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Enhancing and utilizing the Production of Keratinase Enzymes from *Bacillus amyloliquefaciens* for the Recycling of Feather Waste

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***Abstract*— The use of the microbial keratinase enzyme to remediate keratinous waste is an attractive approach in green processing and biotechnology, aiming to eliminate waste accumulation in the environment and recycle it into economical or nutritional products. This study aims to enhance the environmental and nutritional conditions of the growth medium for a keratinolytic bacterium isolated from the soil of a poultry farm in Basrah Province, Iraq, to increase its keratinase production. Subsequently, the keratinase will be used to convert feathers into a poultry feed supplement (feather meal). In the present study, the bacterial isolate was identified as *Bacillus amyloliquefaciens* strain 19E2. The optimal conditions for keratinase enzyme production were determined by evaluating various factors, including temperature, pH, carbon sources, nitrogen sources, and inoculum size. The keratinase activity before optimizing the environmental and nutritional conditions was measured at 42.1 U, after applying the best at 37°C, pH 8, tryptone as the preferred nitrogen source, glucose as the preferred carbon source, and an inoculum size of 1 ml during a 48-hour incubation period, the keratinase activity increased to 129.3 U. This *Bacillus amyloliquefaciens* strain 19E2 was then utilized to convert 25 g of feathers into 8 g of feather meal, which was tested by FTIR and found to be a rich source of amino acids and short peptides for use as a nutritional supplement.**

Keywords— Feather meal, Keratinolytic bacteria, keratinase activity, Optimal conditions, *Bacillus amyloliquefaciens*.

I. INTRODUCTION

Poultry farming has experienced substantial changes in recent years, primarily driven by population growth and the increasing demand for protein sources, with chicken meat emerging as one of the most important and accessible options. This increase in production has also led to a rise in by-products and waste generated by the industry, which need to be disposed of safely [1]. Among these by-products, feathers are particularly problematic due to their long-term environmental stability [2]. Composed primarily of keratin protein (91%), along with 1% fat and 8% water, feathers consist mainly of β-keratin. Additionally, they are rich in essential amino acids, including tryptophan, methionine, and lysine [3, 4].

Keratin residues are stable in the environment and resist degradation. Consequently, they are often disposed of through random methods, such as burning, which releases toxic gas emissions. The toxic emissions from burning feathers are higher than those produced by coal combustion plants [5]. Another method of disposal is landfilling, which also harms the environment. This approach requires large areas, emits unpleasant odors and toxic gases like ammonia and hydrogen sulfide, and contaminates soil and groundwater [6]. Thermochemical treatments of feather waste are more effective than previous methods because they convert feathers into nutritional supplements, specifically feather meal, for livestock and poultry feed. Although these methods effectively break down keratin, they come with significant economic costs and environmental hazards due to the use of strong acids and bases. Furthermore, the soluble substances produced often have low nutritional value, as they can degrade essential amino acids and create undesirable compounds like cysteine, histidine, and lysinoalanine, rendering them cost-ineffective [7].

Biotechnology methods are increasingly utilized to treat keratinous residues, such as feathers, due to their efficiency, environmental safety, and low cost. Keratinase, an enzyme that breaks down the resilient structure of feathers, is produced by various microorganisms in diverse environments. This degradation process converts feathers into peptides and amino acids, resulting in a high-nutritional-value substance that is also economically viable. As a result, it has a wide range of industrial and commercial applications [8].

The keratinase produced by *Bacillus licheniformis* ER-15 efficiently degrades feathers into feather meal within 8 hours at a pH of 8 and a temperature of 50 °C, with shaking at 150 rpm. This process This process yields a product rich in essential amino acids, with a digestibility of 73% as demonstrated in laboratory tests in laboratory tests [9]. Under optimal growth conditions, *Bacillus licheniformis* was able to degrade 10 grams of white chicken feathers within 48 hours, producing a substance

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that retained all the essential amino acids present in the original feathers. The protein content and digestibility of the degraded feathers were 88.7% and 78.0%, respectively, compared to 13.4% and 6.8% in the unprocessed feathers [10]. Furthermore, the keratinolytic bacterium *Bacillus amyloliquefaciens* KB1, isolated from a chicken farm, proved to be the most effective at degrading feathers under optimal growth conditions. This results in soluble proteins and peptides reaching 260 mg/g, along with an increased concentration of amino acids compared to the manufactured feather meal broth [8].

