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Research article

Alkaline keratinase from *Bacillus* sp. DRS4 efficiently biodegrades chicken feathers to synthesize improved keratin/bacterial nanocellulose-based bioplastics

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ABSTRACT

Chicken feathers represent an abundant and sustainable resource that can be harnessed for multiple value-added products. Bioplastic reinforced with bacterial nanocellulose was synthesized using enzymatically digested chicken feathers. A highly efficient keratinolytic bacterium, identified as *Bacillus* sp. DRS4 through biochemical characterization and 16S rRNA gene sequence analysis, was isolated from deposit soils of Lake Chitu in Ethiopia. *Bacillus* sp. DRS4 was able to completely degrade chicken feathers within 48 h. Optimization of the physicochemical parameters increased the enzyme yield from *Bacillus* sp. DRS4 by 30%. The enzyme showed optimal keratinolytic activity at 37 °C and pH 11, hydrolyzing white chicken feathers in 72 h and providing hydrolysates with a total protein content of 251.145 mg/mL. Further, the mechanical and thermal properties of a bioplastic made from hydrolysates and reinforced with bacterial nanocellulose were assessed. The bioplastic exhibited a remarkable tensile strength of 5.769 MPa and reached a melting temperature of 127.5 °C, suggesting that bacterial nanocellulose acts as an effective stabilizer. Fourier Transform Infrared spectroscopy (FTIR) analysis revealed additional peaks in BNC-reinforced plastic films, indicating a binding interaction that enhanced the bioplastic properties. Overall, *Bacillus* sp. DRS4 is a potential strain for alkaline keratinase production and a promising candidate for upgrading chicken feathers into high-value-added products.

1. Introduction

Keratins in various vertebrate epidermal structures, such as feathers, hair, horns, nails, hooves, and wool, are generated mainly as waste in poultry, livestock, animal husbandry, and other industries. The poultry industry generates tons of feathers yearly [1], creating substantial waste management issues. In hair and wool, keratin is tightly packed into α-helices; in feathers, β-sheets. Additionally, the protein chains are cross-linked by cysteine bridges, hydrogen bonds, and hydrophobic interactions, which lead to outstanding mechanical stability and resistance to proteolytic hydrolysis. Current feather waste management practices, including incineration, landfilling, hydrothermal, and thermochemical methods, have side effects related to the spread of disease, pollution, and the loss of proteins that could be used to manufacture value-added products. However, feathers can be used to manufacture several value-added

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products through a facile valorization process involving microbial keratinase.

Keratinases (E.C. 3.4.21.24/99.11) are hydrolase enzymes that can exert sulfitolytic and proteolytic activity on recalcitrant keratinous waste. Numerous bacteria can produce keratinase, including *Bacillus subtilis* [2], *Arthrobacter* sp. NFH5 [3], *Bacillus amyloliquefaciens* [4], *Stenotrophomonas maltophilia* [5], and *Streptomyces* sp. isolate SCUT-3 [6]. Microbial keratinase is considered a green catalyst for producing keratin hydrolysate from chicken feathers, thereby contributing to eco-friendly solid waste management. Keratin hydrolysate is a more affordable and usable raw material that may be combined with synthetic or natural polymers to produce various high-value products, such as bioplastic film, biodegradable materials, nitrogen-rich fertilizers, animal feed, and bioenergy [4, 7].

Bioplastics, primarily made from renewable resources, are gaining significant interest due to their degradability, environmental friendliness, and sustainability. Researchers are actively developing bioplastics from hydrolyzed keratin and other additives as a sustainable alternative to traditional thermoplastics. This approach aims to reduce environmental pollution caused by feather waste. With this framework, Dou et al. used an alkali extraction and acid precipitation method to extract hydrolyzed feather keratin from chicken feathers, producing uniform, translucent, and robust bioplastics [8]. Sharma et al. also employed chemical extraction methods to obtain keratin powder from feathers and created a bioplastic film by mixing it with glycerol and microcrystalline cellulose [9]. To develop a more environmentally friendly and safer method for keratin extraction, researchers have utilized a thermostable-alkaline keratinase from *Bacillus halodurans* SW-X [7]. This enzyme produces white chicken feather hydrolysate, which can then be used to create good-looking bioplastics when combined with starch [7]. Here, keratinases play a crucial role in converting feathers into keratin hydrolysate, which can be further combined with natural polymers to enhance the strength and mechanical properties of the resulting bioplastic material.

While bioplastics offer great potential, their wider adoption is limited due to the need for enhanced material properties. Blending keratin hydrolysate with other polymers like starch, cellulose, and chitin is one approach to creating durable and eco-friendly bioplastics. Interestingly, materials with nanoscale properties offer unique advantages for various applications. In the realm of bioplastics, nanoscale materials can improve their strength, damage resistance, and versatility. One example is bacterial nanocellulose (BNC), a biopolymer produced by various bacteria [10]. BNC possesses outstanding characteristics, making it a valuable material for strengthening bioplastics. These characteristics include high purity, biocompatibility, high crystallinity(70–80%), strong thermal and mechanical stability, and easily tunable surface chemistry [11,12].

The present study isolated a native keratinolytic bacterium, *Bacillus* sp. DRS4, and optimized the physicochemical parameters to enhance both keratinase yield and the efficacy of feather degradation. The main goal of this study was to develop sustainable bioplastic films by combining keratin hydrolysate and bacterial nanocellulose. Bioplastic films with improved tensile strength and favorable thermal properties were synthesized using keratin hydrolysate and bacterial nanocellulose. This approach addresses the environmental footprint associated with traditional plastic production and usage.

2. Materials and methods

2.1. Preparations of chicken feather meal and keratin powder

Pre-treatment of chicken feathers for removing blood and fat components was conducted as described in Ref. [13]. Whole chicken feather waste that was obtained from a local poultry slaughterhouse was washed extensively with warm water, soaked for 24 h in a mixture of chloroform and methanol (1:1), and then incubated overnight in a mixture of chloroform, methanol and acetone (4:3:1). Afterward, the feathers were rinsed with plenty of distilled water 4 to 5 times to remove any remaining solvent and then dried at 50 °C overnight. The defatted feathers were ground using an electric blender and then stored at room temperature for subsequent use in screening for keratinase-producing bacteria. To extract keratin powder, white chicken feathers were immersed in 1000 mL of 0.5 M sodium sulfide for 6 h with continuous agitation at ambient conditions. Soluble keratin was first centrifuged for 10 min at 12,000 rpm and then precipitated from the supernatant using 70% ammonium sulfate. The residue was washed four times with distilled water, dried at 40 °C, and used as chicken keratin powder in keratinase assay experiments. Further, whole feathers were used to identify keratinase-producing bacteria.

