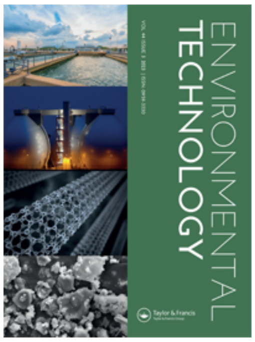


Environmental Technology

ISSN: 0959-3330 (Print) 1479-487X (Online) Journal homepage: www.tandfonline.com/journals/tent20

Chryseobacterium aquifrigidense keratinase liberated essential and nonessential amino acids from chicken feather degradation

Amahle Bokveld, Nonso E. Nnolim, Tennison O. Digban, Anthony I. Okoh & Uchechukwu U. Nwodo

To cite this article: Amahle Bokveld, Nonso E. Nnolim, Tennison O. Digban, Anthony I. Okoh & Uchechukwu U. Nwodo (2023) *Chryseobacterium aquifrigidense* keratinase liberated essential and nonessential amino acids from chicken feather degradation, Environmental Technology, 44:3, 293-303, DOI: 10.1080/09593330.2021.1969597

To link to this article: https://doi.org/10.1080/09593330.2021.1969597

ENVIRONMENTAL TECHNOLOGY
2023, VOL. 44, NO. 3, 293–303
https://doi.org/10.1080/09593330.2021.1969597

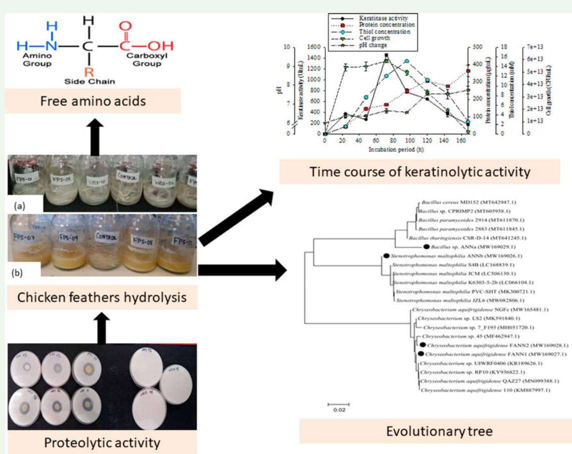
Chryseobacterium aquifrigidense keratinase liberated essential and nonessential amino acids from chicken feather degradation

Amahle Bokveld[a,b], Nonso E. Nnolim [a,b], Tennison O. Digban[a,b], Anthony I. Okoh[a,b] and Uchechukwu U. Nwodo[a,b]

[a]SAMRC Microbial Water Quality Monitoring Centre, University of Fort Hare, Alice, South Africa; [b]Applied and Environmental Microbiology Research Group (AEMREG), Department of Biochemistry and Microbiology, University of Fort Hare, Alice, South Africa

ABSTRACT

Keratinous biomass valorization for value-added products presents a high prospect in ecological management and the advancement of the bio-economy. Consequently, soil samples from the poultry dumpsite were collected. The bacteria isolated on the basal salt medium were screened for keratinolytic activity. The potent chicken feathers degrading bacteria were identified through 16S rRNA gene sequencing and phylogenetic analysis. Fermentation process conditions were optimized, and the amino acid compositions of the feather hydrolysate were likewise quantified. Ten (10) proteolytic bacteria evaluated on skimmed milk agar showed intact chicken feather degradation ranging from 33% (WDS-03) to 88% (FPS-09). The extracellular keratinase activity ranged from 224.52 ± 42.46 U/mL (WDS-03) to 834.55 ± 66.86 U/mL (FPS-07). Based on 16S rRNA gene sequencing and phylogenetic analysis, the most potent keratinolytic isolates coded as FPS-07, FPS-09, FPS-01, and WDS-06 were identified as *Chryseobacterium aquifrigidense* FANN1, *Chryseobacterium aquifrigidense* FANN2, *Stenotrophomonas maltophilia* ANNb, and *Bacillus* sp. ANNa, respectively. *C aquifrigidense* FANN2 maximally produced keratinase (1460.90 ± 26.99 U/mL) at 72 h of incubation under optimal process conditions of pH (6), inoculum side (5%; v/v), temperature (30°C), and chicken feather (25 g/L). The feather hydrolysate showed a protein value of 67.54%, with a relative abundance of arginine (2.84%), serine (3.14%), aspartic acid (3.33%), glutamic acid (3.73%), and glycine (2.81%). *C. aquifrigidense* FANN2 yielded high keratinase titre and dismembered chicken feathers into amino acids-rich hydrolysate, highlighting its significance in the beneficiation of recalcitrant keratinous wastes into dietary proteins as potential livestock feed supplements.



ARTICLE HISTORY
Received 22 May 2021
Accepted 9 August 2021

KEYWORDS
Keratinase; bio-recycling; keratinous biomass; amino acids; protein hydrolysate

Introduction

Keratin, an insoluble fibrous protein, is primarily found in feathers, wool, hair, and other epidermal appendages [1]. It withstands physical degradation due to a vast array of intramolecular and intermolecular forces in the biopolymer, including disulfide bonds, hydrogen bonds, and hydrophobic interactions that confer it with a unique three-dimensional structure [2]. The high keratin content in some agro-industrial wastes accounts for

CONTACT Uchechukwu U. Nwodo UNwodo@ufh.ac.za SAMRC Microbial Water Quality Monitoring Centre, University of Fort Hare, Private Bag X1314, Alice 5700, South Africa; Applied and Environmental Microbiology Research Group (AEMREG), Department of Biochemistry and Microbiology, University of Fort Hare, Private Bag X1314, Alice, Eastern Cape 5700, South Africa
Supplemental data for this article can be accessed https://doi.org/10.1080/09593330.2021.1969597

their recalcitrance and persistence in the environment. Therefore, keratinous wastes biomass are increasingly dumped in landfills or subjected to open-air burning, and this waste management approach occasions a significant degree of an environmental nuisance [3]. The avian feathers constitute about 5–10% of a grown bird weight and are the most universally generated keratinous wastes [4]. Globally, chicken consumption is significantly increasing due to its nutritional value [5].

Consequently, millions of tons of chicken feathers are generated annually as waste with no attractive economic value [6]. The high crude protein content of feather keratin suggests that the biomass could be a source of cheap and quality protein for animal husbandry. Fundamentally, agro-industrialists have converted the chicken feathers into feather meals through pressure cooking and grinding. The feedstock produced via these methods lacks adequate nutritional values due to the unavailability of some essential amino acids and loss of heat-labile nutrients [7].

