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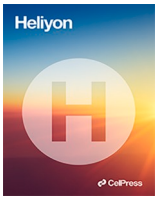

Research article

Microbial production of keratinase from *Bacillus velezensis* strain MAMA: A novel enzyme for eco-friendly degradation of keratin waste

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ABSTRACT

Keratin waste has become an increasingly serious environmental and health hazard. Keratin waste is mainly composed of keratin protein, which is one of the most difficult polymers to break down in nature and is resistant to many physical, chemical, and biological agents. With physical and chemical methods being environment damaging and costly, microbial degradation of keratin using keratinase enzyme is of great significance as it is both environment friendly and cost-effective. The aim of this study was to extract and purify keratinase from bacterial species isolated from the soil. Among the organisms, an isolate of *Bacillus velezensis*, coded as MAMA could break down chicken feathers within 72 hours (h). The isolated strain produced significant levels of keratinase in mineral salt medium by supplying chicken feathers as the sole source of nitrogen and carbon. Feather deterioration was observed with the naked eye, and enzyme activity was evaluated using a spectrophotometric assay. Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and zymography results revealed that the keratinase protein produced by *Bacillus velezensis* had a molecular weight between 40 and 55 kilodalton (kDa).

1. Introduction

The myriad activities of human civilization have caused enormous buildup of solid waste in the environment. Due to overpopulation, management and disposal of solid waste has become a demanding issue for different societies of the world [1]. Keratin waste from slaughterhouses, leather tanneries, the wool industry, and textile factories is a major source of solid waste [2], with the USA, Brazil, and China being the main producers of over 40 million tons of keratin byproducts, annually [3]. Common waste product in poultry slaughterhouses, consist of 2–5 percent (%), sulphur, 15–18 % nitrogen, 3.20 % minerals, 1.27 % fat, and 90 % protein [3]. Keratin waste accumulates and causes environmental issues such as odors and infectious agents in the air, water, and soil. Improper disposal of poultry feathers can lead to human health problems, such as chlorosis and fowl cholera, owing to pathogenic microflora [4]. Wastewater with keratin pollutes the environment and harms people living nearby, and causes acidification, eutrophication, and

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species loss [1]. Feathers can be processed into starting materials, thereby reducing their environmental impacts. Feathers can be converted into clothes, decorations, medical devices, fertilizers, dusters, bedding materials, and feedstocks through washing and chemical treatment [5]. However, these processes require a large amount of energy, and the nutrients in the feathers are not preserved. Therefore, the economic conversion of feather waste into valuable products is of great interest to researchers. Chemical hydrolysis of feathers yields proteins and amino acids but requires high energy and affects product quality. Microbial degradation of feathers is possible, with many microorganisms being able to break down keratin. Studies suggest that feathers can be converted into bio fertilizers via microorganisms, providing a cost-effective way to use keratin from these wastes [5]. Keratinase enzymes produced by microorganisms are possible alternatives, resulting in more useful and toxin-free products [6]. Keratinase is a proteolytic enzyme that breaks down peptide bonds in keratin [7]. Breaking keratin with microbial keratinase can help to convert waste into valuable products for a more sustainable economy. The exact mechanism by which keratinase enzymes break down keratin is not clearly understood. However, it is thought to involve the breaking of peptide bonds and reduction of disulfide bridges [8]. Keratinases are serine and metalloproteases or serine metalloproteases [9]. Bacteria and fungi have been found to be good decomposers of keratin, by producing keratinolytic enzymes. Various bacteria and fungi can break down keratin, a tough protein found in the hair, feathers, and nails. Feather-degrading bacteria have been isolated from soil and poultry waste, including *Streptomyces* and *Bacillus* species as well as *Arthrobacter*, *Microbacterium*, and *Kocuria rosea*. Some thermophilic and alkaliphilic strains of *Bacillus*, *Fervidobacterium*, *Thermoanaerobacter keratinophilus*, *Nesternkonia*, and *Nocardiopsis* have been found to have keratinolytic activity. Additionally, Gram-negative bacteria such as *Vibrio* species *Xanthomonas maltophilia*, *Stenotrophomonas* species, and *Chryseobacterium* species have been isolated from decomposing chicken feathers [10]. *Aspergillus*, *Penicillium*, *Fusarium*, *Microsporum*, *Trichoderma*, and *Chrysosporium* were the most common keratinolytic fungi [11]. Identifying and isolating microorganisms that produce high-yield keratinase enzymes can assign high value to the keratinous waste biomass by significantly adding to the bio-economy through production of protein hydrolysates, nutrient alternates for microbial media, plant growth hormones, organic fertilizers and substrates for production of commercially important enzymes [12]. Although difficult to cultivate, these strains can still provide keratinase genes for expression in other production hosts. Microorganisms adapted to keratin-only environments are the best sources of keratinases. Strains can be screened for keratinolytic potential using qualitative or semi-quantitative methods and then characterized using molecular and biochemical techniques. Many keratinases have been studied, but the perpetuity to discover new keratinases with improved properties, such as higher catalytic efficiency, increased stability at high temperatures and pH levels, and greater resistance to feedback inhibition, remains far from over. Isolation methods and techniques, such as cloning genes from metagenomes and protein engineering, can expand enzyme diversity. Our study describes the production and purification of a keratinolytic protease from the *Bacillus velezensis* strain MAMA that grows well on feathers as the sole source of carbon and nitrogen. The strain can degrade feathers, and the enzyme can be used to convert keratin waste into value-added products, thereby reducing the threat to the environment and human health.