The current study aims to utilize the keratinolytic bacteria Bacillus licheniformis, which has been identified and characterized as the most effective isolate for keratinase production, to degrade white chicken feathers. This process will transform the feathers into feather meal, enhancing both nutritional value and environmental conditions.

II. MATERIALS AND METHODS

A. Isolation and Identification of Utilized Bacterium in the Study

The bacterium used in the current study was isolated from a soil sample collected in a poultry field in the Al-Dair area, north of Basrah City, Iraq, as reported by Al-Amery and Alyousif [11]. Its identification was accomplished using molecular techniques, specifically through sequence analysis of the 16S ribosomal DNA gene with universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3 ') and 1492R (5'-GGTTACCTTGTTACGACTT-3').

B. Preparation of Chicken Feathers

White feathers collected from the Saadoun Saleh poultry field in the Basrah governorate were thoroughly washed several times with tap water. To remove fat, the feathers were soaked for two days in a chloroform and methanol mixture (1:1 V/V), followed by an additional two-day soak in a chloroform, acetone, and methanol mixture (3:1:4 V/V/V), with the solution refreshed daily. Finally, the feathers were washed multiple times with tap water to eliminate any solvent residue and then dried for three days at 50 °C [12].

C. The Primary and Secondary Screening of Keratinolytic Bacterium

The initial screening to assess the keratinolytic ability of the bacterial isolate was performed by streaking it on skim milk agar. The positive isolate produced a transparent halo around its colony on the agar. Following this, a secondary screening was conducted in which the bacterial isolate was activated in the nutrient broth. Then, 1 mL of the activated isolate was added to 20 ml of modified mineral salt medium (MSM), which contained NaCl (0.5 g/L, KH_2PO_4 (0.7 g/l), K_2HPO_4 (1.4 g/L), $MgSO_4 \cdot 7H_2O$ (0.1 g/L), and feathers (2.5 g/L). This mixture was incubated for 72-hours [13].

D. Keratinase Enzyme Assay

The activity of the keratinase enzyme was evaluated by mixing 4 mL of pH 10 buffer, 20 mg of feather, and 1 mL of diluted keratinase enzyme. The mixture was incubated in a water bath at 60 °C for 60 minutes. To stop the reaction, 4 ml of 5% TCA was added. A control sample was prepared without the diluted keratinase enzyme, using 1 ml of 5% TCA to halt the reaction instead. After incubation at room temperature, the mixture was centrifuged at 4000 rpm for 20 minutes. The absorbance of the supernatant was measured at 280 nm using a UV-VIS spectrophotometer, where an absorbance value of 0.01 corresponds to 1 unit of enzyme activity [9].

E. Enhancing The Activity of Keratinase Enzyme

Experiments were conducted to assess how various cultural conditions influence the production and activity of the keratinase enzyme. The conditions evaluated included carbon sources, nitrogen sources, pH, temperature, and inoculum sizes [13].

F. The effect of Temperature on the Production of Keratinase Enzyme

The optimal temperature for producing keratinase enzyme was determined by incubating the culture media at three different temperature levels (25, 37, and 40 °C) at a pH of 7 for 72-hours [13].

G. The Effect of pH on the Production of Keratinase Enzyme

The optimal pH for producing the keratinase enzyme was determined through experiments conducted at pH levels ranging from 6 to 10. To maintain the desired pH, 0.1 N HCl or 0.1 N NaOH was used, and incubation occurred at 37 °C for 72-hours [14].

H. The Effect of Organic and Inorganic Nitrogen Sources on the Production of Keratinase Enzyme

Various organic nitrogen sources, such as tryptone and yeast extract, along with inorganic sources like NH4Cl and (NH4)2SO4, have been assessed to determine the most effective nitrogen source for enhancing keratinase enzyme production. A concentration of 0.1% was incorporated into the growth medium, which was maintained at 37 °C and pH 7 for 72-hours [13].