Some microbes' ability to thrive on keratinous wastes gives clues to exploring potent keratinase producers to recycle these intractable agro-wastes into valuable products. Fungi with keratinolytic potentials are mainly within the confines of dermatophytes; although, a few non-dermatophytes have been isolated and characterized [8]. Therefore, the non-pathogenic bacteria are the organisms of choice for valorizing these low-cost renewable keratinous biomass. *Bacillus* sp., *Streptomyces* sp., *Chryseobacterium* sp. *Arthrobacter* sp., among others, have been reported to demonstrate efficient keratin degradation potentials [9]. Microbes degrade keratin through keratinolytic protease production only or in synergy with cell-associated sulfitolytic system [10]. Biodegradation of keratinous biomass yields protein hydrolysates with a potential application in livestock feed formulation [11,12]. The attribute of bio-recycling of keratin-rich wastes presents enormous advantages over other valorization techniques. In addition to the production of high-value products, the approach is sustainable and environmentally friendly. The prospect of microbial keratinase in the bio-economy is boundless. Therefore, this study explored the potential to isolate novel keratinase-producing bacteria from poultry dumpsites, improve the fermentation conditions, and analyze the amino acids from the feather hydrolysate generated.

Materials and methods

Samples collection and keratinous substrate preparation

Soil samples and chicken feathers were collected from Fort Cox poultry farm (with geographical coordinates 32° 46′ 0″S 27° 2′ 0″E) in the Raymond Mhlaba Municipality, Eastern Cape Province of South Africa, using sterile sampling bags. The samples were transported to the Applied and Environmental Microbiology Research Group (AEMREG) laboratory at the University of Fort Hare, Alice, for further analysis. The soil samples were analyzed within six hours of collection.

The preparation of keratin substrate from chicken feathers was done following the method described by Nnolim et al. [13]. The chicken feathers were washed thoroughly and rinsed with distilled water and dried under a constant air stream. The feathers were further placed in an oven for 48 h at 60°C in order to achieve a constant weight. Subsequently, the feathers were pulverized into a fine powder and stored in an airtight container at room temperature until further use.

Isolation of bacterial strains

Bacteria isolation was carried out using constituted basal salt medium (BSM) prepared with the following constituents; 0.3 g/L K_2HPO_4, 0.4 g/L KH_2PO_4, 0.2 g/L $MgCl_2$, 0.22 g/L $CaCl_2$, 0.2 g/L NH_4Cl and 10 g/L chicken feather powder (CFP) [14]. Approximately 50 mL of the BSM were dispensed into 250 mL bottles sterilized by autoclaving (Already Enterprise Inc., Taiwan). After sterilization, the medium pH was aseptically adjusted to 6.5 and then inoculated with 0.5 g of the soil samples. The bottles were incubated in a rotary shaker (Labotec IncoShake (Pty) Ltd, South Africa) for five days at 30°C. Solid media plates were prepared using the BSM and bacteriological agar (15 g/L). The media was supplemented with 80 mg/L of nystatin (an antifungal agent). After the incubation, serial dilutions of the broth were done to achieve (10^{-7}), and 100 μL from each culture broth spread plated onto the CFP agar plates. The plates were incubated at 30°C for 48 h. Distinct colonies were sub-cultured to obtain a pure culture.

Inoculum preparation

A single colony of the axenic cultures was transferred into microtubules containing sterile normal saline. The tubes were vortexed, and each sample's optical density was adjusted to 0.1 at 600 nm (equivalent to 0.5 McFarland's standard) after baseline calibration with sterile saline. This bacterial suspension served as fresh inoculum for other experiments.

Proteolytic activity screening

Evaluation of isolates for proteolytic activity followed the method described by Riffel and Brandelli [15]. Skimmed milk agar (SMA) plates were prepared with the following constituents; BSM, bacteriological agar (15 g/L), and skimmed milk powder (10 g/L). The media was sterilized by autoclaving, cooled, and poured into Petri plates. Bacterial isolates were screened for the ability to hydrolyze casein in SMA. About 5 µL of the inoculum was placed onto the SMA plates and incubated for 48 h at 30°C. After incubation, the plates were examined for halo zone formation. Isolates with profound hydrolysis of SMA were considered proteolytic and were selected for further evaluation.

Assessment of proteolytic isolates for keratinolytic activity

Fifty millilitres (50 mL) of BSM was dispensed into 250 mL bottles and autoclaved with intact chicken feathers (10 g/L). The media's pH was aseptically adjusted to 6.0, and the bottles were inoculated with 2% (v/v) of the prepared bacterial suspension and incubated for 96 h in an orbital shaker (150 rpm) at 30°C. Feather degradation was monitored as phenotypic impairment of the intact chicken feathers. After the incubation period, the broth was analyzed for the following parameters; keratinase activity, change in fermentation medium pH, broth protein concentration, sulfhydryl group concentration, and percentage feather degradation.

Keratinase activity assay

A modified version of the method described by Jaouadi et al. [16] was employed to determine keratinase activity using chromogenic keratin azure (Sigma-Aldrich, MO, USA). The reaction mixture contained 500 µL crude enzyme and 500 µL of Tris-HCl buffer (pH 8, 100 mM) with 10 mg keratin azure. The mixture was incubated in a water bath for 1 h at 50°C. After that, the tubes were placed on ice for 10 min to stop the reaction. The reaction mixture was centrifuged at 12,500 rpm for 10 min to separate the supernatant from the undegraded substrate. About 300 µL of the reaction mixture was pipetted into a microtiter plate, and then absorbance was measured at 595 nm using the SYNERGYMx 96 wells microplate reader (BioTek, USA) to determine the amount of azo dye released. The reaction mixture containing only crude enzyme and buffer without the keratin azure served as control. One-Unit (U) of keratinase was defined as the amount of enzyme causing 0.01 absorbance increases under the standard assay protocol described.

Protein concentration quantification

The protein concentration of the cell-free extract was determined using the method described by Bradford [17]. Bovine serum albumin (BSA) was used as a standard protein.

Change in pH determination

The change in initial medium pH after the fermentation period was determined using the Jenway pH metre (Bibby Scientific Ltd, UK).

Determination of thiol concentration

A method described by Ellman was used to determine the presence of free sulfhydryl groups in the fermentation broth [18]. Ellman's reagent, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB; 4 mg), was measured into 1 mL phosphate buffer (0.1 M, pH 8) and vortexed to dissolve. After that, 500 µL distilled water was mixed with 250 µL crude enzyme and 50 µL of DTNB solution. The mixture was allowed to stand at room temperature for 5 min for full-colour development, and the yellow colouration developed as a result of the reaction of DTNB with free sulfhydryl groups to give 2-nitro-5-thiobenzoic acid (TNB) was monitored at 412 nm.

Determination of percentage of chicken feathers degraded

The percentage of chicken feather degradation was determined using the weight loss method [13]. The Whatman no. 1 filter paper was used to filter various fermentation media. After filtration, the recovered chicken feathers were oven-dried and weighed. The approach for the calculation of the percentage of feather degradation is as shown in Equation (1):

$$\text{Percentage (\%)FD} = \frac{Wi - Wf}{Wi} * 100 \qquad (1)$$

FD = Feather degradation; W_i = Initial dry weight of the feathers; W_f = Final dry weight of feathers.

Molecular identification of keratinase producing bacteria

The potent keratinase-producing bacterial isolates' genomic DNA was extracted using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. 16005). The 16S target region was amplified using OneTaq® Quick-Load® Master Mix (NEB, Catalogue No. M0486), and the set of oligonucleotides 27f (5′-

AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-CGGTTACCTTGTTACGACTT-3′) served as forward and reverse primers, respectively. The PCR products were electrophoresed and gel extracted with the Zymo-clean™ Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit v3.1, BRD3100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analyzed on the ABI 3500 xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific). A BLAST search was conducted to retrieve similar sequences from the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and a phylogenetic tree was conducted in MEGA X [19].