2.2. Enrichment of keratinolytic bacteria

The isolation of keratinolytic bacteria from the collected samples was conducted via enrichment using feather meal broth medium as outlined in Ref. [7]. Briefly, 1 g of soil sample was inoculated into 250 mL flask containing 50 mL of feather meal broth consisting of (g/L); $MgSO_4.7H_2O$ 1, K_2HPO_4 1, KH_2PO_4 1, NaCl 1, $CaCl_2.2H_2O$ 0.1, yeast extract 0.1 and white feather meal 10. The medium was autoclaved at 121 °C for 15 min. Before inoculation, the pH was adjusted to 10 with sterile 1 M Na_2CO_3. After two days, 100 μL of serially diluted samples (10^{-3} to 10^{-7}) were spread plated onto Tryptone Soy Agar (TSA) medium containing (g/L); pancreatic digestion of casein 15, enzymatic digestion of soybean 5, sodium chloride 5 and agar 15 and adjusted to pH 10. The inoculated samples were incubated at 30 °C for 48 h [14]. Morphologically distinct bacterial isolates were stored on TSA slants at 4 °C for further testing.

2.3. Primary screening for identification of proteolytic bacteria

Morphologically distinct bacterial isolates that were selected from TSA agar were further tested for their ability to produce protease enzymes by spotting individual isolates onto sterile skim milk agar (SMA) plates containing (g/L): skim milk powder 28, casein

enzymatic hydrolysate 5, dextrose 5, yeast extract 2.5, and agar 15. After cooling, the pH of the SMA medium was adjusted to 10 with separately autoclaved 1% (v/v) sodium carbonate. The inoculated plates were incubated at 30 °C for 24 h. Protease-producing bacteria were identified by a clear zone around their colonies and further screened based on their proteolysis ratio (PR) [15]. Bacterial isolates with the highest PR were subcultured onto TSA agar plates, purified, and preserved in a 30% glycerol solution at −80 °C.

2.4. Secondary screening of bacterial isolates for keratinolytic activity

To further identify keratinolytic bacteria, isolates with the highest PR were cultured in minimal broth media containing (g/L): $MgSO_4{\cdot}7H_2O$ 1, K_2HPO_4 1, KH_2PO_4 1, NaCl 1, $CaCl_2{\cdot}2H_2O$ 0.1, yeast extract 0.1, and pH 10 [4]. For this, 1 mL of test microbe from an overnight culture was added to a test tube containing a sterile single white chicken feather and 9 mL of sterile minimal broth medium. After a week of incubation at 30 °C, the tubes were visually inspected for feather deterioration. Additionally, feather degradation rates were assessed through weight loss measurements. Feather weight loss was measured by incubating them in a 250-mL Erlenmeyer flask containing 100 mL of sterilized minimal medium supplemented with white feather meal (10 g/L) at 30 °C. Non-hydrolyzed chicken feathers were harvested after 48 h of incubation and filtered through Whatman filter paper No.1. Residual feathers were thoroughly washed with deionized water to remove bacterial cells and other soluble materials. Then, residual feathers were further oven-dried overnight at 60 °C until they reached a constant weight, and the degradation rate was determined using an analytical laboratory weighing balance [16]. The degree of feather degradation was calculated and expressed as a percentage, as shown in Equation (1).

$$\text{Percentage of feather degradation } (\%) = \frac{\text{Initial Weight - Final Weight}}{\text{Initial Weight}} \times 100 \quad (1)$$

Keratinolytic bacterial isolates showing maximum feather degradation were selected for further experimental studies. Additionally, microbially digested and undigested feathers were analyzed using a scanning electron microscope (SEM) (JCM-6000Plus, Jeol Japan) with an accelerating voltage of 15 kV. A gold coating was applied in an Argon atmosphere with 25 mA and 0.3 MPa conditions.

2.5. Cultural and biochemical characteristics of keratinolytic bacteria

The most efficient keratinolytic bacterium was further characterized in shape, color, size, elevation, margin, and surface. Gram staining and biochemical tests, including catalase test, methyl red (MR) test, Voges-Proskauer (V.P.) test, and citrate test, were also performed as described in Ref. [17].

2.6. Molecular identification of keratinolytic bacterium

Bacterial DNA extraction and PCR amplification were performed for keratinolytic bacterium as described in Ref. [17]. Briefly, bacterial genomic DNA was extracted using a DNA extraction kit (QIAGEN QIAamp DNA Mini Kit) according to the manufacturer's instructions and eluted in 100 μL of TE buffer. The eluted DNA was used as template DNA in the polymerase chain reaction (PCR) to amplify the 16S rRNA gene. The PCR mix consisted of 10 μL master mix (contains 10x Taq buffer, 10 mM dNTPs, 25 mM $MgCl_2$, 1 μL Taq DNA polymerase), 1 μL of each bacterial 16S universal rRNA primer (forward primer 27-F (5-ACAGTTTGATCCTGGCTCAG-3) and reverse primer 1492-R (5-GGGTTACCTTGTTACGACTT-3)), 2 μL genomic DNA and 6 μL PCR grade water. PCR amplifications were performed with a thermal cycler (model: DW-B960). For this, the standard PCR reaction conditions were maintained (initial denaturation at 94 °C for 5 min; 35 cycles consisting of template denaturation at 94 °C for 1 min, primer annealing at 55 °C for 40 s, and primer extension at 72 °C for 1 min and a final extension at 72 °C for 10 min). A gel documentation system visualized the PCR products after gel electrophoresis on 1% agarose gel. The 16S rRNA gene was sequenced using an automated sequencer in Germany, following manufacturer protocol (Eurofins, DE).