2. Material and methods

2.1. Sample collection

Samples were collected from different areas in Peshawar. Different sampling sites were selected because a large quantity of keratinous waste was dumped on a regular basis. Soil samples were collected in sterile Falcon Tubes of 50 milliliters (mL) at a depth of approximately 3–4 centimeters (cm). The samples were shifted to the Main Research Laboratory, Institute of Integrative Biosciences (IIB) within 24 h after collection and stored at 4 degree Celsius (°C) for further analysis.

2.2. Substrate collection and processing

Chicken feathers collected from local shops in Peshawar were used as substrate in this study. For cleaning, the feathers were placed in tap water for 1 h and then washed thoroughly. This process was repeated several times, followed by subjecting the feathers to air-drying overnight at ambient temperature. The feathers were then defatted using 1 % weight by volume (w/v) sodium lauryl sulfate (SLS), treated with tap water, and rinsed with distilled water. After washing with distilled water, the chicken feathers were placed in a hot air oven and dried at 60 °C for two days. Dried chicken feathers were cut into small pieces of approximately 3 × 3 cm and used as substrates for enzyme production. For enzyme activity assays, these feathers were ground to powder in a Silver Crest® Powder Machine for use as a substrate in enzyme activity assays.

2.3. Isolation of proteolytic bacteria

To isolate protease-producing bacteria, soil samples were enriched in mineral salt medium as reported [13] with slight modification containing 0.05 % Sodium Chloride (NaCl), 0.03 % Dipotassium hydrogen phosphate (K_2HPO_4), 0.04 % Potassium dihydrogen phosphate (KH_2PO_4), 0.01 % Magnesium sulfate heptahydrate ($MgSO_4{\cdot}7H_2O$) and 1 % Chicken feathers; pH 7 at 37 °C for three weeks. After enrichment, 1 mL of each sample was transferred to new flasks containing feather-enriched medium. This process was repeated three times. The flask sample was serially diluted to 10^{-4}, then inoculated on skim milk agar plates using the spread plate method. Skim milk agar plates were prepared by mixing 3 grams (g) yeast extract, 15 g agar, 5 g NaCl in a flask, and distilled water up to 400 mL and lastly the pH was adjusted to 7 using 1 molar (M) Hydrochloric acid (HCl) and 1 M Sodium hydroxide (NaOH). The medium was sterilized in an autoclave at 121 °C for 20 minutes (min). Concurrently, skim milk solution was prepared using 10 g skim milk in 100 mL distilled water and autoclaved separately for 5 min. The medium was allowed to cool to 60 °C before adding sterilized skim milk

solution. The mixture was then poured into sterilized Petri dishes and allowed to solidify. After inoculation of the samples on skim milk agar plates, the plates were incubated at 37 °C for 24 h, and the plates were then checked for hydrolysis zones around the colonies. The positive organisms were isolated for additional studies.

2.4. Isolation of keratinase producing bacteria

All protease-positive bacterial strains were incubated in feather-enriched medium with feathers as the sole source of carbon and nitrogen for 1 week. The degradation of feathers was visually analyzed. Bacteria that were able to completely degrade feathers were considered the best keratinase-producing bacteria and were used in further experiments.