Optimization of fermentation conditions for enhanced keratinase production

The effect of fermentation conditions on keratinolytic activity of selected isolate FPS-09 was studied using a one variable at a time (OVAT) approach. The initial medium pH varied from 3.0 to 9.0 at an interval of 1 unit. Similarly, the incubation temperature effect on keratinase production was studied by varying the temperature from 25 to 50°C at an interval of 5°C. The standardized bacterial suspension was used at a range of 1%–7% (v/v), while the chicken feathers concentration in the fermentation medium was varied from 0.5% to 3.5% to determine the optimal concentration that enhanced extracellular keratinase production.

Time course assay

Keratinase production time course for the bacterial isolate was through fermentation with optimized conditions, and aliquots were aseptically withdrawn at 24 h interval to determine the analytical parameters, including pH change, protein concentration, thiol concentration, and keratinase activity.

Hydrolysate amino acid profile

Submerged state fermentation was carried out at optimized conditions using chicken feathers as the only carbon and nitrogen source. After that, the residual substrates and cell biomass were separated from the broth by centrifugation. The hydrolysate was concentrated by lyophilization, and amino acid constituents were analyzed using pre-column derivatization, high-performance liquid chromatography (HPLC) separation, and detection using a fluorescence detector [20].

Statistical analysis

The datasets generated from triplicate experiments were analyzed, and the results were presented as mean and standard deviation. The statistical difference was compared at $P < 0.05$, and the analysis was conducted in the Statistical Package for the Social Science (SPSS) version 23.

Results

Isolation and screening of bacterial strains for protease activity

Twenty-two morphologically distinct bacterial isolates were recovered from the soil samples collected from the poultry dumpsite. The evaluation of these isolates for proteolytic activity showed that 10 out of 22 isolates hydrolyzed skimmed milk in solid media (Supplementary Figure 1), at variable degrees ranging from 11.5 ± 16.26 mm for WDS-02 to 25.75 ± 1.06 mm for FPS-07 (Table 1). The promising bacterial isolates were selected and subsequently assessed for their efficiency in the dismemberment of structurally intact chicken feathers in BSM.

Evaluation of the protease producing isolates for keratinolytic potentials

The evaluation of proteolytic isolates for keratinolytic potentials showed that they degraded intact chicken feathers in variable degrees (Supplementary Figure 2), ranging from 33% for WDS-03 to 88% for FPS-09 (Table 2). The isolates' extracellular keratinase production ranged from 224.52 ± 42.46 U/mL for WDS-03 to 834.55 ± 66.86 U/mL for FPS-07. The quantitation of the thiol-containing groups liberated during chicken feathers biodegradation showed a variation in thiol

Table 1. Proteolytic isolates with respective diameters of halo zone on skimmed milk agar

Isolate	Halo zone (mm)
FPS-04	15 ± 1.41
FPS-05	17.25 ± 0.35
FPS-06	18.75 ± 1.77
FPS-07	25.75 ± 1.06
FPS-09	21.25 ± 0.35
FPS-12	18.25 ± 1.06
WDS-02	11.5 ± 16.26
WDS-03	19 ± 1.41
WDS-06	18.5 ± 0.71
WDS-10	23.25 ± 0.35

Table 2. Evaluation of the proteolytic bacterial isolates for keratinolytic potentials

Isolate code	pH	Protein concentration (mg/ml)	Feather degradation (%)	Keratinase activity (U/mL)	Thiol concentration (mM)
FPS-01	7.4	0.02 ± 0.01	69	628.18 ± 52.71	2.22 ± 0.37
FPS-07	8.2	0.25 ± 0	81	834.55 ± 66.86	0.22 ± 0.08
FPS-09	8.3	0.17 ± 0.01	88	742.73 ± 14.14	2.27 ± 0.09
WDS-06	8.0	ND	72	610.91 ± 56.57	2.76 ± 0.79
FPS-12	7.7	0.1 ± 0	62	283.63 ± 1.26	1.5 ± 0.22
FPS-05	8.0	0.04 ± 0.01	66	414.54 ± 56.57	1.36 ± 0.2
WDS-10	7.5	ND	40	330.91 ± 0	1.66 ± 0.06
FPS-11	7.9	0.04 ± 0.01	34	534.54 ± 107.99	0.18 ± 0.12
FPS-08	7.9	ND	67	482.73 ± 11.56	0.1 ± 0.03
WDS-03	7.2	ND	33	224.52 ± 42.46	ND

ND – Not detected.

concentration, ranging from 0 to 2.76 ± 0.79 (mM) for WDS-03 and WDS-06, respectively. Furthermore, the study of change in medium pH after fermentation indicated a drift from an initial pH of 6.0 to a final pH of 7.2 for WDS-03 to pH 8.3 for FPS-09 (Table 2). Additionally, the total protein content quantified from the cell-free broth ranged from 0 mg/mL for FPS-08, WDS-03, WDS-06, WDS-10 to 0.25 ± 0 (mg/mL) for FPS-07.

Molecular identification of the potent keratinolytic bacteria

Based on the isolates' keratinolytic efficiency, the identity of FPS-07, FPS-09, FPS-01, and WDS-06 was confirmed using 16S rDNA sequencing and phylogenetic analyses. The results indicated that isolates FPS-07 and FPS-09 had over 99% of sequence homology with *Chryseobacterium aquifrigidense*, including *Chryseobacterium aquifrigidense* QAZ27 (accession no. MN099388.1) and *Chryseobacterium aquifrigidense* 110 (accession no. KM7997.1); hence, they were, respectively identified as *Chryseobacterium aquifrigidense* FANN1 and *Chryseobacterium aquifrigidense* FANN2 (Figure 1). On the other hand, FPS-01 showed 100% of sequence similarity with *Stenotrophomonas maltophilia*, including *Stenotrophomonas maltophilia* S4B (accession no. LC168839.1) and *Stenotrophomonas maltophilia* JZL6 (accession no. MW082806.1). Therefore, FPS-01 was identified as *Stenotrophomonas maltophilia* ANNb (Figure 1). Furthermore, the BLAST hit showed that isolate WDS-06 partial sequence had high sequence homology (100%) with *Bacillus* spp., including *Bacillus cereus* MD152 (accession no. MT642947.1), *Bacillus thuringiensis* CSR-D-14 (accession no. MT641245.1), and *Bacillus paramycoides* 2883 (accession no. MT611845.1). Following the WDS-06 nucleotide sequence's incomplete discrimination with other related *Bacillus* spp., it was identified as *Bacillus* sp. with a suffix ANNa (Figure 1). The nucleotide sequences of the isolates *Chryseobacterium aquifrigidense* FANN1, *Chryseobacterium aquifrigidense* FANN2, *Stenotrophomonas maltophilia* ANNb, and *Bacillus* sp. ANNa were submitted to the GenBank under the accession numbers MW169027.1, MW169028.1, MW169026.1, and MW169029.1, respectively.