The raw DNA sequences (DNA chromatogram) were viewed, edited using the BioEdit software, and converted into FASTA format for phylogenetic analysis. Consequently, poor-quality sequence products were trimmed from the 3′ and 5′ sequence ends and assembled using MEGA X. Then, using BLASTn the sequence data were compared to the NCBI (http://www.ncbi.nlm.nih.gov) database, and the phylogenic analysis was performed. The phylogenetic tree was built using MEGA X with bootstrap values of 1000 replications using the maximum likelihood method [18] and the Kimura 2 parameter model [19].

2.7. Optimization of keratinase production parameters

The keratinase production by a selected bacterial strain was examined with various culture conditions, such as pH, temperature, inoculum size, agitation speed, and incubation period, using the one-variable-at-a-time (OVAT) approach. Therefore, the effects of pH on keratinase production were studied by adjusting the pH of the medium in a range from 6 to 12 using 1 M sodium carbonate and 1 M hydrogen chloride. The fermentation was carried out at 35 °C for 48 h. The fermented broths were centrifuged at 6000 rpm for 10 min, and then cell-free supernatants were used for the enzyme activity assay. Likewise, the effect of temperature on keratinase production was investigated by inoculating bacterial culture into sterile feather meal broth at optimal pH and incubating at various temperatures (25, 30, 35, 40, and 45 °C), with the enzyme activity for each temperature being measured independently. Moreover, different inoculum volumes (1, 2, 3, 4, and 5%) in feather meal broth were also used to determine the appropriate inoculum size required for maximum production of keratinase. Also, the effect of agitation speed on keratinase production was determined by incubating the

selected isolate in feather meal broth at an agitation speed range of 75–200 rpm. The time course of keratinase production was determined at 24 h intervals during an incubation period of 96 h under previously optimized conditions.

2.8. Keratinase assay

The keratinase activity was determined by the modified method of [20] using 0.1% keratin solution prepared by dissolving 0.1g of keratin in 100 mL of 50 mM Glycine-NaOH buffer, pH 11. 200 μL of the crude or partially purified enzyme and 800 μL keratin solution were mixed and incubated in a water bath at 37 °C for 30 min. The control was prepared by incubating the enzyme solution with 1 mL trichloroacetic acid (TCA) without adding keratin solution. The reaction was stopped by adding 1 mL of 10% TCA. The mixture was later centrifuged at 10,000 rpm for 10 min. Then, 1 mL of supernatant was added to 2.5 mL of a 0.5 M Na_2CO_3 solution. Finally, 0.5 mL of Folin reagent was added to each tube, vortexed, and left for 30 min at room temperature. The absorbance of the supernatant was determined at 600 nm using a UV–Vis spectrophotometer, and the released amino acids were estimated using a tyrosine standard curve. One unit of keratinase activity was ascertained as the amount of enzyme that increased one μmol of tyrosine from keratin in 1 min under defined standard assay conditions. Also, the concentration of protein in the crude enzyme was estimated according to Lowry's assay method [21] using bovine serum albumin as a standard protein.

2.9. Production and partial purification of alkaline keratinase

The production of keratinase enzyme using the most efficient isolate was carried out by inoculating overnight culture into 500 mL of conical flask containing 250 mL of 10 g/L feather meal broth and incubating it under previously optimized conditions. At the end of fermentation, the broth was centrifuged (6000 rpm, 15 min) to separate cells and the supernatant. The cell-free supernatant was subjected to 80% ammonium sulfate precipitation with continuous shaking by a magnetic stirrer. The precipitated enzyme was harvested by centrifugation at 10,000 rpm for 15 min at 4 °C, suspended in 50 mM phosphate buffer (pH 7.5), and then dialyzed [22].

2.10. Characterization of partially purified alkaline keratinase

The keratinase enzyme extracted from the most efficient isolate was partially purified and characterized for pH and temperature stability. The effect of pH on keratinase activity was carried out in buffers of different pH, including citrate buffer (pH 4–6), sodium phosphate buffer [7,8], and glycine-NaOH (pH 9–12). To evaluate the effect of pH on keratinase enzyme stability, the enzyme was incubated in buffers with different pH levels [4–12] at 37 °C for 30, 60, and 120 min. Then, the keratinase and residual activity were measured under standard assay conditions. Likewise, the effect of temperature on the enzyme activity was conducted following a standard keratinase assay in temperatures ranging from 25 to 45 °C for 30 min. To investigate the temperature stability, the enzyme was incubated at different temperatures for 15, 30, and 60 min, and the residual activity was measured under standard assay conditions.

2.11. Production of keratin hydrolysate from chicken feather waste

Keratin hydrolysate from white chicken feathers (WCF) was produced using dialyzed keratinase as described in Ref. [7] with slight modifications. Briefly, white chicken feather (10 g/L) was added to a 0.05 M sodium carbonate buffer pH (10.6) at 55 °C for 30 min. After cooling, to initiate feather degradation, 1 mL of dialyzed keratinase enzyme solution was added to 100 mL solution containing the substrate. The mixture was then incubated at 37 °C and 125 rpm for time points of 0, 24, 48, and 72 h. After complete degradation, the enzyme was inactivated by boiling for 10 min. Keratin hydrolysate suspension was filtered through an 80 μm sieve to remove insoluble feather fragments and neutralized by 85% H_3PO_4. The total protein concentration of the hydrolysate was estimated using Lowry's method [21].

2.12. Preparation of keratin-based bioplastic film

The keratin hydrolysate was used to synthesize a bioplastic film as described in Ref. [23] with slight modification. An in-house nanocellulose-producing bacterium was used to extract BNC, as described in Ref. [10]. To prepare bioplastic film, 60 mL of keratin hydrolysate, glycerol (1%), and BNC (0.2%) were mixed under vigorous magnetic agitation at 60 °C for 5h [9]. The aliquot was spread over a Petri plate and dried in an oven at 60 °C for 24h. Later, the synthesized bioplastic was separated from the Petri plates and stored for further analysis.

2.13. Characterization of keratin-based bioplastic film

The thermal properties of the synthesized bioplastic were studied using Differential Scanning Calorimetry (DSC, SKZ1052, China). The functional groups in bioplastic were characterized by Fourier Transform Infrared (Nicolet Evolution-300) spectroscopy with a frequency range of 4000 cm^{-1} to 500 cm^{-1}. The tensile strength of keratin-based bioplastic film was measured using a Texture Analyzer and calculated using Equation (2).