2.5. Colony morphology and microscopy

The keratinolytic protease-producing bacteria were checked for colony morphology, such as shape, margin, elevation, size, appearance, transparency, texture, and pigmentation, as an initial step towards characterization. The strain was further characterized by Gram staining and microscopic features.

2.6. Molecular characterization and identification

The DNA was isolated using the cetyltrimethylammonium bromide (CTAB) method [14]. Polymerase Chain Reaction (PCR) was performed to amplify the 16S ribosomal RNA (rRNA) region of the keratinase-producing bacteria using universal primers (27F: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R: 5′-GGTTACCTTGTTACGACTT- 3′). Initially, the reaction mixture was denatured at 95 °C for 5 min. Following denaturation, the temperature was lowered to 58 °C for 1 min during the annealing. The temperature was then raised to 72 °C for 1 min for extension, and PCR was repeated for 35 cycles, followed by a final extension step at 72 °C for 5 min. Upon completion of the PCR, the samples were loaded onto an agarose gel to analyze the amplified DNA. All reagents were kept on ice during PCR. New England Biolabs (NEB) Monarch PCR and DNA clean-up kits were used to purify PCR products. PCR product of the 16S rRNA gene of the isolated strain was sent for sequencing to the Advanced Center for Genomic Technologies, Khyber Medical University, Hayatabad, Peshawar, Pakistan. The sequencing results were edited using “Chromas” to remove the primer regions and poor base quality regions. The edited sequencing results were then compared with known sequences in the National Centre for Biotechnology Information (NCBI) database using nucleotide Basic Local Alignment Search Tool (BLAST), and a phylogenetic tree was constructed by NJ (neighbor joining) method in Molecular Evolutionary Genetics Analysis Version 11 (MEGA 11.0).

2.7. Optimization of cultural conditions for keratinase production

Keratinase production by the bacterial isolate was examined by examining the substrate and culture conditions. The results of each experiment were performed in duplicate.

2.7.1. Effect of temperature on keratinase production

To determine the optimal temperature for enzyme production, the culture was incubated at various temperatures (32, 37, 42, 47, and 52 °C) for 3 days, and enzyme activity was measured.

2.7.2. Effect of pH on keratinase production

To determine a suitable pH for enzyme production, the culture was incubated at the optimum temperature at different pH values (6, 6.5, 7, 7.5, 8, 8.5,9 and 9.5) for 3 days, and enzyme activity was measured.

2.7.3. Effect of substrate concentration on keratinase production

To assess the effect of substrate concentration on enzyme production, the culture was incubated at the optimal temperature and pH for three days at varying substrate concentrations (1.5, 2.5, 5, 7.5, and 10). The enzyme activity was then measured. The substrate concentration was expressed as weight percentage relative to the total volume of the culture media.

2.7.4. Effect of inoculum size on keratinase production

The influence of inoculum size on enzyme production was examined by incubating the culture at the optimal temperature, pH, and substrate concentration for 3 days at varying inoculum sizes (1, 2, 3, 4, 5, 6, 7, 8, 9, and 10). Enzyme activity was measured, and the inoculum size was presented as volume percentages relative to the total volume of the culture media.

2.8. Keratinase production at optimum condition

For enzyme production, overnight bacterial culture broth was used under optimum conditions in a shaking incubator at 150 revolutions per minute (rpm) for 5 days, and the degradation of feathers was observed visually. The experiments were performed in duplicate. A control containing mineral media and chicken feathers was also prepared. Enzyme activity was analyzed for 5 days at different time intervals to determine the maximum enzyme production for protein purification.

2.9. Enzyme activity analysis

Enzyme activity was analyzed using method [15] with slight modifications. 50 mL falcon tube was taken and a 40 mL sample was taken from the enzyme production flask into the falcon tube. The Falcon tubes were centrifuged at 8000 rpm for 15 min. The culture supernatant was transferred to a new falcon tube and labeled. The experiment was performed for each enzyme production flask. 0.01 g chicken feather powder was added to a 1.5 mL Eppendorf tube followed by 0.2 mL enzyme solution and keratinase buffer (10 mM (mM) Calcium Chloride ($CaCl_2$), 150 mM NaCl, and 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 8.0) until the volume reached 1 mL. The tubes were then incubated for 1 h at 40 °C. The reaction was terminated by boiling the sample for 3 min at 100 °C. The reaction sample was then centrifuged at 6000 relative centrifugal force (RCF or g) for 1 min. 50 microliters (μL) of supernatant were taken from the reaction tube and transferred to a new Eppendorf tube. Then, 50 μL of keratinase buffer and 50 μL of 2 % ninhydrin solution were added to the new tube. A new tube was incubated at 100 °C for 10 min. The tube was then centrifuged at 6000×*g* for 1 min. 75 μL supernatant were taken from the tube and added to 96 well microtiter plate followed by 125 μL of ethanol. Absorbance was measured at 570 nanometers (nm). One unit (U/mL) of enzyme activity was defined as equal to the concentration of enzyme that is sufficient to produce 1 micromole (μmol) free amino acids per mL per minute using glycine as a standard in the above conditions.

