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# Characterization of Highly Active Keratinase Procuder *Bacillus cereus* KK69 for Biological Degradation of Feather Waste

**Kerem Kaya, Emrah Nikerel***

Genetics and Bioengineering Department, Faculty of Engineering, Yeditepe University, İstanbul, Turkey

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## Abstract

A *Bacillus cereus* species, with highly active keratinase have been isolated from chicken feather waste. The keratinase is used to valorise feather containing approximately 90% protein, mostly keratin which is hard-to-degrade and insoluble in water. Although there are many old methods for decomposing feather such as incineration, burying, and chemically, enzymes have shown that are to be useful. This study focuses on characterizing the microorganism and its keratinase enzyme where the microorganism that produces enzyme can degrade more than 90% of the initial chicken feather in 2 days. Optimum working conditions of the enzyme is determined. Enzyme shows maximum activity at pH 9 and 50ºC. Bioinformatic analysis of *Bacillus cereus* KK69 genome revealed that there are many possible proteases for degradation of feather. Comparing to literature, this microorganism have displayed that produces highly active keratinase. Beside proteases, industrially important other enzymes also have been screened from annotations.

**Keywords:** *Bacillus cereus*, industrial biotechnology, chicken feather degradation, protease, keratinase enzyme

## 1 Introduction

Enzymes are the Swiss-army-knives of (industrial) biotechnology for sustainable bio-economy. They are used for various applications such as food, feed, waste management, detergents, pulp and paper, dairy, textile, etc. [1]. For this, industrially important enzymes represent a significant-market, nearly $4.6 billion in 2014, $4.9 billion in 2015, and $5.0 billion in 2016 and by 2021 the market is expected to reach $6.3 billion [2]. In several applications, enzymes have shown to be an "alternative technology" in industry. For instance, in detergent industry enzymes (protease, lipase, amylase, and cellulase) have had been used to place surfactants (ethoxylated alcohol, linear alkyl benzene sulfonate and sodium soap), resulting saving energy due to low temperature operation and being environmentally benign [3]. Green(er) industry concepts, wide industrial application areas are using enzymes as an alternative [4, 5], and economic concerns lead global enzyme market to grow every year.

Microbial production of enzymes is performed using either natural hosts or available industrial host cells, also referred as "workhorses" e.g. *Escherichia coli*, *Saccharomyces cerevisiae*, *Aspergillus niger*, typically equipped with genetic material encoding the enzyme of interest using well-established protocols of recombinant DNA technology. Complementary to this, natural producers, isolated usually from extreme environments are important to unravel the genetic diversity present in nature, are highly specific to its substrate and their enzyme production is relatively inexpensive. Isolates either should possess desired properties of production hosts or these should provide unique and useful genetic material. Industrial workhorses should grow fast, be safe and "easy-to-cultivate", produce fast with minimal by-products, sustain to harsh bioprocess conditions and should not produce toxic side products during fermentation. Especially with the advent of genome editing technologies, the efficiency of bioprocesses greatly improved [6, 7]. Additionally, using advanced approaches such as directed evolution [8] potentially increases product yield of natural hosts.

World population and nutritional demand is increasing day-by-day. As one result of this, poultry consumption increases every year, resulting further in poultry waste. Due to Food and Agriculture Organization statistical database (FAOSTAT, [9]), in last 55 years world live poultry amount of stock increased around 20.47 billion per annum

***Corresponding Author:** Genetics and Bioengineering Department, Faculty of Engineering, Yeditepe University, İstanbul, Turkey. E-mail: emrah.nikerel@yeditepe.edu.tr.

worldwide (1961, 4.35 billion; 2016, 24.82 billion) Feather waste is hard-to-dispose, becomes a significant pollution problem, since it potentially contains pathogenic microorganisms, and has effluvium for local area [10]. Feather contains up to 90% by weight keratin [11] which is a structural protein present in several life forms for protection and covering via skin, wool, fur, baleen, hair, spines, quills; defence and aggregations via horns, claws, nails, beaks, teeth, slimes; motions via hooves and feathers [12].