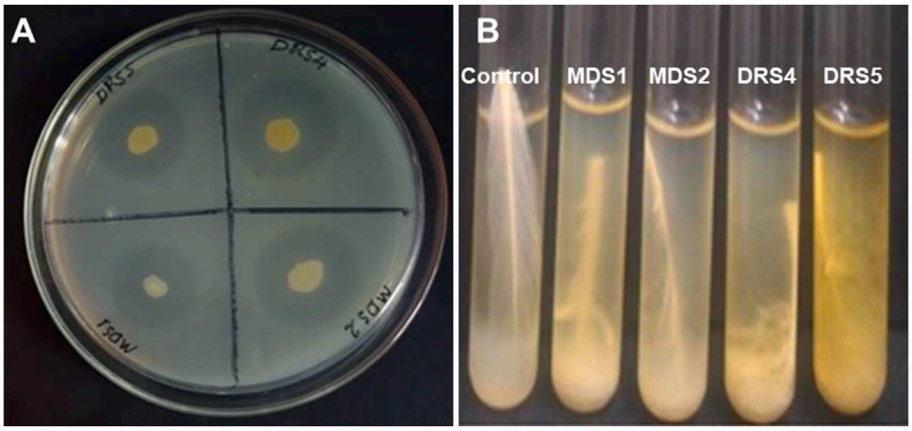

Fig. 1. (A) Screening general proteolytic bacteria in a spot test method. (B) Screening of keratinolytic bacteria using feather in test tube method. Isolates were incubated at 30 °C for 7 days.

Table 1
Chicken feather degradation efficiency of the bacterial isolates.

Bacterial isolates	Degradation efficiency (%)
MDS1	20 ± 0.632
MDS2	40 ± 0.665
DRS4	81.06 ± 0.155
DRS5	64.8 ± 0.1527

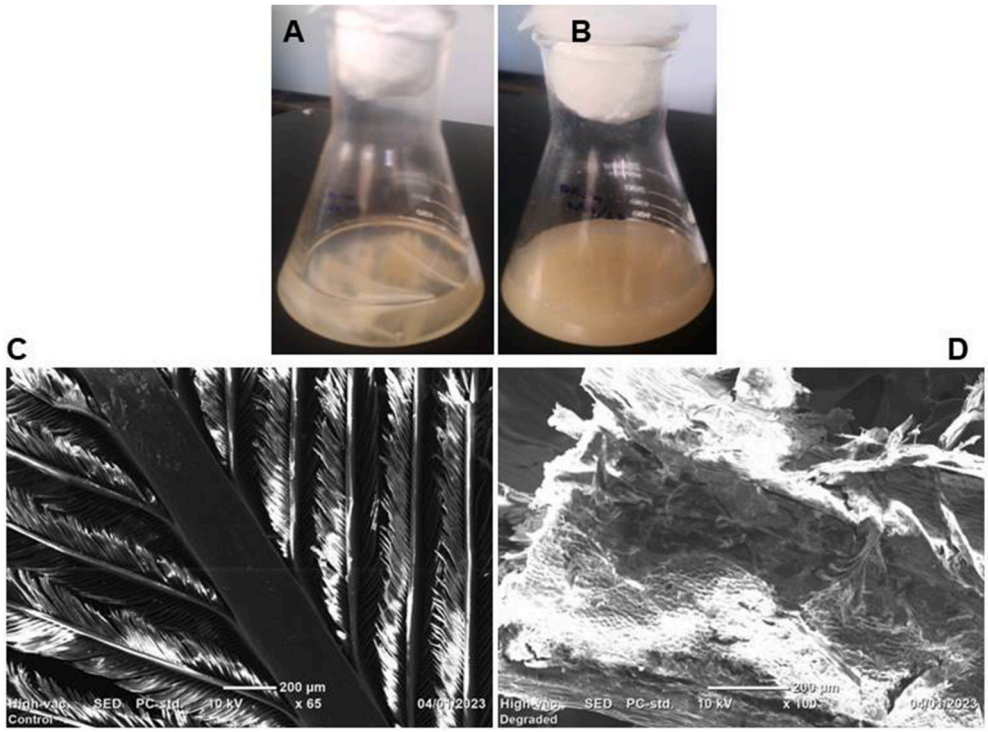

Fig. 2. (A) A control without the bacteria at 48 h of culturing. (B) Chicken feathers were completely degraded after 48 h of incubation by the selected isolate (DRS4) at 30 °C and pH 11. (C) Scanning electron microscopy micrograph of control sample without bacteria. (D) Scanning electron microscopy micrograph showing the disintegration of feather barbules after 48 h of incubation by the DRS4 isolate.

Table 2
Morphological and biochemical characteristics of the keratinolytic bacterial isolate (DRS4).

Parameters	Morphological and biochemical characteristics
Nature	Mucoid
Shape	Circular
Margin	Entire
Color	Yellowish
Optical	Opaque
Gram staining	Positive
Catalase test	Positive
Citrate test	Positive
Methyl red test	Positive
Triple sugar iron test	Negative
Manitol salt agar test	Positive
Voges Proskauer test	Positive
Starch hydrolysis test	Negative

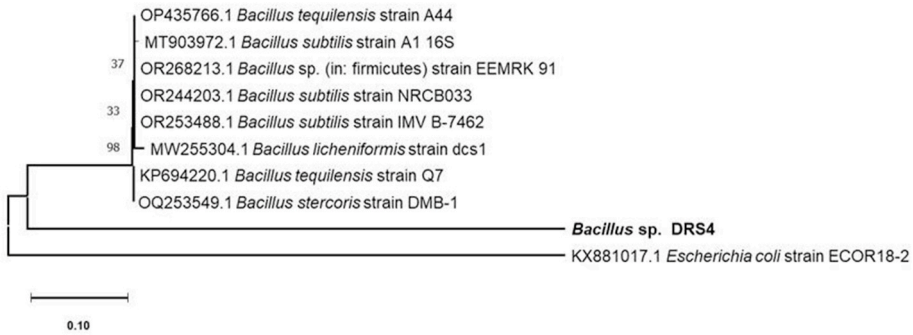

Fig. 3. Phylogenic tree of 16S rRNA gene sequences showing the relationships between strain DRS4 and other related strains.

$$\text{Tensile strength (Mpa)} = \frac{\text{Maximum force(N)}}{\text{t(m)} \times \text{W(m)}} \quad (2)$$

t represents thickness and W represents the width of bioplastics.