I. *The Effect of Carbon Sources on the Production of Keratinase Enzyme*

To identify the optimal carbon source for enhancing keratinase enzyme production, different carbon sources, including glucose, sucrose, and starch, were separately added to the production medium at a concentration of 0.1%. The incubation was conducted at 37°C and pH 7 for 72-hours [14].

J. The Effect of Inoculum Sizes on the Production of Keratinase Enzyme

Various inoculum volumes (0.5, 1, 3, and 5 mL) were examined in the media to determine the optimal volume for maximizing keratinase enzyme production at 37°C and pH 7 over 72-hours [14].

K. Processing Feather Meal

Feather meal processing was carried out using the method described by Tiwary and Gupta [9], with slight modifications to prepare the feathers raw as livestock feed. A total of 25 grams of feathers were processed and mixed with 250 ml of mineral salts medium (MSM) at a pH of 7 followed by-sterilizing in an autoclave. Once cooled to room temperature, the medium was inoculated with 20 mL of a bacterial isolate that had been pre-activated in nutrient broth. The mixture was then incubated for 96 hours in a shaking incubator at 37°C. After incubation, the medium containing the degraded feathers was dried in an oven at 50°C for 72-hours, and the dried material was subsequently finely ground for FTIR analysis

L. Feather Meal Tested Using Fourier Transform Infrared Spectroscopy (FTIR)

Screening was conducted to identify effective groups of feathers before and after degradation using an FTIR device. Infrared spectrum analysis was conducted with the Shimadzu FTIR at the Department of Chemistry, College of Science, University of Basrah. Approximately 1 mg of degraded and dried feathers was ground with 100 mg of KBr to form a powder, which was then pressed into thin pellets. Measurements were taken with the FTIR device over a wavenumber range of 4000 to 400 cm^{-1}.

III. RESULTS AND DISCUSSION

A. Isolation and Identification of Utilized Bacterium in the Study

The bacterial isolate used in the current study was previously identified by Al-Amery and Alyousif [10] through PCR techniques and sequence analysis of the 16S ribosomal DNA gene. This isolate was identified as *Bacillus amyloliquefaciens* strain 19E2. *Bacillus amyloliquefaciens* strain 19E2 is a versatile bacterium with various applications, characterized by several key features. Biochemically, it produces a diverse array of enzymes, including amylase, protease, lipase, and chitinase, enhancing its potential for applications in bioremediation and biocontrol. Physiologically, strain 19E2 thrives in aerobic conditions and can form spores, allowing it to endure harsh environments. Additionally, it is closely related to another strain of *Bacillus amyloliquefaciens* known as F9.

B. The Primary and Secondary Screening of Keratinolytic Bacterium

The primary screening of the keratinolytic bacterium was performed using a skim milk agar medium. *Bacillus amyloliquefaciens* strain 19E2 showed positive results, forming clear zones around its colony, as depicted in Fig. 1. Following this, strain 19E2 underwent a secondary screening to assess its ability to degrade feathers in a mineral salt medium (MSM). The results indicated that *Bacillus amyloliquefaciens* strain 19E2 possesses significant feather biodegradation potential, with the highest keratinase activity recorded at 42.1 U/mL, as shown in Fig. 2.

C. Improving the Production of Keratinase Enzyme from Bacillus amyloliquefaciens Strain 19E2

The optimal conditions for producing the keratinase enzyme were determined by evaluating This study examined various variables, focusing on three temperature values: 25, 37, and 40 °C, to evaluate keratinase production. The results revealed that the optimal temperature for producing keratinase from *Bacillus amyloliquefaciens* strain 19E2 was 37 °C, resulting in keratinase activity values of 42.1 U/mL, as shown in Table 1. Although the degradation of feathers can be enhanced by breaking disulfide bonds, this process usually occurs at higher temperatures [15]. These findings support a previous study by Alahyaribeik et al. [16], which also found that the optimum keratinase activity for *Bacillus pumilis* and *Rhodococcus erythropolis* occurred at 37 °C.