Fermentation conditions optimization

The fermentation process conditions of *C. aquifrigidense* FANN2 were optimized for improved keratinase production in a submerged state fermentation. The pH optimization results indicated that *C. aquifrigidense* FANN2 produced keratinolytic protease at all the pH evaluated, with optimal keratinase activity at pH 4.0–6.0 (Figure 2a); there was no significant difference in enzyme yield at the three pH conditions. However, after pH 6.0, the extracellular keratinase activity of *C. aquifrigidense* FANN2 decreased consistently.

The effect of inoculum size on keratinase production was evaluated from 1% to 7% (v/v). The results showed that *C. aquifrigidense* FANN2 keratinase production increased with increasing starter culture concentration, reaching optimum at 5% inoculum size with the keratinase activity of 793.64 ± 39.85 U/ml (Figure 2b). The subsequent increase in the size of the inoculum caused a consistent decline in enzyme productivity by *C. aquifrigidense* FANN2.

The incubation temperature was studied from 25°C to 50°C, and *C. aquifrigidense* FANN2 demonstrated maximum keratinase activity of 739.99 ± 38.56 U/ml at 30°C (Figure 2c). However, the enzyme production was significantly affected by relatively high temperatures as the isolate displayed 92.72 ± 5.14 U/mL keratinase activity at 50°C.

The effect of chicken feathers concentration on extracellular keratinase secretion by *C. aquifrigidense* FANN2 was assessed from 5 g/L to 35 g/L (Figure 2d). *C. aquifrigidense* FANN2 showed remarkable keratinase synthesis and secretion between 5 and 30 g/L, with optimal enzyme production achieved at chicken feather concentrations ranging from 15 g/L to 30 g/L (Figure 2d). Above 30 g/L of chicken feathers, the enzyme production drastically decreased.

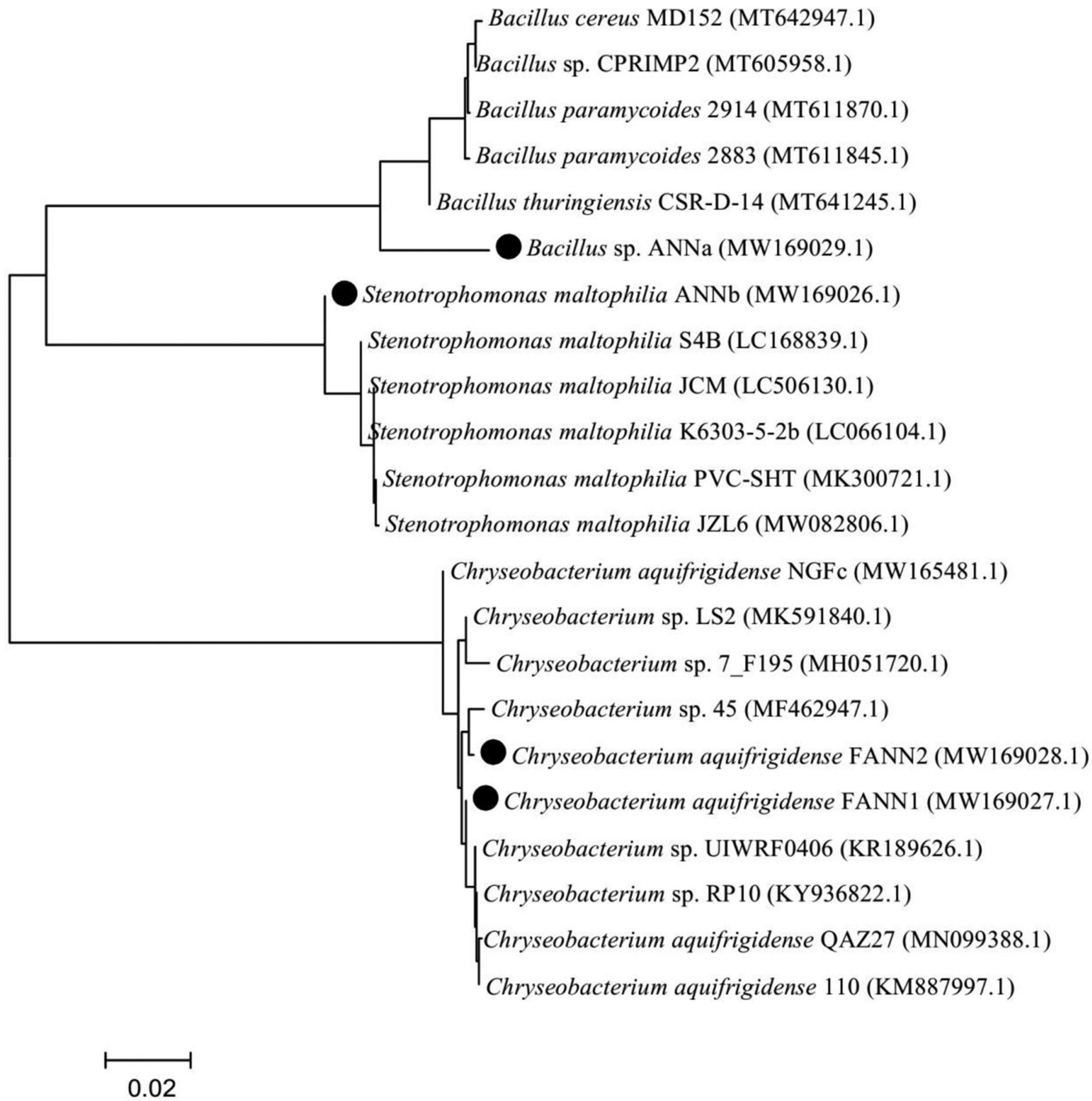


Figure 1. A phylogenetic tree showing the keratinolytic bacteria under investigation (indicated with black dots) and other related sequences from the NCBI GenBank. The accession numbers are presented in parenthesis.

Keratinase production time course

The time course profile of keratinase activity by *C. aquifrigidense* FANN2 showed that 373.64 ± 6.42 U/mL of keratinase production was obtained at 24 h of fermentation (Figure 3). The enzyme concentration decreased at 48 h (269.09 ± 7.71 U/mL) and peaked at 72 h with the keratinase activity of 1460.90 ± 26.99 U/mL. Beyond 72 h of incubation, the keratinase yield decreased consistently, and the least enzyme activity (179.99 ± 20.57 U/mL) was obtained at 168 h of incubation. The highest bacterial count was obtained at 72 h, corresponding with the optimal incubation time for the extracellular keratinase production (Figure 3). After 72 h, the viable cell population consistently declined with an increasing incubation period. The total protein content of the cell-free broth showed constant increment with increasing incubation period, and the maximum concentration of 364.02 ± 34.52 μg/mL was recorded at 168 h of fermentation (Figure 3). Furthermore, the quantification of the free sulfhydryl groups (thiols) liberated during chicken feathers biodegradation showed the accumulation of remarkable concentration of thiol groups in the fermentation medium following the extension of the fermentation process. The thiol concentration reached the climax (15.16 ± 0.08 mM) at 96 h of incubation and after that decreased consistently with increasing fermentation period. Moreover, the medium's pH changed from a weakly acidic condition with a pH