2.10. Keratinase purification

A chromatography column with a diameter of 2.5 cm and height of 50 cm, containing Sephadex G-75, was used as the gel filtration matrix. Eppendorf tubes were used as containers to collect the fractions during chromatography. The cell-free culture supernatant was collected, and the protein was precipitated at 80 % ammonium sulfate saturation on ice. The sample was then transferred to a refrigerator for 24 h. The sample was centrifuged at 8000 rpm for 15 min, and the supernatant was discarded. The pellet was suspended in 10 mL buffer (20 mM Tris-HCl and NaCl at pH 8.0) and loaded onto the column. The elution was allowed to proceed under gravity, and fractions (1.5 mL) were collected. A total of 80 fractions were collected, and for each fraction, enzyme activity and total protein estimation were performed using the Lowry protein estimation assay [16]. The active fractions were pooled and analyzed for purity and molecular weight using Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS- PAGE) [17]. Furthermore, for zymography, the method reported [18] was used, with slight modification of using 12% resolving gel in the experiment.

3. Results

3.1. Isolation of protease producing bacteria

Bacterial colonies capable of forming zones of hydrolysis of various sizes were observed on skim milk agar plates when a sample of the enriched culture was plated after serial dilution. There were various zones of hydrolysis observed after serial and spread plate methods on skim milk agar plates, but only 21 isolates were selected with maximum zones of hydrolysis (Table 1), in which the strain MAMA produced the maximum zone of hydrolysis (Fig. 1).

3.2. Isolation of best keratinolytic protease produce bacteria

Among the 7 strains with maximum zone of hydrolysis on the skim milk agar media, the strain MAMA was only able to completely degrade feathers in 72 h and was considered as best keratinase producing bacteria (Fig. 2).

3.3. Colony morphology and microscopy

The strain MAMA exhibited an irregular shape with a circular margin and increased elevation. Its size is medium, and it appears to be glistening. The strain appeared opaque with a smooth texture and cream-white pigmentation. The Gram staining results revealed

Table 1
Bacterial isolates and zone of hydrolysis measurements.

No.	Strain Name	Diameter (cm)	No.	Strain Name	Diameter (cm)
1	MAMA	1.5	12	DGDA	0.8
2	MAMB	0.6	13	DGDB	0.6
3	MAMC	0.4	14	DGDC	0.4
4	MAMD	0.4	15	DGDD	0.6
5	MAME	0.6	16	DP1	1
6	MAMF	0.4	17	DP2	1
7	DGD1	0.6	18	DP3	0.8
8	DGD2	1	19	DP4	0.6
9	DGD3	0.6	20	DPA	0.6
10	DGD4	0.6	21	MK1	0.8
11	DGD5	0.4			

Fig. 1. Zone of hydrolysis by Strain MAMA on skim milk agar plate.

Fig. 2. Strain MAMA feather degradation in 3 days.

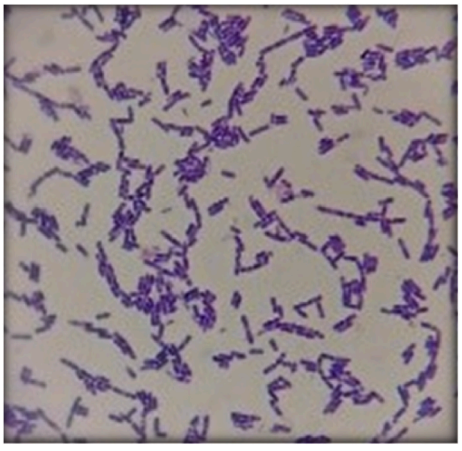

Fig. 3. Strain MAMA visualization after Gram staining.

that this strain was gram-positive with microscopic features of *Streptobacillus* (Fig. 3).