2.14. Data analysis

All experiments of this work were done in triplicate. The data are expressed as mean ± standard deviation, and statistically analyzed by OriginPro 2023 software. Duncan's multiple range tests examined significant differences ($p \leq 0.05$) between means.

3. Results and discussion

3.1. Isolation and screening of keratinolytic bacteria

Sixty-eight bacterial isolates recovered from enrichment cultures were screened for protease production. Among them, 18 (26.47%) showed a clear zone around the colony when cultured on SMA (Fig. 1A), indicating the ability to hydrolyze casein present in skim milk, which is related to the secretion of proteolytic enzymes [14]. The proteolytic isolates were further examined for their ability to produce keratinolytic enzymes by culturing them in 10 mL of minimal broth supplemented with a single feather (Fig. 1B). After seven days of incubation, only four of the 18 isolates (MDS1, MDS2, DRS4, and DRS5) showed feather degradation, indicating their ability to utilize chicken feathers as a carbon and nitrogen source [24]. Each isolate with keratinolytic activity was cultured in basal salt medium (BSM) to further investigate the extent of feather degradation. The ability of isolates to degrade feathers varied from 20% to 81%, with DRS4 having the highest degrading capacity and an associated weight loss of 81 ± 0.15% (Table 1). Scanning electron microscopy (SEM) micrographs also revealed the colonization of DRS4 on feathers as well as the disintegration of feather barbules after 48 h of incubation (Fig. 2A–D), corroborating the results of the broth studies; therefore, DRS4 was selected for further analysis.

3.2. Identification and biochemical characteristics of keratinolytic bacterium

Next, bacterial isolate DRS4 was cultured on nutrient agar to study its morphological and cultural characteristics. The isolate was a Gram-positive, motile, rod-shaped bacterium that formed yellowish, smooth, round, opaque colonies with a slimy texture on nutrient

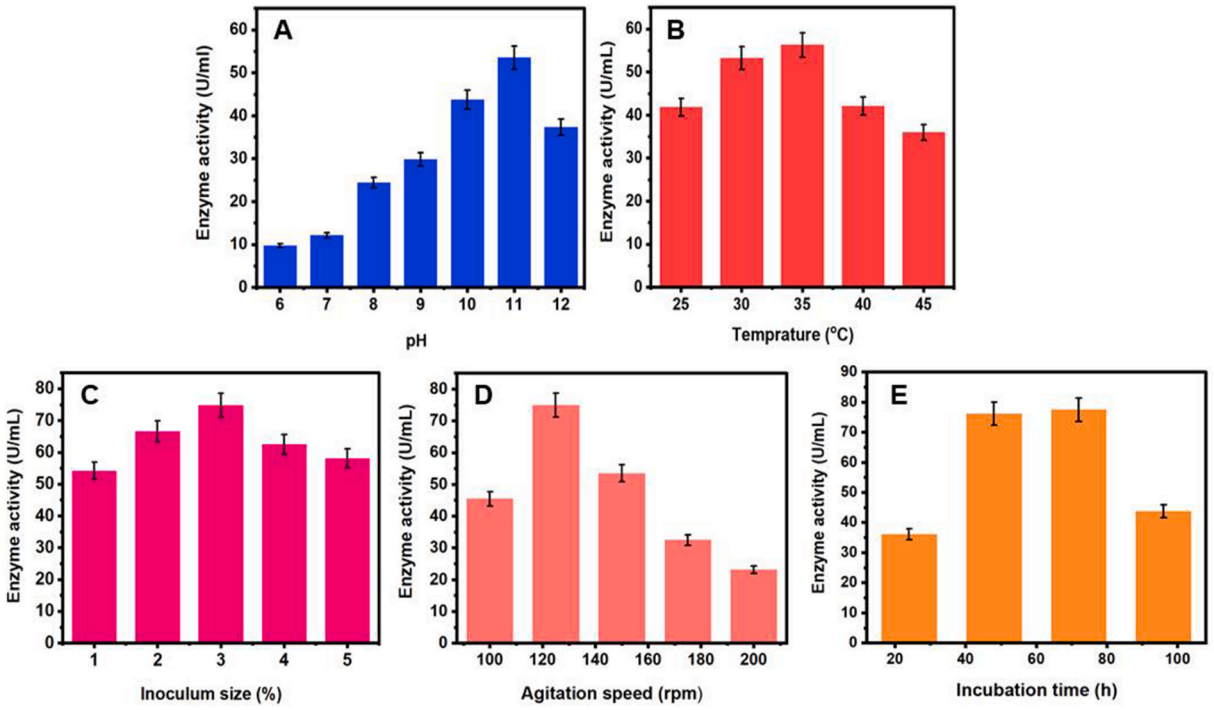

Fig. 4. The effect of physicochemical parameters on keratinase production. (A) pH: (B) Temperature: (C) Inoculum size: (D) Agitation speed and (E) Incubation time.

agar (Table 2). The DRS4 bacterium showed positive results in numerous biochemical tests, including catalase, citrate, methyl red, mannitol, and Voges-Proskauer (Table 2). To taxonomically assign the keratinolytic bacterium, a region that carries much of the diversity information was PCR-amplified and sequenced. The 16S rRNA sequence analysis revealed that the DRS4 belongs to the genus *Bacillus*, showing 99.78% similarity to several species of *Bacillus*. Thus, partial 16S rDNA gene sequences from members of the *Bacillus* sp. were used to construct the phylogenetic tree by including additional isolates from NCBI (Fig. 3). The phylogenetic tree of the strains is shown in Fig. 3. Therefore, isolated DRS4 formed a cluster with *B. subtilis*, named *Bacillus* sp. DRS4.