Conventional feather decomposition is carried out using either incineration or burying. Beside these methods, thermal [13], chemical, and enzymatic hydrolysis is performed [14] to process feather and reclaim its nutritional value.

Keratinases or keratinolytic proteases are enzymes dominantly noted as serine or metallo-proteases that can degrade keratin containing substrates [15]. In its essence, keratinases constitutes a sub-group of proteases or peptidases, which in turn are enzymes that hydrolyse proteins or peptides. Keratinases is often extracellularly produced but also cell-wall bound [16, 17] and intracellular [16, 18] types have been reported. Microbial sources for producing keratinases are generally isolated from poultry wastes, soil, lakes or hot springs [11]. Several fungi are also reported to produce keratinase: *Aspergillus fumigatus* [19], *Aspergillus oryzae* [20], *Doratomyces microsporum* [21], *Trichophyton* sp. [22]. Alternatively, bacterial keratinases are also extensively studied: *Bacillus licheniformis* [23], *Bacillus subtilis* [24], *Bacillus megaterium* [25], *Pseudomonas* sp. [26], *Thermoanaerobacter keratinophilus* [27]. Keratinolytic activity is typically measured as percent degradation of known initial feather over time. **Table 1** summarizes previous reports on feather degradation (or keratinase production) via *Bacillus* species.

The keratinase enzyme has been extensively studied for its characteristic (e.g. molecular weight) as well as in terms of its optimum working conditions, e.g. temperature, pH. The reported range for molecular weight is between 18 to 240 kDa [28], and the optimum working conditions are between 6 and 12.5 for pH and mesophilic to thermo stable (between 40-75°C) for temperature, while seldomly acidic keratinases has also been reported [28].

The applications of keratinases is well beyond feather decomposition, with various uses in e.g. feed, fertilizer, detergent and leather industry, prion degradation, cosmetics, and pharmaceutics [29]. Therefore, the isolation, identification and characterization keratinase producers represents both scientific and industrial significance.

In the light of the above, the aim of this paper is to report firstly the isolation and genomic and biochemical characterization of new microorganisms with keratinolytic activity. Secondly, the produced enzyme is also characterized in its optimum conditions as well as hydrolysis kinetics.

## 2. Material and Methods

### *2.1. Isolation and Growth Media*

Following Cai et al. (2009), isolation of microorganisms was performed using 10 g/L of chicken feather added in 0.9% (w/v) NaCl solution and incubated at 37°C for 1 week [24]. To screen microorganisms with keratinolytic activity, 300 µL samples were taken from the saline solution and transferred to feather containing agar plates. The feather plates were prepared by using (in g/L): NaCl (0.5), $KH_2PO_4$ (1.4), $K_2HPO_4$ (0.7), $MgSO_4$ (0.1), chicken feather (10), and agar (10) at pH 7.2. After two days of incubation at 37°C, colonies were streaked to Tryptic Soybean Agar (TSA) plates for observing single colonies. Liquid media to produce the keratinase enzyme, as well for degradation of feather in broth was prepared similar to feather agar, except omitting agar. Inocula of isolated selected bacteria for culture media were grown in Tryptic Soybean Broth (TSB, HiMedia M011). All chemicals used in this work are either from Sigma-Aldrich or Merck unless otherwise stated. In all fermentations for feather degradation, temperature, initial pH and rotation of shaker (New Brunswick™ Innova® 44) kept at 37°C, 7.2 and 150 rpm, respectively.