3.4. Molecular identification and characterization

DNA was extracted from the strain MAMA using the CTAB method and visualized on a gel documentation system using a 1 kb molecular marker as a reference. After genomic DNA isolation, the 16S rRNA gene region was amplified using universal primers (27F and 1492R) and the PCR product was purified and analyzed using a gel documentation system. The amplified 16S rRNA gene segment from the strain MAMA was sequenced at the Khyber Medical University, Peshawar. Sequencing results were analyzed using the NCBI BLAST. BLAST analysis revealed that the Strain MAMA had the maximum similarity (96.05 %) with *Bacillus velezensis* (MK928419.1). After BLAST analysis, a phylogenetic tree was constructed using the NJ method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown below the branches (Fig. 4). The evolutionary distances were computed using the Maximum Composite Likelihood method. All ambiguous positions were removed for each sequence pair (pairwise deletion). There were 1628 positions in the final dataset. Evolutionary analyses were conducted using MEGA 11.

3.5. Optimization of culture conditions for keratinase production

Different conditions, such as temperature, pH, substrate concentration, and inoculum size were analyzed, and enzyme activity was determined.

3.5.1. Effect of temperature on keratinase production

The effect of temperature on keratinase production was studied. The graph illustrates the relationship between various temperatures and corresponding levels of enzyme production. The graph displays the temperature (°C) on the x-axis and enzyme activity (U/mL) on the y-axis. Each data point represents the average enzyme activity obtained from two independent replicates. As the temperature increased, there was a noticeable trend in enzyme production. At lower temperatures, the enzyme production remained relatively low. Maximum enzyme production was observed at 37 °C and 42 °C, after which enzyme activity started to decline with further temperature increments (Fig. 5a).

3.5.2. Effect of pH on keratinase production

The effect of pH on enzyme activity was investigated by measuring keratinase production at various pH values. These results revealed a clear relationship between pH and keratinase production. At pH 6.5, enzyme activity was found to be relatively low. As the pH increased to 7, keratinase production significantly increased, suggesting that the enzyme activity approached its optimal range. The highest keratinase production was observed at pH 8. However, as the pH was further increased to 8.5 and 9.5, there was a noticeable decline in keratinase production, indicating a decrease in enzymatic activity (Fig. 5b).

3.5.3. Effect of substrate concentration on keratinase production

The impact of substrate concentration on enzyme production was examined to assess the enzyme response to varying substrate levels. These results demonstrated a notable relationship between substrate concentration and enzyme production. At lower substrate concentrations, enzyme activity was relatively high. As the substrate concentration increased, enzyme production increased proportionally. A peak in enzyme production was observed for 2.5 % substrate (w/v), respectively. However, after further increasing the substrate concentration, enzyme production plateaued and eventually declined (Fig. 5c).

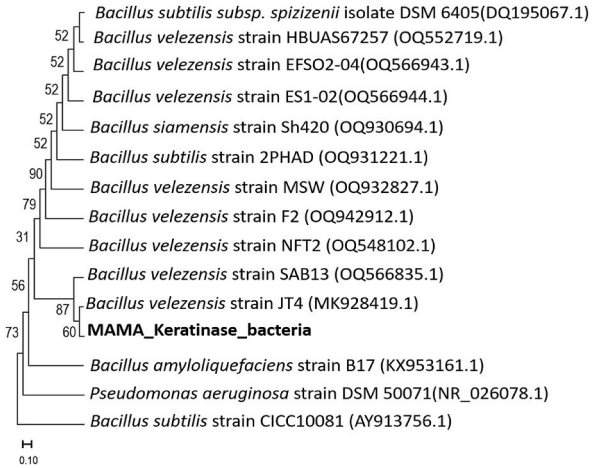

Fig. 4. *The* 16S rRNA gene phylogenetic tree of Strain MAMA.