3.3. Optimization of keratinase production parameters

During the study of keratinase production from *Bacillus* sp. DRS4, several physicochemical factors were investigated, including pH, temperature, inoculum size, agitation speed, and incubation period. This study assessed the impact of pH on the production of keratinase by *Bacillus* sp. DRS4 in the pH range of 6–12. The results indicated that keratinase production increased with increasing pH levels, reaching its peak at pH 11, with a keratinase activity of 53.57 ± 2.67 U/mL (Fig. 4A). However, beyond this pH, the activity decreased. These findings suggest that *Bacillus* sp. DRS4 produces alkalophilic keratinase, which makes the keratin easier to break down by the keratinase enzyme [25,26]. Interestingly, most bacteria, actinomycetes, and fungi have the highest keratinase production in the neutral to slightly alkaline pH range [25,26]. In contrast, other bacteria, including *Bacillus subtilis* DP1 [2] and *Bacillus pumilus* GRK [27], achieved maximum keratinase production at pH 10.

The incubation temperature is another factor influencing keratinase production; thus, its impact was investigated at temperatures between 30 °C and 45 °C and pH 11. *Bacillus* sp. DRS4 showed the highest keratinase activity (56.27 ± 2.81 U/mL) at 35 °C (Fig. 4B). Similarly, many alkaliphilic bacteria, such as *Bacillus* sp. MBRL 575 [16] and *Bacillus subtilis* DP1 [2], attained optimal keratinase production at mesophilic conditions (30–37 °C). On the other hand, some keratinolytic bacteria exhibit optimal keratinase activity at higher temperatures, ranging from 40 to 70 °C. Examples include *Bacillus weihenstephanensis,* which shows peak activity at 40 °C [28], and *Fervidobacterium islandicum* AW-1, which achieves optimal activity at 70 °C [29].

Further, different inoculum sizes (1–5%) were also evaluated to optimize keratinase production by the *Bacillus* sp. DRS4. The highest level of keratinase production, reaching 74.83 ± 3.741 U/mL, was achieved at a 3% (v/v) inoculum size (Fig. 4C). As the inoculum size increased beyond 3%, enzyme production gradually decreased. This decline is likely due to a combination of faster bacterial growth and depletion of nutrients in the medium [30].

Likewise, the effect of agitation speed on keratinase production by *Bacillus* sp. DRS4 was tested at various speeds between 100 and 200 rpm under previously optimized conditions. *Bacillus* sp. DRS4 exhibited the highest keratinase activity (74.8 U/mL) at 125 rpm under these conditions (Fig. 4D). Next, incubation times ranging from 24 to 96 h were employed to investigate how they affect keratinase production under optimal conditions. The highest keratinase production (77.39 ± 3.86 U/mL) by *Bacillus* sp. DRS4 was

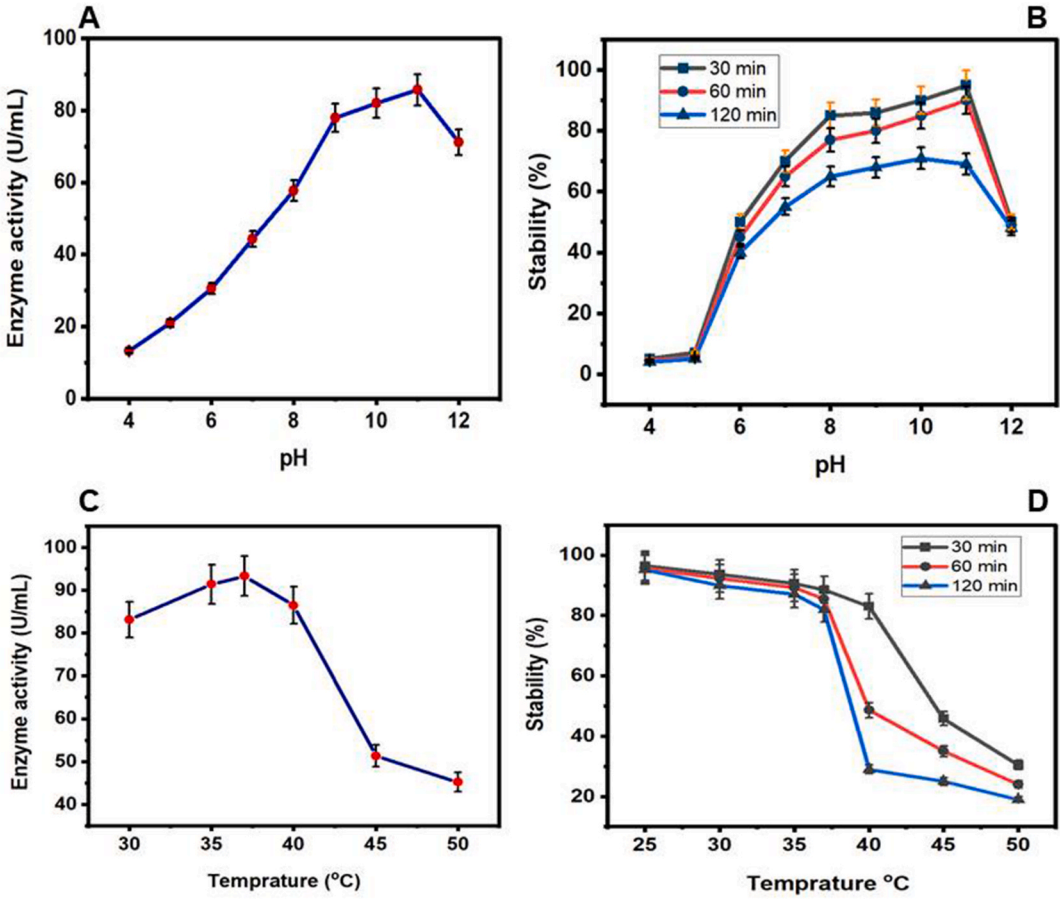

Fig. 5. The effect of pH on enzyme activity (A) and stability (B) and the effect of temperature on enzyme activity (C) and stability (D).

observed at 72 h, when chicken feathers served as the sole carbon and nitrogen source (Fig. 4E). After 72 h, a gradual loss of enzyme activity was observed, likely due to enzyme breakdown and feedback inhibition [31]. Overall, the enzyme yield increased by 30% after applying all optimized parameters for fermentation.