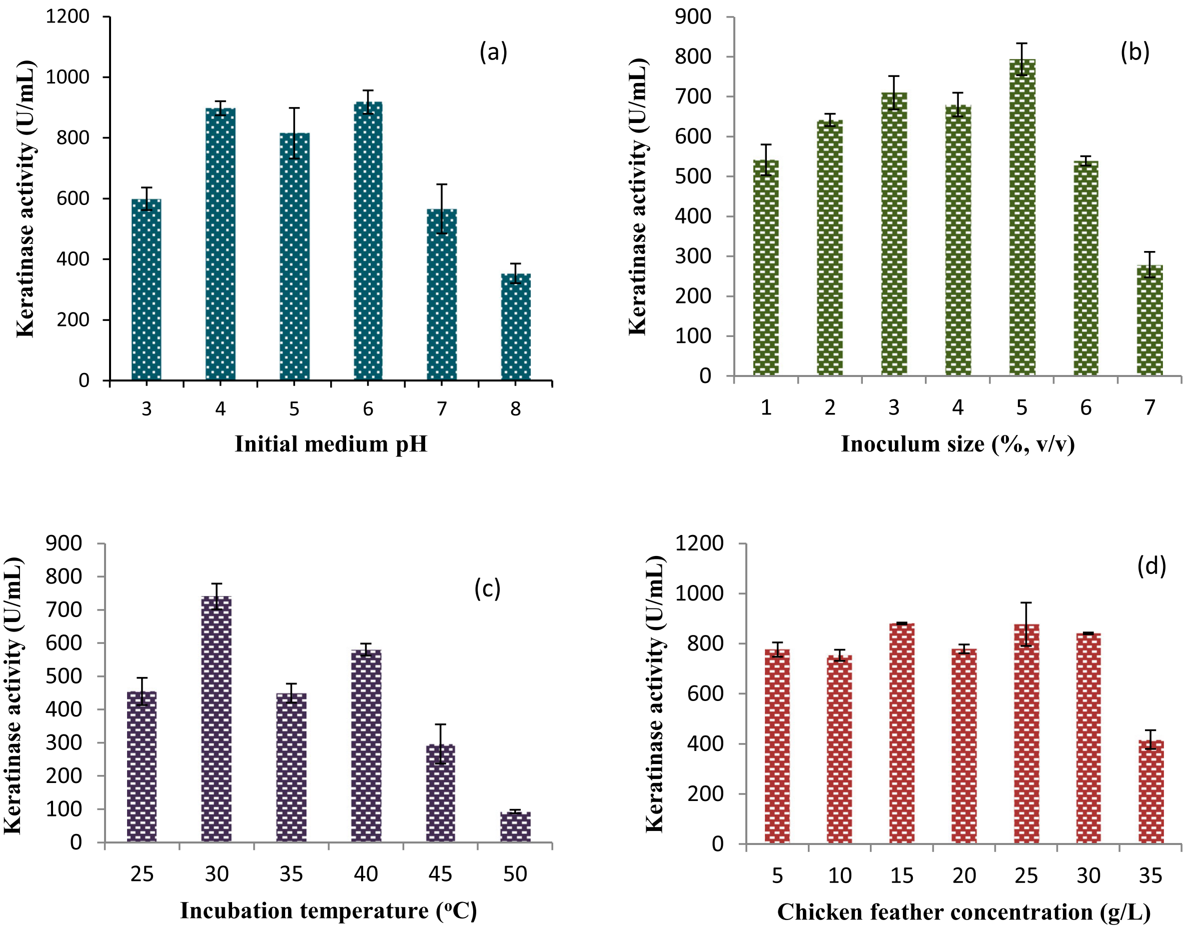


Figure 2. The effect of fermentation process conditions (a) initial medium pH (3–8), with optimum a(b) inoculum size (1–7%), (c) incubation temperature (25–50°C), and (d) chicken feather concentrations (5–35 g/L) on keratinase production by *C. aquifrigidense* FANN2. Each bar represents the mean and standard deviation of triplicate experiments.

value of 6.0 at the start of fermentation to 7.74 ± 0.19 after 168 h of incubation.

Chicken feather hydrolysate's amino acid analysis

Analysis of the protein hydrolysate produced during chicken feathers fermentation with keratinase-producing *C. aquifrigidense* FANN2 showed a protein value of 67.54% and the presence of essential and nonessential amino acids in various concentrations (Figure 4). The amino acids in relatively higher abundance include arginine (2.84%), serine (3.14%), aspartic acid (3.33%), glutamic acid (3.73%), and glycine (2.81%). However, HO-proline, methionine, tyrosine, tryptophan, and cysteine occurred in lower concentrations of 0.16%, 0.68%, 0.91%, 0.53%, and 0.56%, respectively.

Discussion

The poultry waste dumpsite has become of considerable interest in exploring keratinase-producing microbes [21]. Consequently, protease-producing bacteria were isolated from a local poultry farm waste dumpsite using the culture-based technique. This approach has effectively selected promising wild-type keratinolytic bacteria [15]. Quantitative evaluation of the isolates for keratinase activity led to a hierarchical selection and identity confirmation of four isolates that effectively degraded structurally intact chicken feathers and considerably secreted keratinolytic protease. The identification of these isolates based on 16S rDNA sequencing and phylogenetic analysis showed two *C. aquifrigidense*, *Bacillus* sp. and *S. maltophilia*. Similar bacterial isolates with efficient keratin-degrading potentials were characterized from diverse ecological niches [13,22–24]. Based on the isolates' keratinolytic activity, *C. aquifrigidense* FANN2 was selected for further studies.

Construction of the optimal fermentation conditions indicated that the isolates maximally produced keratinases at a weakly acid condition that contradicts many reported studies that keratinolytic bacteria optimally produce keratinase from neutral to alkaline pH spectrum [25–27]. Medium pH influences enzyme synthesis and