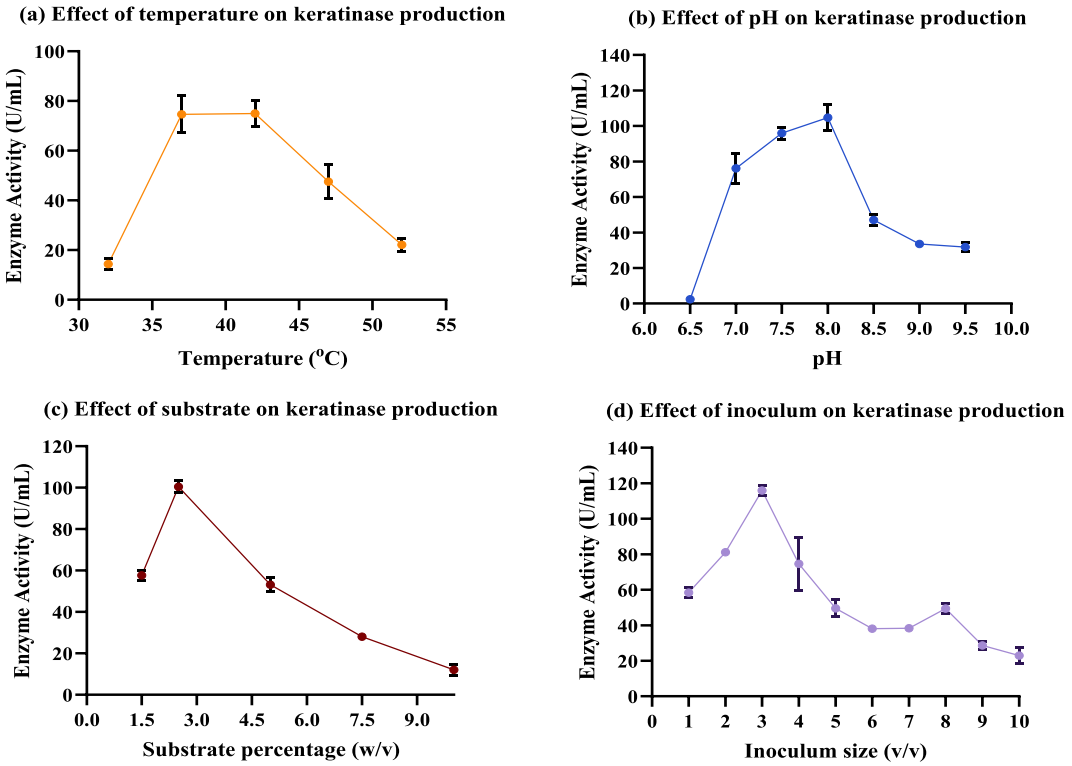

Fig. 5. (a) Effect of temperature on keratinase production (b) Effect of pH on keratinase production (c) Effect of substrate concentration on keratinase production (d) Effect of inoculum size on keratinase production.

3.5.4. Effect of inoculum concentration on keratinase production

It was observed that inoculum concentration plays a very important role in keratinase production. Although all other conditions were kept optimum for production, the inoculum percentage still had a significant impact on enzyme production. The maximum amount of enzyme was produced when 3 % inoculum was added to the enzyme production flasks (v/v) (Fig. 5d).

3.6. Keratinase production at optimum conditions

New reaction flasks were used to analyze keratinase production under optimum conditions for several days. Maximum keratinase production was observed on day 4 (108 h) at 166 U/mL (Fig. 6).

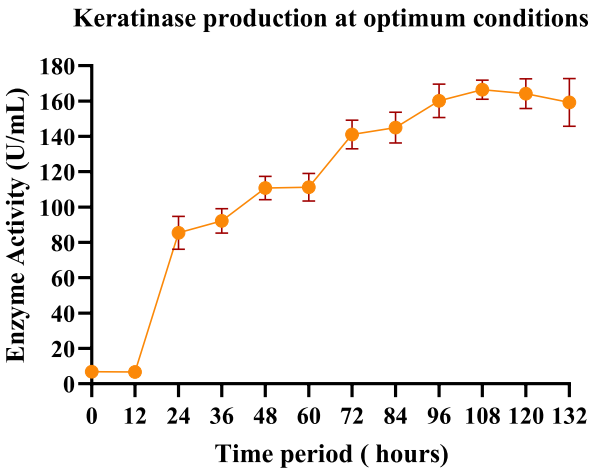

Fig. 6. Keratinase production at optimum conditions.

3.7. Keratinase purification

Eighty fractions were collected in a total of 1.5 mL. Protein concentration and enzyme activity were analyzed using Bovine Serum Albumin (BSA) as a standard (Fig. 7).