Table 1: *Bacillus* sp. literature on feather degradation

| Microorganism | Degradation Percentage | Time | Reference |
|---|---|---|---|
| *Bacillus cereus* B5esz | 72.1% | 10 days | [24] |
| *Bacillus cereus* KB043 | 78.16% | - | [40] |
| *Bacillus subtilis* FDS15 | 79.33 % | 21 days | [37] |
| *Bacillus* sp. FK 46 | 85% | 5 days | [39] |
| *Bacillus pumilus* FH9 | 97.8% | 72 hours | [16] |

### *2.2. Screening of Proteolytic or Keratinolytic Activity*

Skim milk agar was used for primary screening proteolytic activity of the isolates, following Alnahdi, 2012 [30] with minor modifications. Skim milk (100 g/L) and agar (20 g/L) are separately prepared, mixed at equal volume and autoclaved at 121°C for 5 minutes. The isolates were transferred to skim milk agar and the proteolytic activity was assessed by visually inspecting the clear zones on the plate. Standard protease activity was also measured using Sigma Aldrich's Technical Bulletin [31] based on determination of tyrosine released from casein upon hydrolysis with protease. 130 µL 0.65% Casein solution dissolved in Glycine buffer (pH 9) and 25 µL enzyme containing sample is mixed and incubated for 10 minutes at 37ºC. This is followed by addition 130 µL 110 mM trichloroacetic acid (TCA) and incubation of the mixture for 20 minutes at 37ºC. The mixture is then centrifuged at 10,000 rpm for 5 minutes. Released tyrosine from casein is quantified using Folin's reagent. 250 µL supernatant was added to solution that contains 625 µL 500 mM $Na_2CO_3$ and 125 µL Folin's Reagent and then incubated for 30 minutes at 37ºC. After incubation samples were read at 660 nm in spectrophotometer (Thermo Scientific Genesys 10S UV-VIS). To correct for tyrosine originating from hydrolysed feather, each sample have a read blank that TCA is included before casein is added. In addition to the above proteolytic activity, keratinolytic activity has been assessed by quantifying degraded feather during fermentation relative to initially present amount per volume per time.

### *2.3. Determination of kinetic parameters for keratinolysis*

The kinetic parameters ($K_M$ and $v^{max}$) of a standard Michaelis-Menten equation ($v = v^{max}S/(S + K_M)$) for keratinolysis are estimated using kinetic data on feather degradation with different initial feather levels. The keratinolytic activity vs. initial feather concentration data has been fit to the model, using non-linear optimization tools, minimizing the squared error between the data and the model, corrected for the standard deviation in the experimental error, taking thereby the experimentally measured variation into account. The initial value for the minimization problem has been provided by the calculated parameters of a Lineweaver-Burk plot [32].

### *2.4. SDS-PAGE for molecular weight determination*

The molecular weight of enzyme's is estimated by SDS-PAGE [33]. Gel, prepared using polyacrylamide resolving gel 12% (w/v) and polyacrylamide stocking gels 5% (w/v). Coomassie Brilliant Blue R-250 used for staining. The resulting bands on SDS-PAGE is used to determine the molecular weight of the protein, which is estimated with Pageruler™ Prestained Protein Ladder (Thermo Scientific 26617).

### *2.5. Whole Genome Sequencing and Bioinformatics analyses*

Whole genome of isolated microorganism is obtained using High Pure PCR Template Preparation Kit (Roche, Cat. No: 11 796 828 001). Sequencing is performed by microbesNG. (https://microbesng.uk). de novo sequence assembly is done using velvet and CLC. Annotation is performed using RAST servers (http://rast.nmpdr.org/rast.cgi, [34]). Upon annotation, the resulting gene list is filtered for possible keratinase coding genes, including peptidases, proteases, together with the corresponding nucleotide sequence. The sequence has been translated into amino acid sequence and expected molecular weight of the enzyme is calculated taking into account the molecular weights of the amino acids and the chain length.

## 3 Results and Discussion

Chicken feather has been incubated in saline solution and cultures from this solution have been spread to feather-agar, whereby isolated were selected and screened for their activities on skim milk agar (Figure 1). Selected isolates based o clear-zone assay performance, a single colony was selected and tested for its feather degradation performance in liquid medium. Preliminary identification of the colony via Fatty Acid Methyl Ester (FAME) analysis (data not shown) pointed that the isolate was *Bacillus cereus*.