Fig. 1: The clear zones around the *Bacillus amyloliquefaciens* strain 19E2 colonies on the skim milk agar

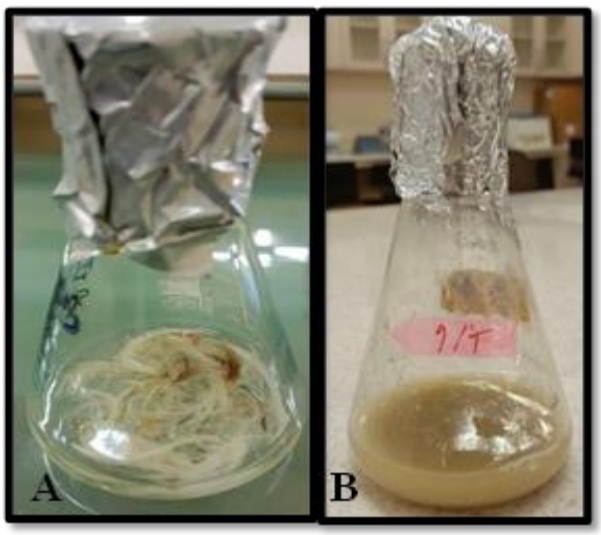

Fig. 2: Secondary screening of *Bacillus amyloliquefaciens* strain 19E2for the production of keratinase. A: The control flask containing MSM without bacterial isolate, B: Flask containing MSM with *Bacillus amyloliquefaciens* strain 19E2.

The pH of the medium influences the reaction mixture and aids the movement of nutrients through the bacterial cell membrane [13]. In this study, we tested various pH values: 6, 7, 8, 9, and 10. The optimal pH for *Bacillus amyloliquefaciens* strain 19E2 was determined to be 8, at which point the keratinase enzyme activity reached 45.4 U/mL. Conversely, at the lowest pH of 6, keratinase enzyme activity dropped to 2.5 U/mL, as shown in Table 1. These results are consistent with findings from Mukhtar et al. [17], which suggested that pH values of 7 and 8 optimize keratinase enzyme activity in bacterial isolates of the Bacillus genus. Additionally, it has been noted that most bacteria produce keratinase and effectively degrade feathers in alkaline conditions, as higher pH levels convert cysteine residues into lanthionine, making them more accessible to the keratinase enzyme. Therefore, an alkaline pH is anticipated to enhance keratin degradation [18].

Table 1: The effect of different variables on keratinase activity (U) of *Bacillus amyloliquefaciens* strain 19E2

Variables		Keratinase activity (U) of *Bacillus amyloliquefaciens* strain 19E2
Temperature	25°C	20.6
	37°C	42.1
	40°C	35.2
pH	6	2.5
	7	42.1
	8	45.4
	9	24.9
	10	23.9
Nitrogen Sources	tryptone	58.5
	yeast extract	51.9
	NH_4Cl	13.3
	$(NH_4)_2SO_4$	17
Carbon Sources	glucose	50
	sucrose	39.3
	starch	27.5
Inoculum sizes	0.5ml	35.4
	1ml	42.1
	3ml	34.8
	5ml	28.7

In the current study, we assessed four nitrogen sources to identify the most effective one for keratinase enzyme production over a 72-hour lag period. The results showed that keratinase activity in the *Bacillus amyloliquefaciens* strain 19E2 varied with the nitrogen source used. Tryptone proved to be the most effective, yielding a keratinase activity of 58.5 U/mL. In contrast, the inorganic nitrogen sources, ammonium chloride and ammonium sulfide, inhibited keratinase activity, resulting in reductions to 13.3 U/mL and 17 U/mL, respectively, as illustrated in Table 1. These findings are consistent with a previous study by Akhtar et al. [14] which investigated yeast extract, tryptone, and aluminum sulfate as individual nitrogen sources in bacterial media. Yeast extract significantly enhanced keratinase production in *B. cereus* and *Pseudomonas* sp., while tryptone had a more modest effect. Conversely, aluminum sulfate inhibited keratinase production in both *B. cereus* and *Pseudomonas* sp. This inhibition is likely due to its characteristics as a weak acidic salt. Aqueous solutions of aluminum sulfate and aluminum chloride lower the pH of the medium, which hinders keratinase production by keratinolytic bacteria that thrive in moderate to alkaline conditions.