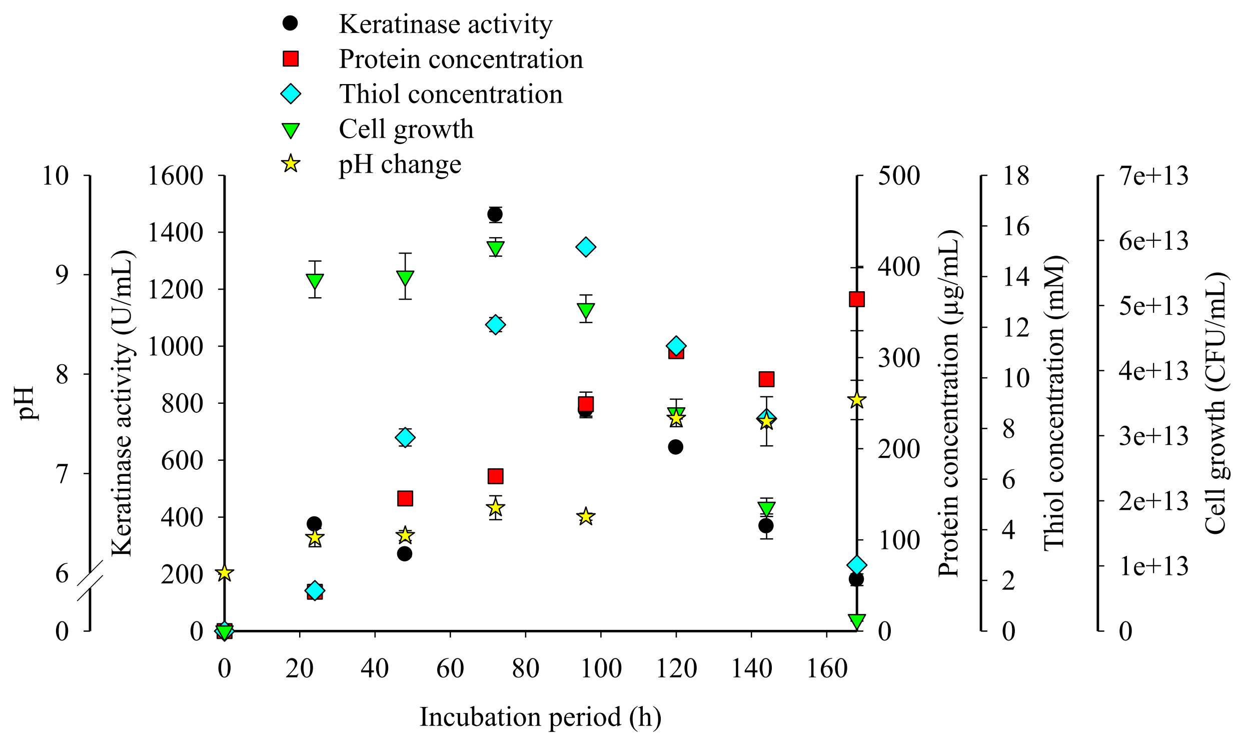


Figure 3. Time course profile of keratinolytic activity of *C. aquifrigidense* FANN2 at optimal fermentation conditions. Each point represents the mean and standard deviation of triplicate experiments.

transportation across the cell membrane [21,28]. Hence, the attainment of optimal pH conditions may enhance the metabolic efficiency of microorganisms toward metabolite production. A similar pH condition for optimal extracellular keratinase secretion has been demonstrated by other keratinolytic bacteria [29,30].

The fermentation process's initial inoculum concentration is an essential factor that influences bacterial growth and enzyme production. However, beyond the optimal inoculum size, keratinase production decreased. The decrease in keratinase production at higher inoculum concentrations may be attributed to constraints

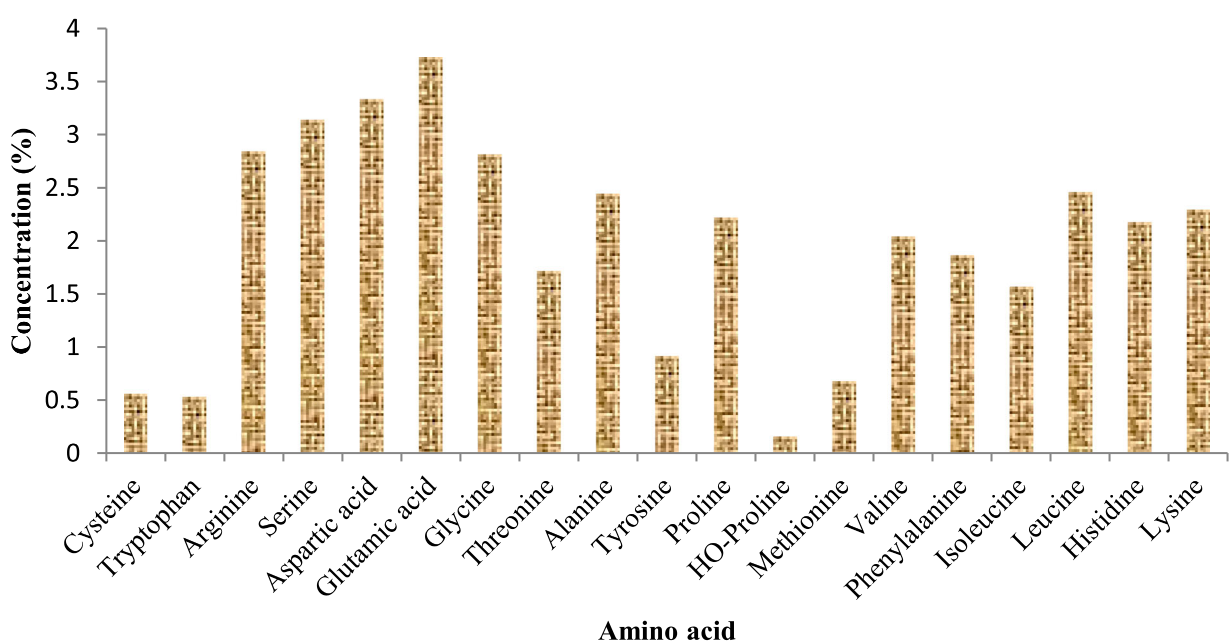


Figure 4. Amino acid profile of *C. aquifrigidense* FANN2 generated feather hydrolysate.

that include quick depletion of nutrients at exponential growth phase, low media aeration, and a high accumulation of toxic by-products of microbial metabolism [21]. Similarly, Sharma and Kango established that 5% inoculum size was ideal for extracellular keratinase production by *Ochrobactrum intermedium* [27]. In contrast to the present finding, Yusuf et al. [31] and Akhter et al. [32] documented optimal keratinase production at a respective inoculum size of 2% and 3% (v/v); and a further increase in the concentration of the starter culture adversely affected feather degradation and keratinase production. Additionally, the differences in inoculum size optima for keratinase production by various bacterial isolates may also be ascribed to the strain peculiarities or disparity in fermentation vessels' size.

C. aquifrigidense FANN2 showed maximum keratinase production at the mesophilic condition. Incubation temperature significantly controls microbial cell growth and the expression of secondary metabolites. The isolation source of bacterial strains could also influence their optimal activity towards optimal growth and secretion of the metabolite of interest. Enhanced keratinase activity at a moderate temperature condition by *C. aquifrigidense* FANN2 is attractive from biotechnological and industrial perspectives. Literature accounts of keratinase production at moderate temperatures by keratinolytic bacteria have been comprehensively documented [9,33].

Keratinase production is generally effectuated in the presence of keratinous substrate by bacterial producers [13]. Hence, keratinase biosynthesis is highly regulated, and such regulation determines keratinase titre in culture media [34]. The concentration of keratinous biomass could trigger commensurable keratinase secretion for the bioconversion of keratin into bioavailable products. These products serve as nutrients to sustain the bacteria's carbon and nitrogen requirements [35]. However, as observed from the present study, a higher concentration of keratinous biomass might lead to the accumulation of copious degradation products in the media, which may signal for downregulation of the keratinase biosynthetic pathway. Other reports have linked the fermentation medium's viscosity and poor aeration as the significant factors that prompt reduction in extracellular keratinase production when a higher concentration of keratinous substrates is added to fermentation media [25,36]. The utilization of cost-effective chicken feathers as a sole source of carbon and nitrogen by *C. aquifrigidense* FANN2 indicates its significance in sustainable development.