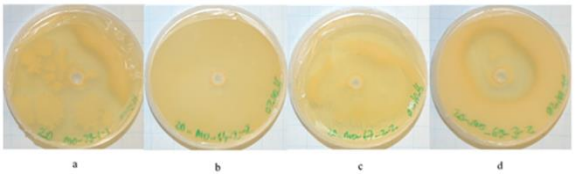


Figure 1: Protease activity on skim milk agar. a) 29 b) 44 c) 67 d) 69 numbered isolates

### *3.1. Biochemical characterization of Bacillus cereus KK69 strain*

As first step, being the main feature of the new isolate, the extent of feather degradation has been characterized (Figure 2 - Left). Feather degradation over time was measured to be up to 82%, 92%, and 93% on 1st, 2nd, and 3rd day respectively (Figure 2 - Right), which is far larger than the mechanical effect for feather degradation, assessed by incubating the feather at same conditions without inoculate, as 17% on 3rd day.

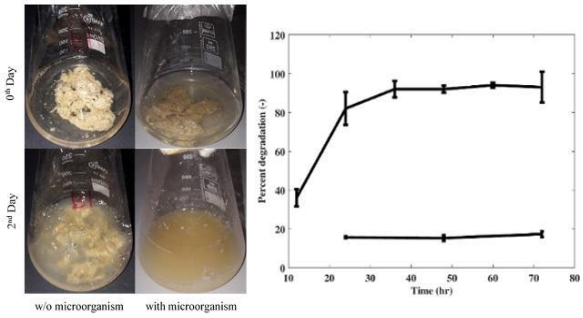


Figure 2: Typical feather degradation in two days. (Left) Conditions: 37°C, 150 rpm; 50 mL working volume, 20 g/L feather and (Right) the extent of feather degradation as percentage of feather over days.

Later, the effect of initial feather concentration as substrate on the hydrolysis kinetics is assessed, which yielded typical Michaelis-Menten type curve (Figure 3 - Top). Using the resulting hydrolysis data (Figure 3 - Bottom), the parameters of the irreversible Michaelis-Menten equation are estimated as Km is 14.4 gFeather /L and Vmax is 0.60 gFeather/L/hr.

The feather degradation experiments were followed by standard protease activity assays to assess the effect of hydrolysis time, pH and temperature on protease activity. In doing so, we first define the standard enzyme production and assay conditions as 48h fermentation at 37°C, 150 rpm and initial pH 7.2 with 20 g/L initial feather concentration and the protease assay as 10 minutes hydrolysis at pH 9 and temperature 37°C using casein as substrate. Under these conditions, we measured the protease activity to be 0.17±0.02 U/mL. We compare the effect of each process variable to this base-case scenario.

### *3.2. Effect of initial feather concentration for protease activity*

Protease activity is measured over time on different starting feather concentrations. At 80 g/L highest activity was measured as 0.909 U/mL (49 h), at 40 g/L 0.669 U/mL (32 h), 20 g/L 0.289 (24 h). Here, it should be noted that, available feather consisting mainly of keratin, would result in free tyrosine in the medium, upon keratinolytic activity from the microorganism. This might interfere with protease activity measurements, which are themselves based on quantification of tyrosine released from casein upon proteolytic activity. Therefore, extra care must be taken to take this into account by correctly selecting the blank during protease assay.