Carbon sources are crucial for the growth of microorganisms and the supply of energy for reproduction and biomass production. In this study, three carbon sources were examined: glucose, sucrose, and starch. The results indicated that glucose was the most effective carbon source in the growth medium for *Bacillus amyloliquefaciens* strain 19E2, significantly increasing keratinase enzyme activity to 50 U/mL. In contrast, sucrose and starch had a limited impact on keratinase production, as shown in Table 1. The variations in the effects of these sugars may be due to their chemical structures and how readily bacteria can utilize them. Monosaccharides, made up of a single molecule, are less complex than disaccharides, which consist of two molecules, while disaccharides are simpler than polysaccharides [19].

The most effective parameters were selected to increase the production of keratinase enzyme. These optimal conditions included a temperature of 37 °C, a pH of 8, glucose as the preferred carbon source, tryptone as the preferred nitrogen source, and an inoculum size of 1 mL. Testing these conditions together resulted in maximum keratinase enzyme production, reducing the lag phase from 72-hours to 48-hours. The activity of keratinase from *Bacillus amyloliquefaciens* strain 19E2 increased from 42.1 U/mL to 129.3 U/mL. Several studies, including those by Kumar et al. [20] and Uttangi and Aruna [21], have demonstrated similar enhancements in keratinase activity produced by bacteria.

D. Processing Feather Meal

Twenty-five grams of feathers were degraded in 250 ml of liquid mineral salt medium (MSM) by the *Bacillus amyloliquefaciens* strain 19E2 over 96-hours of incubation in a shaking incubator. The degraded feathers were then dried at room temperature, yielding a final weight of 8.7 grams of feather meal, as illustrated in Fig. 3.

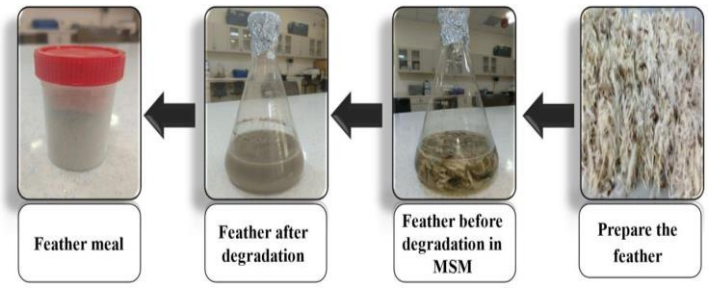

Fig. 3: Processing father meal

E. Feather Meal Tested Using Fourier Transform Infrared Spectroscopy (FTIR)

The active groups in feathers were measured both before and after degradation. Changes in the composition of multiple peptides were analyzed using FTIR, comparing the results to those of the active groups in feathers before degradation (Fig. 2, 3). The FTIR results indicated that the symmetrical CH3 stretching vibrations were responsible for the transmission bands observed in the 3000–2800 cm^{-1} range [22, 23]. In the 1700–1600 cm^{-1} range, a strong transmission band was attributed to C=O stretching (amide I) [24]. Additionally, the N-H bending and C-H stretching contributed to the formation of the amide II transmission band in the 1580–1540 cm^{-1} range [25].