3.4. Effect of pH and temperature of keratinase activity and stability

Enzymes with broad pH activity are valuable for both commercial and industrial applications. To study the effect of pH on the activity of the partially purified alkaline keratinase, the enzyme activity was tested across a pH range of 4–12 using different buffer systems. The partially purified alkaline keratinase showed activity across a broad pH range, with the highest activity observed at pH 11 (Fig. 5A). While a relatively low activity (13.16 ± 0.65 U/mL) was observed at pH 4, the activity steadily reached 44.24 ± 2.21 U/mL at pH 7. Furthermore, the enzyme retained over 80% of its activity even after 60 min of incubation at a pH range of 8–11 (Fig. 5B), indicating its stability in this alkaline pH range. In contrast, the enzyme showed decreased stability at lower pH values after 60 min of incubation. These findings suggest that this enzyme may be advantageous for feather hydrolysis at higher pH values due to its stability and activity in that range.

Next, enzyme assays were conducted at temperatures ranging from 25 to 45 °C to examine the effect of temperature on the activity of partially purified keratinase. As illustrated in Fig. 5C, the results revealed that keratinase activity increased as the temperature increased, with a peak at 37 °C. Additionally, the enzyme retained more than 70% of its activity even after 120 min of incubation at temperatures between 25 and 37 °C (Fig. 5D). Similar results were reported for an alkaline keratinase from *Bacillus subtilis* DPI, which exhibited activity across a broad pH [8–12] and temperature (20–50 °C) range, with optimal values at pH 10 and 37 °C [2].

3.5. Production of keratin hydrolysate and keratin-based bioplastic

Chicken feathers are an excellent source of hydrolyzed keratin protein, usable in various applications like bioplastic films, biodegradable materials, nitrogen-rich fertilizers, animal feed, and bioenergy [4,7]. In this study, the dialyzed alkaline keratinase from DRS4 was utilized to fully degrade WCF within 72 h under optimized culture conditions (Fig. 6). The keratin hydrolysate contained

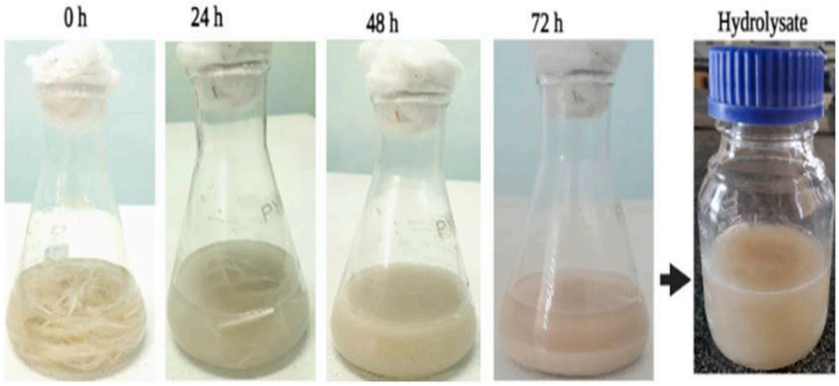

Fig. 6. Degradation of white chicken feathers by purified keratinase enzyme to generate keratin hydrolysate.

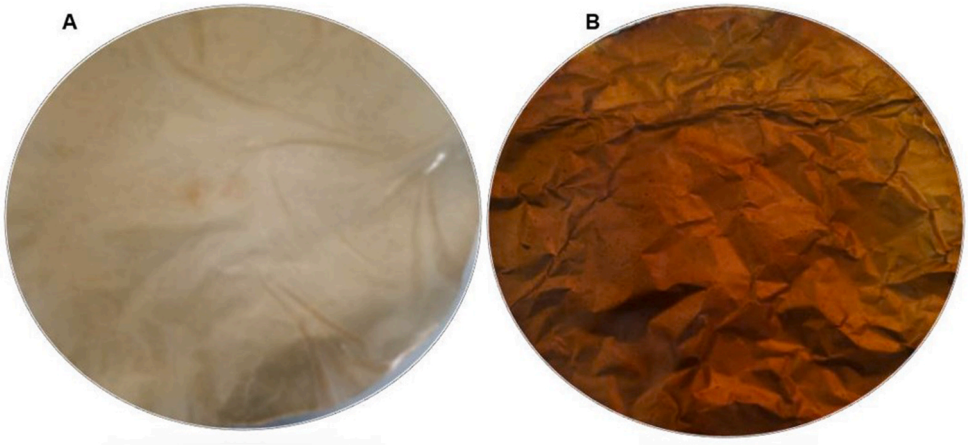

Fig. 7. Keratin-based bioplastic films synthesized from feather hydrolyzate and (A) glycerol (1%): (B) glycerol (1%) and bacterial nanocellulose (0.2%).

251.145 mg/mL of protein, indicating the efficacy of keratinase from DRS4 in breaking down the WCF. Furthermore, the obtained keratin solution was combined with glycerol and BNC to manufacture bioplastic films (Fig. 7A–B), and its characteristics were subsequently analyzed.

3.6. Characterization of keratin-based bioplastic films

3.6.1. Tensile strength

Bioplastics crafted solely from keratin hydrolysate exhibit fragility with inadequate strength and mechanical properties. Glycerol is a widely employed plasticizer known to enhance film flexibility by optimizing its concentration [9]. Consequently, a low glycerol concentration (1%) exhibited a higher tensile strength value of 0.722 MPa, which decreased to 0.256 MPa with an increase in glycerol concentration up to 5% (Fig. 8A), aligning with prior findings [23,32]. Sanyang et al. examined the impact of different plasticizers at various concentrations (ranging from 0% to 45% w/w) on the tensile strength and thermal properties of sugar palm starch films [32]. Their findings indicated that the tensile strength of plasticized sugar palm starch films decreased with higher concentrations of plasticizers.

Ramakrishnan et al. also showed a decrease in both the tensile strength and Young's Modulus of keratin bioplastic films with higher glycerol concentrations [23]. When films contain less than 20% glycerol, they are more likely to be fragile and may exhibit an uneven texture and appearance [8]. This can affect the overall quality and durability of the films, making them unsuitable for desired applications and requiring additional reinforcement. Remarkably, incorporating BNC (0.20%) improved the tensile strength of bioplastic to 5.769 MPa (Fig. 8A). In a related study, Alashwal et al. explored the development of bioplastic films by leveraging different combinations of keratin extracted from chicken feathers and cellulose [33]. The bioplastic films exhibited good surface morphology, high crystallinity, and good thermal properties. Amin et al. produced bioplastics using starch, vinegar, and glycerol, with the tensile strength ranging from 3.55 to 3.95 MPa [34]. Moreover, BNC has an advantage in bioplastic production due to its high tensile strength and better mechanical properties [35].