Time course study showed that *C. aquifrigidense* FANN2 had a relatively short timeline for maximum extracellular keratinase production at improved fermentation conditions. Keratinolytic protease optimal production at a reduced incubation period is highly desirable as it would ensure a constant enzyme supply for sustainable bioprocesses. Optimal keratinase production by bacterial strains has been reported within a similar timeline [35,37,38]. Conversely, *Chyseobacterium* sp. kr6 [34], *Chryseobacterium* sp. RBT [36] and *Pedobacter* sp. 3.14.7 [7] maximally produced keratinase after 20, 48, 192 h of incubation, respectively. The difference in optimal production time among the keratinolytic bacterial strains might be attributed to the medium composition and strain variations. Detection of free sulfhydryl groups in the keratin supplemented fermentation medium indicates efficient disulfide bonds' reduction properties of the keratinolytic system [39]. The remarkable concentration of thiol groups quantified using the fermentation broth suggests the isolate's relevance in the valorization of recalcitrant keratinous agro-industrial wastes. It is worth noting that the optimal thiol concentration liberated from chicken feathers biodegradation is comparatively higher than most similar studies. Jeong et al. [11] and Gurav et al. [36] reported a similar timeline of 96 h for the optimal generation of free sulfhydryl groups by *Bacillus subtilis* S8 and *Chryseobacterium* sp. RBT but at a respective lower concentration of 15.5 ± 0.2 µM and 2.144×10^{-4} M. The growth of keratinolytic *C. aquifrigidense* FANN2 on keratinous chicken feathers led to an increase in the fermentation medium's pH. This observation has been attributed to the accumulated ammonia originating from the deamination of the degradation products [40,41].

Furthermore, the amino acid profiling of the feather hydrolysate indicated a higher abundance of nonessential amino acids, including arginine, serine, alanine, aspartic, and glutamic acids, than the essential amino acids including valine, phenylalanine, leucine, histidine, and lysine. Notably, the protein value of the feather hydrolysate was higher than the total amino acids concentration, and this discrepancy may be due to other nonprotein nitrogenous compounds in the protein hydrolysate if factored in. Similarly, Yusuf and colleagues reported a remarkable abundance of nonessential amino acids, including glutamic acid, serine, and proline, compared to essential amino acids like lysine, methionine, tryptophan, and histidine in a feather hydrolysate generated by *Pseudochrobactrum* sp. IY-BUK1 [12]. *B. subtilis* S8 associated feather hydrolysate contained amino acids of protein in decreasing order of proline > histidine > phenylalanine > cysteine > tyrosine, among others [11]. The specificity of the keratinases from these bacterial strains could have accounted for the observed variation in the amino acid concentrations. The pattern of amino acids obtained from the feather hydrolysate indicates the keratinolytic potentials of *C. aquifrigidense* FANN2 in sustainable recycling keratinous wastes into valuable products that may serve as dietary protein supplements in livestock feed production.

Conclusion

In conclusion, proteolytic bacteria with dexterity for chicken feathers degradation were isolated from poultry dumpsites; and based on the 16S rDNA sequencing and phylogenetic analysis, they were identified as *C. aquifrigidense* FANN1, *C. aquifrigidense* FANN2, *S. maltophilia* ANNb, and *Bacillus* sp. ANNa. *C. aquifrigidense* FANN2 grew on chicken feathers as the only carbon and nitrogen source and showed optimal keratinase production of 1460.90 ± 26.99 U/mL at 72 h with initial medium pH of 6.0 and incubation temperature of 30°C. Furthermore, amino acid profiling of the feather hydrolysates showed an abundance of essential and nonessential amino acids, clear evidence of an effective keratinolysis by *C. aquifrigidense* FANN2. Therefore, *C. aquifrigidense* FANN2 high keratinase titre and amino acids-rich protein hydrolysate generated from chicken feathers decomposition highlight its significance in the beneficiation of recalcitrant keratinous wastes into dietary proteins as potential livestock feed supplements.

Acknowledgments

The Department of Science and Innovation (DSI), and the Technology Innovation Agency (TIA), South Africa, supported this work under SIIP enzyme and microbial technologies (grant number: DST/CON/0177/2018).

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by The Department of Science and Innovation (DSI), and the Technology Innovation Agency (TIA), South Africa [grant number DST/CON/0177/2018].

Data availability statements

The data that support the findings of this study are available from the corresponding author, [UUN], upon reasonable request.

ORCID

Nonso E. Nnolim http://orcid.org/0000-0002-7127-7707

References

[1] Wang B, Yang W, McKittrick J, et al. Keratin: structure, mechanical properties, occurrence in biological organisms, and efforts at bioinspiration. Prog Mater Sci. 2016;76:229–318.

[2] Bragulla HH, Homberger DG. Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia. J Anat. 2009;214:516–559.

[3] Shalaby MM, Samir R, Goma FAZM, et al. Enhanced fusidic acid transdermal delivery achieved by newly isolated and optimized *Bacillus cereus* Keratinase. Biotechnol Rep. 2021;30:e00620. https://doi.org/10.1016/j.btre.2021.e00620.

[4] de Menezes CLA, do Couto Santos R, Santos MV, et al. Industrial sustainability of microbial keratinases: production and potential applications. World J Microbiol Biotechnol. 2021;37:86. https://doi.org/10.1007/s11274-021-03052-z.

[5] Nnolim NE, Nwodo UU. Microbial keratinase and the bioeconomy: a three-decade meta-analysis of research exploit. AMB Express. 2021;11:12. https://doi.org/10.1186/s13568-020-01155-8.

[6] Tesfaye T, Sithole B, Ramjugernath D, et al. Valorization of chicken feathers: characterization of physical properties and morphological structure. J Clean Prod. 2017;149:349–365.

[7] Bezus B, Ruscasso F, Garmendia G, et al. Revalorization of chicken feather waste into a high antioxidant activity feather protein hydrolysate using a novel psychrotolerant bacterium. Biocatal Agric Biotechnol. 2021;32:101925. https://doi.org/10.1016/j.bcab.2021.101925.

[8] Gopinath SC, Anbu P, Lakshmipriya T, et al. Biotechnological aspects and perspective of microbial keratinase production. BioMed Res Int. 2015;140726. https://doi.org/10.1155/2015/140726.

[9] Nnolim NE, Udenigwe CC, Okoh AI, et al. Microbial keratinase: next generation green catalyst and prospective applications. Front Microbiol. 2020a;11:580164. https://doi.org/10.3389/fmicb.2020.580164.

[10] Fang Z, Zhang J, Liu B, et al. Biochemical characterization of three keratinolytic enzymes from *Stenotrophomonas maltophilia* BBE11-1 for biodegrading keratin wastes. Int Biodeterior Biodegrad. 2013;82:166–172.