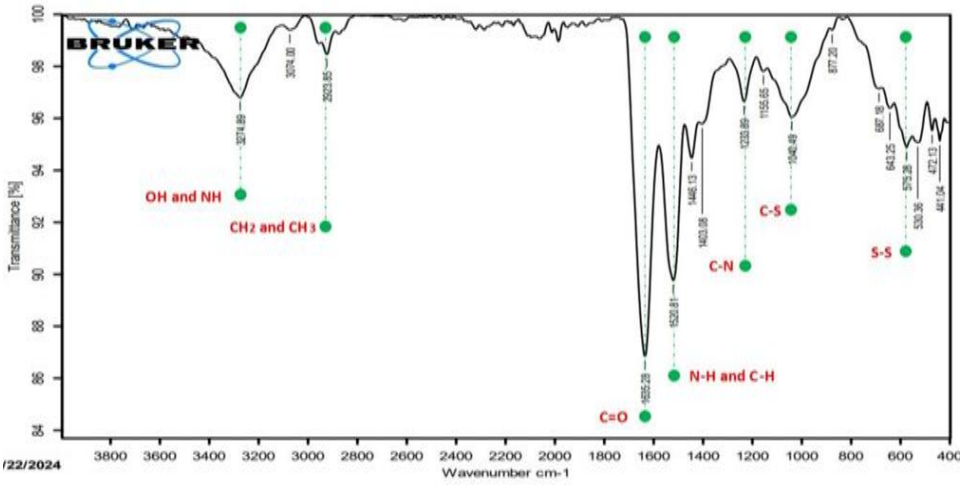

Fig. 4: Results of FTIR for the feather before degradation

The amide III band is a weak feature observed between 1300 and 1220 cm^{-1}, attributed to C-N stretching and N-H bending [26, 27]. Additionally, signals from C-O bending and C-C stretching are also present [28, 29]. Peaks at 990 and 580 cm^{-1} correspond to C–S and S–S bonds, respectively. Notably, the S–S bonds in feathers disappear after degradation, indicating the breakdown of keratin's disulfide bonds [25]. Understanding these bonds is crucial for analyzing protein composition and structural changes [29].

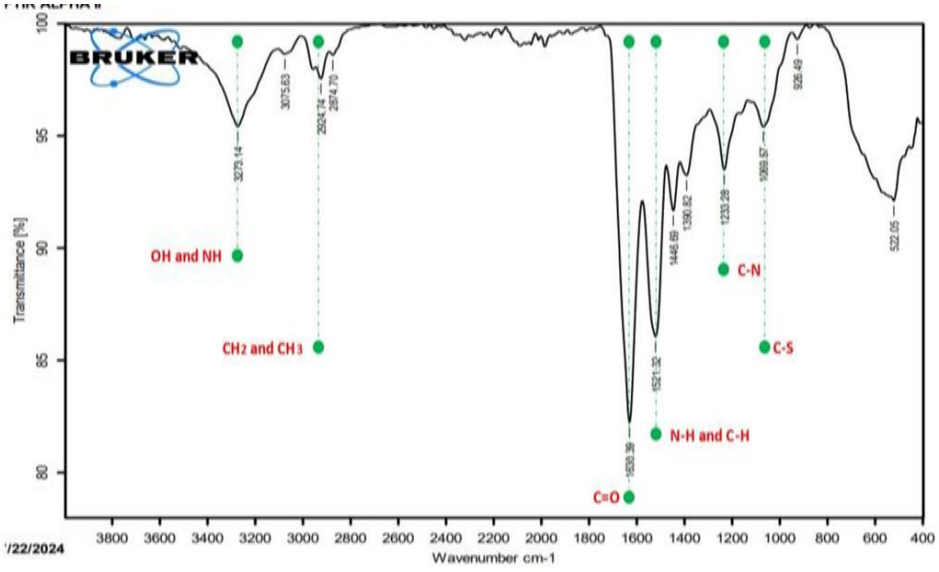

Fig. 5: Results of FTIR for the feather after degradation

This study's results confirm that bacterial degradation of feathers significantly increases the concentration of free functional groups, such as carboxylic acids, compared to undegraded feathers [30]. This increase occurs because feathers are broken down into shorter peptide chains through keratinase activity. As a result, the degraded feather sample is composed of various peptides, while the amino acids remain undamaged during the degradation process [31].

IV. CONCLUSIONS

Microorganisms are affected by various factors in their natural environment, each with specific temperature and pH ranges that optimize their growth and effectiveness. These factors play a crucial role in determining their metabolic potential and enzyme production. Therefore, testing is essential to identify the optimal conditions for growth and to enhance enzyme production in microorganisms. Additionally, the physical and chemical processes used to convert feathers into valuable products are not only costly but also environmentally damaging, as they destroy amino acids in the feathers, diminishing their nutritional value when used as animal feed supplements. However, the environmental pollution caused by feather waste can be alleviated, and the nutritional value of feathers can be improved through remediation by microorganisms that produce keratinase, such as certain bacteria. The findings of the current study highlight the potential for developing new waste management strategies that enhance keratinase enzyme production, thereby contributing to sustainable development in industrial applications and green technologies. While feather waste represents a significant source of protein, there is currently insufficient interest in its utilization or recycling, despite advancements in biotechnology that could create more opportunities in this field.

CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

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