3.7.1. SDS gel electrophoresis

All the active fractions were pooled together and grouped into different "tubes." The process began by creating the first tube, which consisted of fractions ranging from 31 to 35. The second tube was established to include numbers from 36 to 40, whereas the third tube consisted of fraction numbers between 41 and 45. As the process continued, a fourth tube was created covering a range of 46–50. The fifth tube was formed to include numbers ranging from 51 to 55. The sixth tube contained 56–60 tubes, and the seventh tube was established to cover the range of 61–65, followed by the eighth tube, which included numbers ranging from 66 to 70. Finally, the ninth tube was formed, consisting of numbers between 71 and 75, thereby effectively completing the grouping process. All fractions were then lyophilized, and 150 μL buffer was added and then loaded in SDS Gel electrophoresis under reducing conditions. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue dye to visualize the protein bands. The resulting gel showed well-resolved protein bands, indicating successful separation of the proteins based on their molecular weights. The molecular weight marker bands allowed the estimation of the sizes of the separated proteins. Notably, distinct bands were observed in the gel, suggesting the presence of multiple proteins within the analyzed samples; however, some bands were observed in each fraction with different molecular weight (40–55 kDa, 25–35 kDa, and 15–25 kDa) as shown in Fig. 8a.

3.7.2. Zymography

Zymography was performed to detect keratinase. The results revealed the presence of keratinase by showing a clear zone in all fractions at a band located between 40 and 55 kDa revealing that keratinase produced by *Bacillus velezensis* produced a keratinase with a molecular weight between 40 and 55 kDa (Fig. 8b).

4. Discussion

The aim of this study was to isolate and identify bacteria that produce keratinase from soil samples collected from various areas of Peshawar. To increase the likelihood of obtaining keratinase-producing bacteria, an enrichment process was performed, and serial dilutions were performed to reduce the microbial load and facilitate identification. Skim milk agar plates were used to isolate protease-producing bacteria, which were identified by their ability to form zones by breaking the peptide bonds present in cysteine in skim milk media. Among the 21 isolates, the best one was selected based on its ability to degrade feathers within 3 days. This isolate, MAMA, was identified as *Bacillus velezensis* through 16S rRNA gene sequencing. The strain MAMA was then used to produce keratinase under flask conditions by only providing feathers as a source of carbon and nitrogen. The enzyme was able to completely degrade feathers within three days. The maximum keratinase activity was 166 U/mL under optimum conditions, and the enzyme was precipitated with ammonium sulfate. After enzymatic activity, 80 % saturation was observed for the keratinase enzyme produced by *Bacillus velezensis*. The enzyme was further purified using Sephadex gel filtration chromatography and SDS gel electrophoresis was performed, in which several bands were observed. To determine which band is keratinase, zymography was performed, which revealed that keratinase produced by *Bacillus velezensis* is 40 kDa–55kDa. *Bacillus velezensis* has been reported to produce four distinct keratinase enzymes with varying molecular weights. However, in this study, using zymography, the production of a single keratinase enzyme by the *Bacillus velezensis* strain MAMA was observed. The molecular weight of the isolated enzyme did not match that of any of the four keratinases previously reported for *Bacillus velezensis*. This difference suggests that the *Bacillus velezensis* genome may be a host for multiple proteases with the capacity to hydrolyze keratins. Notably, this study employed a higher substrate concentration (2.5 %) for optimal enzyme production, in contrast to the 0.5 % substrate concentration used in the reported characterization of different keratinase genes from *Bacillus velezensis* [19]. One of the previous study have also reported the decrease in keratinase production with high substrate concentration [20]. Interestingly, *Bacillus amyloliquefacians* S13 has been reported to produce a 47 kDa keratinase, along with another keratinase of 28 kDa [21], with the former closely resembling the molecular weight of keratinase produced by *Bacillus velezensis* strain MAMA. Moreover, the optimal pH for the 47 kDa keratinase was reported to be 8, which aligns with the optimal pH observed in this study. Many reported keratinases isolated from *Bacillus* species has shown optimum activity in the neutral to alkaline pH [22–25]. However, a notable difference was observed in the reported temperature requirement for optimal enzyme activity, which was approximately 60 °C for the 47 kDa keratinase [21], which was considerably higher than the findings (47 °C) in this study. Furthermore, studies have reported different strains of *Bacillus* species that produce keratinase enzymes with varied molecular weights, such as 33 kDa in *Bacillus licheniformis*, 25.4 kDa in *Bacillus subtilis*, and 24 kDa in *Bacillus paeudofirmis* [23]. Another study also reported *Bacillus* species that produce keratinase with molecular weight of approximately 40 kDa [26]. These observations raise the possibility of horizontal gene transfer events that facilitate the transfer of keratinase genes across *Bacillus* species. This phenomenon may explain the presence of multiple keratinase genes in certain strains, whereas others only produce a single keratinase gene.