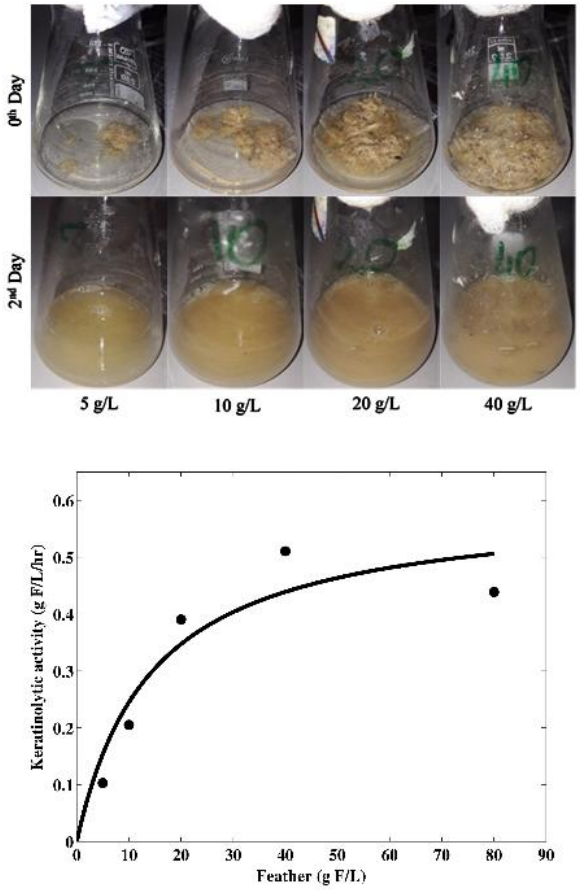


Figure 3: (Top) Feather degradation at different initial feather concentrations and (Bottom) overall keratinase activity on feather degradation, inoculum size 10%, 37°C 150 rpm, 2 days

### *3.3. Effect of Temperature and pH on protease activity*

To assess the effect of pH and temperature on the protease activity, a series of standard protease assays were performed. Temperatures (20°C, 40°C, 50ºC, 60°C, and 80ºC) were performed. The enzyme exhibited highest activity at 50ºC (Figure 4) and at 80ºC, no activity was measured which shows that it is a mesophilic enzyme. As for the pH (7, 8, 9, 10, and 11), highest activity was obtained at pH 9 which shows that it is an alkaline protease.

### *3.4. Genomic characterization of the keratin degrading bacteria*

The isolate has been further characterized by Whole Genome Sequencing. Genomic DNA was isolated and the DNA has been sequenced as a service using Illumina HiSeq 2500 infrastructure. Resulting raw reads (submitted to NCBI database with SRA number: SRR7691678) has the average GC content of 35.3% and have been de novo assembled using CLC Genomics Workbench (with final genome size, N50 and L50 numbers being 5.5 MB, 431108 and 3 respectively) and the resulting 143 contigs have been aligned to NCBI database.