[11] Jeong JH, Jeon YD, Lee OM, et al. Characterization of a multifunctional feather-degrading *Bacillus subtilis* isolated from forest soil. Biodegradation. 2010;21:1029–1040.

[12] Yusuf I, Garba L, Shehu MA, et al. Selective biodegradation of recalcitrant black chicken feathers by a newly isolated thermotolerant bacterium *Pseudochrobactrum* sp. IY-BUK1 for enhanced production of keratinase and protein-rich hydrolysates. Int Microbiol. 2020;23:189–200.

[13] Nnolim NE, Okoh AI, Nwodo UU. Proteolytic bacteria isolated from agro-waste dumpsites produced keratinolytic enzymes. Biotechnol Rep. 2020b;27:e00483. https://doi.org/10.1016/j.btre.2020.e00483.

[14] Nnolim NE, Okoh AI, Nwodo UU. *Bacillus* sp. FPF-1 produced keratinase with high potential for chicken feather degradation. Molecules. 2020c;25:1505. https://doi.org/10.3390/molecules25071505.

[15] Riffel A, Brandelli A. Keratinolytic bacteria isolated from feather waste. Braz J Microbiol. 2006;37:395–399.

[16] Jaouadi B, Abdelmalek B, Fodil D, et al. Purification and characterization of a thermostable keratinolytic serine alkaline proteinase from *Streptomyces* sp. strain AB1 with high stability in organic solvents. Bioresour Technol. 2010;101:8361–8369.

[17] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–254.

[18] Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys. 1959;82:70–77.

[19] Kumar S, Stecher G, Li M, et al. MEGA x: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 2018;35:1547–1549.

[20] Einarsson S, Josefsson B, Lagerkvist S. Determination of amino acids with 9-fluorenylmethyl chloroformate and reversed-phase high-performance liquid chromatography. J Chromatogr A. 1983;282:609–618.

[21] Akram F, ul Haq I, Hayat AK, et al. Keratinolytic enzyme from a thermotolerant isolate *Bacillus* sp. NDS-10: an efficient Green biocatalyst for poultry waste management, laundry and hide-dehairing applications. Waste Biomass Valor. 2021. https://doi.org/10.1007/s12649-021-01369-2.

[22] Hong SJ, Park GS, Jung BK, et al. Isolation, identification, and characterization of a keratin-degrading bacterium *Chryseobacterium* sp. P1-3. J Appl Biol Chem. 2015;58:247–251.

[23] Kang D, Huang Y, Nesme J, et al. Metagenomic analysis of a keratin-degrading bacterial consortium provides insight into the keratinolytic mechanisms. Sci Total Environ. 2021;761:143281. https://doi.org/10.1016/j.scitotenv.2020.143281.

[24] Prajapati S, Koirala S, Anal AK. Bioutilization of chicken feather waste by newly isolated keratinolytic bacteria and conversion into protein hydrolysates with improved functionalities. Appl Biochem Biotechnol. 2021. https://doi.org/10.1007/s12010-021-03554-4.

[25] Verma A, Singh H, Anwar MS, et al. Production of thermostable organic solvent tolerant keratinolytic protease from *Thermoactinomyces* sp. RM4: IAA production and plant growth promotion. Front Microbiol. 2016;7:1189. https://doi.org/10.3389/fmicb.2016.01189.

[26] Alahyaribeik S, Sharifi SD, Tabandeh F, et al. Bioconversion of chicken feather wastes by keratinolytic bacteria. Process Saf Environ Prot. 2020;135:171–178.

[27] Sharma I, Kango N. Production and characterization of keratinase by *Ochrobactrum intermedium* for feather keratin utilization. Int J Biol Macromol. 2021;166:1046–1056.

[28] Sharma KM, Kumar R, Panwar S, et al. Microbial alkaline proteases: optimization of production parameters and their properties. J Genet Eng Biotechnol. 2017;15:115–126.

[29] Abdel-Fattah AM, El-Gamal MS, Ismail SA, et al. Biodegradation of feather waste by keratinase produced from newly isolated *Bacillus licheniformis* ALW1. J Genet Eng Biotechnol. 2018;16:311–318.

[30] Nnolim NE, Okoh AI, Nwodo UU. Elucidation of coding gene and characterization of alkaline metallo-keratinase produced by acidophilic *Bacillus* sp. Okoh-K1 grown on chicken feather. Environ Technol Innov. 2021;21:101285. https://doi.org/10.1016/j.eti.2020.101285.

[31] Yusuf I, Ahmad SA, Phang LY, et al. Keratinase production and biodegradation of polluted secondary chicken feather wastes by a newly isolated multi heavy metal tolerant bacterium-Alcaligenes sp. AQ05-001. J Environ Manage. 2016;183:182–195.

[32] Akhter M, Wal Marzan L, Akter Y, et al. Microbial bioremediation of feather waste for keratinase production: an outstanding solution for leather dehairing in tanneries. Microbiol Insights. 2020;13:1–12.

[33] Srivastava B, Khatri M, Singh G, et al. Microbial keratinases: an overview of biochemical characterization and its eco-friendly approach for industrial applications. J Clean Prod. 2020;252:119847. https://doi.org/10.1016/j.jclepro.2019.119847.

[34] Riffel A, Daroit DJ, Brandelli A. Nutritional regulation of protease production by the feather-degrading bacterium *Chryseobacterium* sp. kr6. New Biotechnol 2011;28:153–157.

[35] Thankaswamy SR, Sundaramoorthy S, Palanivel S, et al. Improved microbial degradation of animal hair waste from leather industry using *Brevibacterium luteolum* (MTCC 5982). J Clean Prod. 2018;189:701–708.

[36] Gurav RG, Tang J, Jadhav JP. Sulfitolytic and keratinolytic potential of *Chryseobacterium* sp. RBT revealed hydrolysis of melanin containing feathers. 3 Biotech. 2016;6:145. https://doi.org/10.1007/s13205-016-0464-0.

[37] Gupta S, Singh R. Hydrolyzing proficiency of keratinases in feather degradation. Indian J Microbiol. 2014;54:466–470.

[38] Hendrick Q, Nnolim NE, Nwodo UU. *Chryseobacterium cucumeris* FHN1 keratinolytic enzyme valorized chicken feathers to amino acids with polar, anionic and non-polar imino side chain characteristics. Biocatal Agric Biotechnol. 2021;35:102109. https://doi.org/10.1016/j.bcab.2021.102109.

[39] Nnolim NE, Nwodo UU. *Bacillus* sp. CSK2 produced thermostable alkaline keratinase using agro-wastes: keratinolytic enzyme characterization. BMC Biotechnol. 2020;20:65. https://doi.org/10.1186/s12896-020-00659-2.

[40] Kumar AG, Swarnalatha S, Gayathri S, et al. Characterization of an alkaline active–thiol forming extracellular serine keratinase by the newly isolated Bacillus pumilus. J Appl Microbiol. 2008;104:411–419.

[41] Tiwary E, Gupta R. Medium optimization for a novel 58 kDa dimeric keratinase from Bacillus licheniformis ER-15: biochemical characterization and application in feather degradation and dehairing of hides. Bioresour Technol. 2010;101:6103–6110.