD 9.2 Billion by 2027 BlueWeave Consulting and Research Pvt Ltd. Available at https://www.globenewswire.com/en/news-release/2021/10/12/2312804/0/en/Global-Industrial-Enzymes-Market-Size-Share-Witnessing-Consistent-Growth-Projected-to-Reach-worth-USD-9-2-Billion-by-2027-BlueWeave.html (Accessed 9th November 2022).

[48] Macedo, A. J., da Silva, W. O. B., Gava, R., Driemerier, D., Henriques, J. A. P., Termignoni, C. (2005): Novel keratinase from *Bacillus subtilis* S14 exhibiting remarkable dehairing capabilites. Applied Environmental Microbiology 71(1): 594–596.