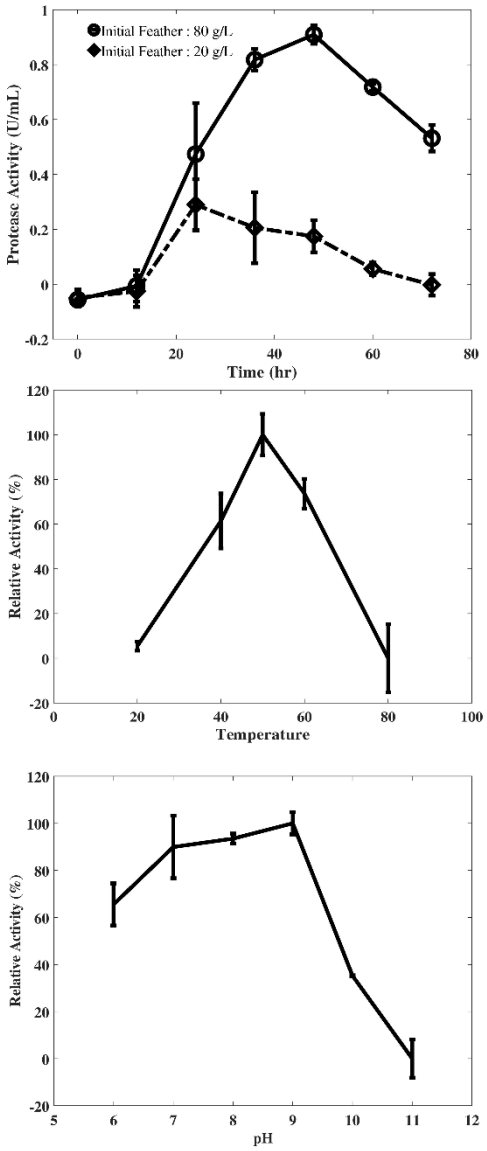


Figure 4: The effect of process parameters on the proteolytic activity. (Top) Initial feather concentration on feather degradation, (Middle) protease assay on temperature and (Bottom) protease assay on pH.

For various DNA segments (including the sequence coding 16S rRNA), the best match was always various *B. cereus* strains, confirming earlier FAME results. Noting the absence of perfect match between the DNA sequence of the isolate and the ones at available databases, we conclude that the feather degrading isolate was *Bacillus cereus* is new isolate and further name it as *Bacillus cereus* KK69 strain. Lastly, we used RAST server to find compare the genome of KK69 strain with previously studied organisms. This further confirmed that the isolate is a Bacillus strain, close to *Bacillus cereus* Q1 strain.

The assembled genome sequence was annotated using RAST server. The annotation pipeline resulted in 5603 coding sequences, 44% being assigned to one of the 482

subsystems. In particular, the subsystem protein degradation contains 56 coding sequences, ranging from metalloendo peptidases (EC3.4.24.-), aminopeptidases (EC3.4.11.-), metallocarboxypeptidases (EC 3.4.17.-) , to serine endopeptidase (EC 3.4.21.-) and omega peptidases (EC 3.4.19.-), indicating the relatively large genomic portfolio of the *B.cereus* KK69 for protease-like enzymes (Nearly 50 different protease-like enzymes are found in the annotation file, data not shown). The sequences of each protease is submitted to SecretomeP 2.0 Server (http://www.cbs.dtu.dk/services/SecretomeP) with the aim to pinpoint the sequence of the gene(s) responsible for the observed extracellular keratinolytic activity [35], which yielded 46 out of 56 proteases being secreted with either classical (signal peptide) or non-classical (e.g. secretion signal is within the protein sequence) secretion.

The annotated genome has also been screened for possible hydrolysis enzymes that can potentially be used in industrial context. With around 200 EC 3.x.x.x enzymes annotated, the genome of *B. cereus* KK69 indeed contains a rich portfolio of hydrolysis enzymes. The list contains various phosphatases (EC 3.1.3.x triphosphatase, alkaline phosphatase, Phosphoglycolate phosphatase), carbohydrate degrading enzymes (EC 3.2.1.x, alpha, beta-gluco or galatosidases, pulluanases, chitinases), lipases (EC 3.1.x.x, monoglyceride, triacylglycerol and Lysophospholipases) as well as other proteases including microbial collegenase (EC 3.4.24.3). Considering that above 15% of the 1310 genes assigned to an EC number is classified as hydrolase, *B.cereus* KK69 can be considered as gene source for later studies on industrially relevant enzymes.

The keratinolytic activity has been detected in culture media, even in the absence of bacteria. To further probe the secreted proteins, the supernatant of the culture media has been loaded to SDS-PAGE gel for determination of Molecular Weights of the secreted proteins. Two bands between 15-25 kDa, two bands between 35-40 kDa, one band between 55-70 kDa, one band between 70-100 kDa, and one band between 100-130 kDa has been observed (Figure 5).

Possible proteases (both intracellular and extracellular) are listed from the genome annotation along with their molecular weight. 56 different possible proteases are detected, their molecular weight ranges between 8-100 kDa, in line with the SDS-PAGE results. One should note that, SDS-PAGE results only monitors the approximate molecular weight of the protein, yet the secreted proteins might also have other, possibly hydrolysis activity.