5. Conclusion

The soil sample collected from Peshawar contained a strain referred to as MAMA, which exhibited the capability to fully degrade chicken feathers within three days. This indicates the presence of keratinolytic bacteria in the soil, capable of breaking down keratin. The Keratinase producing strain MAMA was *Bacillus velezensis* as identified by colony morphology, microscopy and 16S rRNA gene

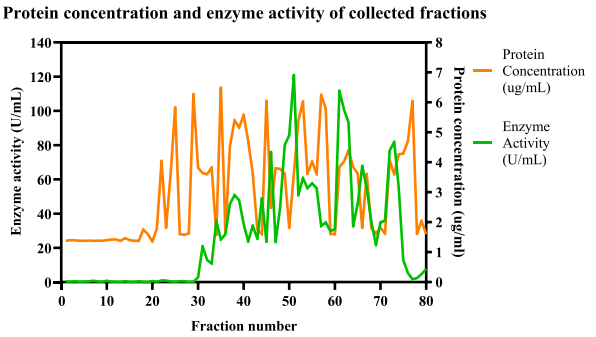

Fig. 7. Protein concentration and enzyme activity of eighty fractions collected using gel filtration chromatography.

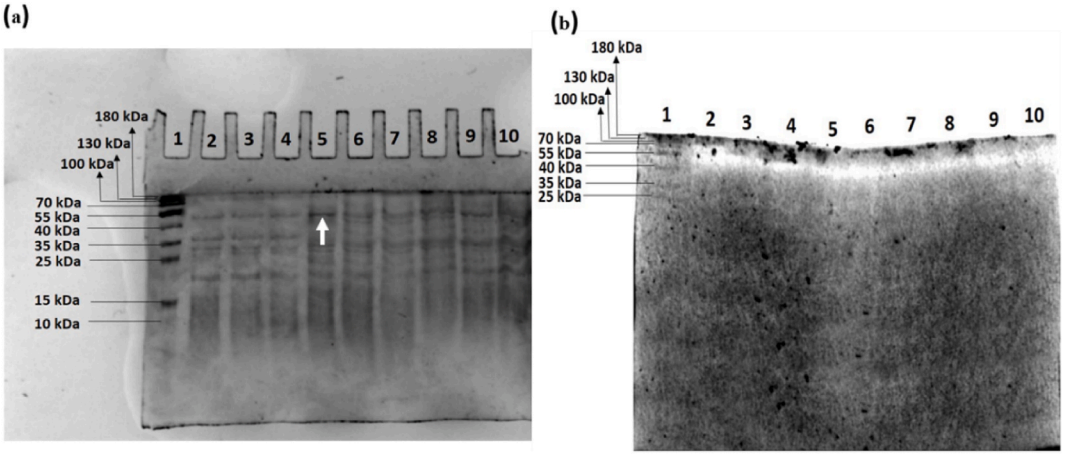

Fig. 8. (a) SDS PAGE results of active fractions (b) Zymography results of active fractions.

sequence analysis. The *Bacillus velezensis* strain investigated in this study exhibited optimal keratinase production at pH 8.0 and temperatures ranging from 37 to 42 °C. The optimal conditions were a substrate concentration of 2.5 % and an inoculum size of 3 %. The maximum enzymatic activity was 166 U/ml. The keratinase enzyme produced by the *Bacillus velezensis* strain MAMA in this study demonstrated the ability to precipitate at 80 % ammonium sulfate concentration and exhibited a molecular weight ranging from 40 to 55 kDa.

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Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article. For those interested in obtaining raw data supporting the study's finding, the corresponding author will make them available upon reasonable request.

CRediT authorship contribution statement

Aimon Khan: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kashif Mehmood:** Visualization, Validation, Supervision, Investigation, Formal analysis, Conceptualization. **Akhtar Nadhman:** Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Data curation. **Sami Ullah Khan:** Writing – review & editing, Resources, Data curation. **Aamer Ali Shah:** Writing – review & editing, Validation, Resources, Methodology, Data curation. **Ziaullah Shah:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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