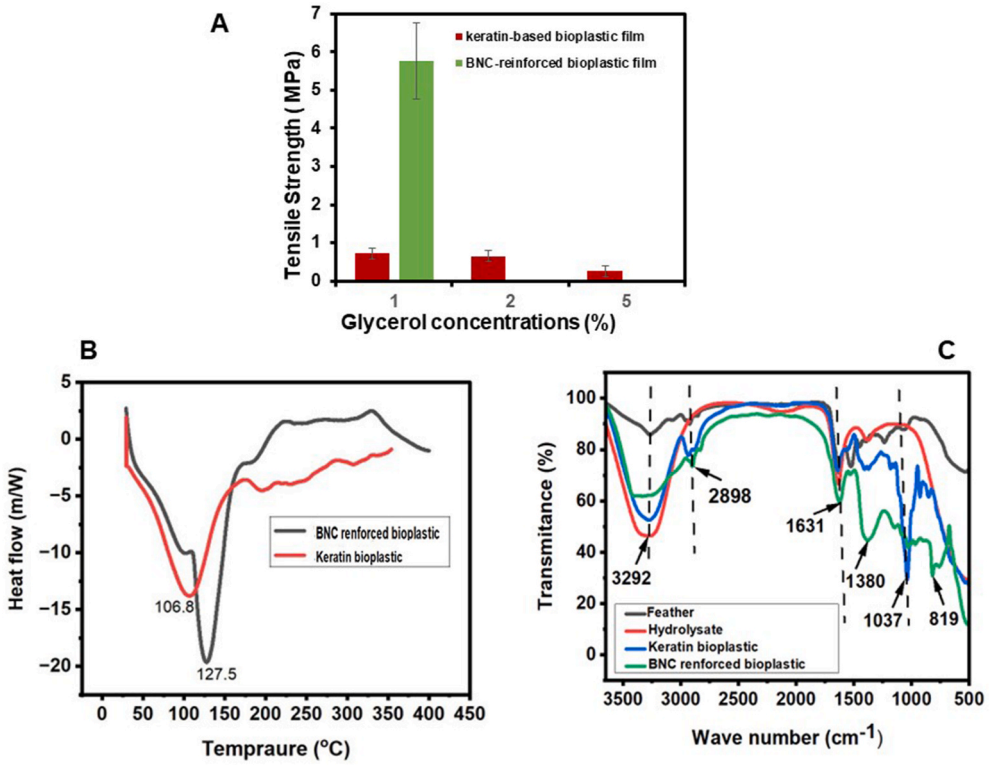

Fig. 8. Characterization of keratin-based bioplastic films. (A) Tensile strength of a bioplastic films with different concentration of glycerol and bacterial nanocellulose. (B) Differential Scanning Calorimeter curves of a BNC-reinforced plastic film and a film without BNC. (C) FTIR spectrums of produced bioplastics.

3.6.2. Differential Scanning Calorimetry

Thermal properties of BNC-reinforced plastic film were investigated across the temperature range of 25–350 °C using a Differential Scanning Calorimetry (DSC). DSC curves illustrated distinct endothermic peaks corresponding to the melting temperature of the films (Fig. 8B). The BNC-reinforced film displayed the highest melting temperature of 127.5 °C, suggesting that BNC-reinforced plastic films are more heat-resistant than other tested bioplastics. Conversely, the keratin-based bioplastic exhibited a relatively lower melting temperature of 106.8 °C, signifying lower thermal stability compared to BNC-reinforced bioplastic films. In a related research study, Amin and colleagues fabricated a composite bioplastic material [34]. They achieved a significant increase in the maximum melting temperature of the bioplastic, reaching 303 °C, by incorporating titanium dioxide (TiO_2) into the material. This approach holds promise for enhancing the thermal properties of bioplastics, which could have wide-ranging applications in various industries.

3.6.3. Fourier-transform infrared spectroscopy analysis of bioplastic

Fourier Transform Infrared spectroscopy (FTIR) was used to identify changes in the chemical composition of the bioplastic films, such as the formation of new functional groups or alterations to existing ones. A peak in the range of 1700-1600 cm^{-1} (Fig. 8C) corresponds to C═O stretching (amide I) [9]. Additionally, broader peaks between 3000 and 3600 cm^{-1} in both keratin and keratin-BNC bioplastics indicated N–H stretching. The keratin bioplastic exhibited a distinct peak at 1037 cm^{-1}, a characteristic signature of glycerol [23]. Peaks at 819 cm^{-1} suggested higher levels of residual cysteine-S-sulfonate bonds (C–S and S–S) [9,33]. The presence of additional peaks in the BNC-reinforced film highlighted the role of BNC as a reinforcing agent within the bioplastic network [33].

4. Conclusion

This study identified four bacterial isolates capable of feather degradation (40%–81%). *Bacillus* sp. DRS4, the most potent isolate, was optimized for keratinase production. DRS4 achieved the highest enzyme activity at 35 °C, pH 11, 125 rpm, and 3% inoculum. Notably, the partially purified keratinase from DRS4 fully degraded chicken feathers within 72 h, generating soluble hydrolysates. These hydrolysates were then used to synthesize keratin-based bioplastic films with glycerol and BNC. The BNC reinforcement significantly improved the film's mechanical and thermal properties. Consequently, *Bacillus* sp. DRS4 alkaline keratinase is not only a suitable enzyme to solve the issue of feather waste in the environment, but it also offers a workable solution to valorize chicken feathers for synthesizing bioplastic with improved properties.

Data sharing statement

The data generated or analyzed during this study are included in the manuscript. The partial 16S rRNA sequence of *Bacillus* sp. DRS4 was submitted to the NCBI, and the accession number was obtained as PP575820.

CRediT authorship contribution statement

Tiruwork Zewudie Admasie: Writing – original draft, Resources, Methodology, Investigation, Conceptualization. **Fantahun Biadglegne:** Writing – review & editing, Validation, Methodology, Conceptualization. **Ebrahim M. Abda:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Conceptualization.

Declaration of competing interest

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

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