## 4 Conclusions

A new bacteria with high keratinolytic activity has been isolated based on both clear zone on skim milk agar and feather degradation performance. This microorganism, *Bacillus cereus* KK69, has been characterized in its degradation kinetics as well as genomic features. The use of bacteria allows processing keratinolytic substances, allowing generation of high-value added products. The bacteria grows optimally between 20-40°C and its enzyme portfolio degrades feather up to 92.42% at 2nd day, which ranks as highly active when compared to the reports in literature. Expectedly, the activity decreases in either end of optimal temperature scale. As for the pH, standard protease activity protocol enzyme have shown its highest activity at pH 9, slightly over pH 8. This shows that enzyme is an alkaline protease which keratinases are working mostly at alkaline conditions. Future directions for this host include, reactor optimizations (constant pH, aeration) for scale-up studies, use in combination (either in co-culture or in tandem) with already known enzyme workhorses, and screening other potentially valuable industrial enzymes on various applications and different keratin sources (feather, hooves etc).

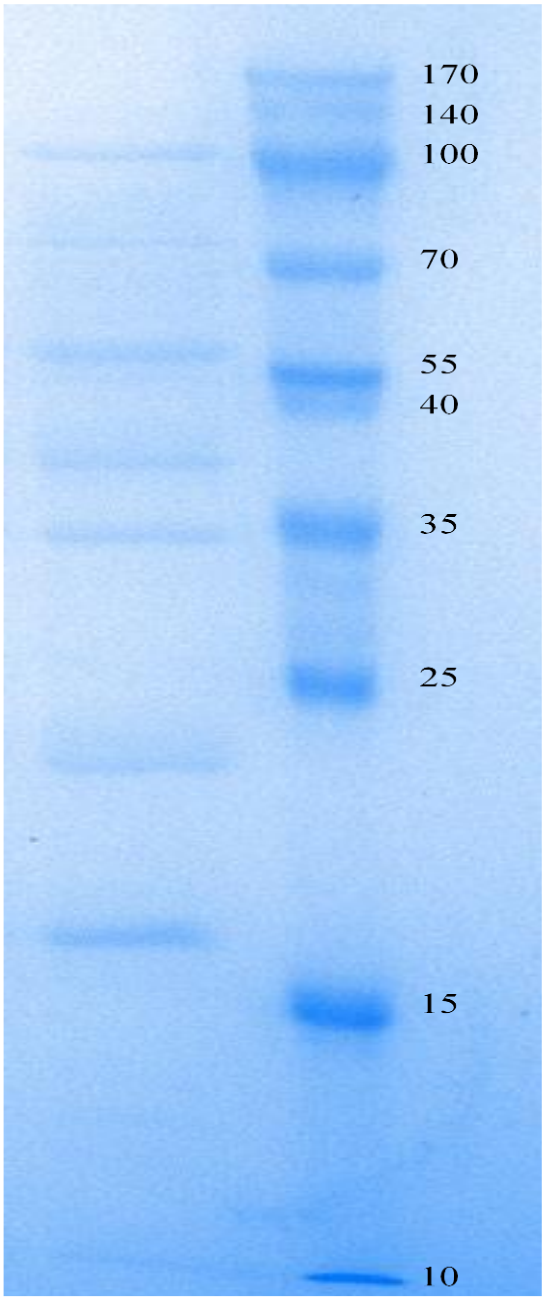


Figure 5: SDS-PAGE analysis of supernatant from degraded feather

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## Ethical issue

Authors are aware of, and comply with, best practice in publication ethics specifically with regard to authorship (avoidance of guest authorship), dual submission, manipulation of figures, competing interests and compliance with policies on research ethics. Authors adhere to publication requirements that submitted work is original and has not been published elsewhere in any language.

## Competing interests

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

## Authors' contribution

All authors of this study have a complete contribution for data collection, data analyses and manuscript writing

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