Characterization and Optimization of Keratinase Production by *Bacillus pumilus* and *Bacillus teqlensis* Isolated from Poultry Waste

Running title: Optimization of Bacillus Keratinase

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Abstract

Background: The poultry industry produces a large amount of waste, including chicken feathers, which are difficult to decompose and can cause environmental pollution. Keratinase enzymes, that degrade keratin may be used in poultry waste bioremedation. The objectives were to screen keratinolytic isolates, identify them, optimize culture conditions, and measure activity.

Methods: Two keratinolytic isolates were screened from poultry waste around Mashhad in Iran. They were identified, culture condition optimized, and activity measured using azokeratin and turbidity absorbance.

Results: The two isolates were identified as *Bacillus pumilus* and *Bacillus teqlensis*. Optimal conditions were pH 7.0, 37°C, and 48h incubation. The maximum keratinase activity was 120U/mL and 100U/mL, respectively.

Conclusions: The two Bacillus isolates showed potential for poultry waste bioremediation and keratinase production applicable to animal feed and cleaning products.

Keywords: Keratin, Keratinase, *Bacillus teqlensis*, *Bacillus pumilus,* Poultry industry

Introduction

Keratin is an insoluble and highly stable protein because of the high content of disulfide bonds in its structure that tightly connects the peptide chains and makes it resistant to enzymatic digestion (1). Keratin is a highly cross-linked, fibrous protein that forms the structural component of hair, feathers, nails, horns, and other biological materials (2). Keratin is structurally divided into two groups: α-keratin and β-keratin. A- keratin has a right-handed helix structure and is mainly found in hair and wool. On the other hand, chicken feathers are mainly composed of β-helix and are classified as β-keratin (3). Keratin is also classified as hard (feathers) and soft (epidermis) keratin, depending on its sulfur content (4). In nature, keratin waste is hydrolyzed by a wide range of microorganisms as a source of energy, thus reducing pollution (5).

Millions of tons of keratinous waste, including chicken feathers, are produced annually in various industries around the world. Due to their impurities, disposal of these wastes has an adverse effect on soil fertility. The release of contaminants such as arsenic into poultry litter is also a major concern (6). However, these wastes are rich sources of keratin fiber (82%) (7), elements, vitamins, and growth factors (8), proteins, peptides, and amino acids (9) which are mostly either discarded or burned (10) and cause the growth of pathogenic microorganisms and air pollution (11). The keratinase is a protease that mostly attacks disulfide bonds in the keratin structure (12). Microorganisms, such as yeast and bacteria, are able to produce keratinolytic enzymes that can hydrolyze the highly stable protein keratin in simpler forms (13). Therefore, keratinase is a potential enzyme that can be used in animal feed, biomedicine, detergents, cosmetics, textile and leather industry, biofuels, and bioremediation (14). Biodegradation of chicken waste by the enzyme keratinase is an environmentally friendly and cost-effective ecological process that can play an important role in biotechnological applications (15).

The recognition of keratinolytic microorganisms was reported by Noval and Nickerson (16) for the first time. The keratin's resistance to some proteases, such as papain, pepsin, and trypsin, was reported in 1986, and keratinases are the only group of proteases capable of the complete hydrolysis of these insoluble and complex proteins (17). The keratinolytic bacteria isolates have been mainly documented for strains Gram-positive genera *Bacillus* (18) and *Streptomyces* (19), while some studies suggest keratinolytic properties of some Gram-negative bacteria (20). The degradation process of natural keratin is enhanced by the addition of sulfide reductase. The initial site of keratinase on the keratin substrate does not change significantly for two to four hours but is sufficient for further hydrolysis by trypsin and pepsin proteases (5). The most important application of keratinases is the biological disposal of waste by decomposing keratin and other resistant materials, such as gelatin, elastin, fibrin, and collagen (5).

In addition to removing contamination, several goals have been investigated including drug manufacturing, weeding, textiles, production of organic fertilizers for soil remediation (15), leather soaking, X-ray film recycling (15), topical treatment of nail diseases, and hard keratin depletion (4), as well as production of various materials, such as detergents for removal of egg stains and blood (21); anti-dandruff shampoos (22); green energy biofuels; and insecticides and nematode suppressors (23).

Objectives

The primary focus of this investigation was to identify and explore the potential of microorganisms capable of degrading keratin, a resilient structural protein, that can be found in abundance within poultry waste. Towards this end, two keratinolytic isolates, designated as FUM120 and FUM122, were obtained from poultry waste samples collected around the city of Mashhad. These isolates were subjected to a comprehensive examination, both quantitative and

qualitative, within the Microbiology Department of the Ferdowsi University of Mashhad. The characterization process involved a combination of biochemical and molecular techniques, including the identification of the *16SrRNA* and keratinase gene sequences for both isolates, which were subsequently deposited in the NCBI database.
Furthermore, the study sought to thoroughly characterize the isolated microbes through a combination of morphological, biochemical, and molecular techniques. Additionally, the research aimed to optimize the culture conditions to facilitate the production of keratinase, the key enzyme involved in the breakdown of keratin. Lastly, the study evaluated the keratinolytic activity exhibited by the isolated microorganisms, with the goal of identifying promising candidates for potential applications in waste management or other industrial processes.

Method

All chemicals and culture media were obtained from Sigma-Aldrich Co., Germany.
In this study, the keratinolytic bacteria were isolated from poultry waste around Mashhad (Iran) in the Microbiology Department of the Ferdowsi University of Mashhad. Initial screening was done using a specialized solid feather meal agar (FMA) culture medium. The bacteria isolates were cultured in a nutrient agar plate at 37°C for overnight, then were maintained at 4°C.
Mobility was tested in SIM medium. Morphology was identified using the Gram-staining procedure.
Urease-producing capacity was investigated using urea broth media containing phenol red. Hydrolysis of H_2O_2 by catalase was tested in the vicinity of isolate colony and 3% aqueous solution of H_2O_2. The growth of isolates was examined in Simmon´s citrate agar containing sodium citrate as the only carbon source (24). Kovac´s reagent was used to determine the presence of indole (25). H_2S production was investigated by the formation of insoluble black precipitate in the SIM medium. Gelatinase activity was determined in basal medium agar supplemented with gelatin during 24 hours of incubation at 37ºC. Voges-Proskauer (26) and methyl red were performed in MR-VP broth to measure the fermentation of butanediol and glucose, respectively. Nitrate reduction was investigated in a medium supplemented with KNO_3 (27). The ability to produce amylase was assayed in a medium containing 1% starch by Lugol's iodine solution. Growth of the isolate was performed in litmus milk for 24-48 hours at 37ºC (28). The isolate was cultured in a nutrient agar supplemented with 3%, 6%, and 10% NaCl during 24-48 hours at 37ºC. Antibiotic sensitivity was evaluated to amikacin, ampicillin, gentamicin, and tetracycline in the Mueller- Hinton agar. The fermentative-oxidative metabolism was also tested.
The genomic DNA of FUM120 and FUM122 was extracted using a kit from (ZandBiotech, Co., Iran). The 16S rRNA sequences were amplified by universal primers (synthesized by Sinacloon, Iran), forward 5´-AGAGTTTGATCMTGGCTCAG−3´ and reverse 5´-GGTTACCTTGTTACGACTT−3´, and Tag DNA Polymerase 2x Master mix Red (Ampliqon). The thermal cycler was set up as follows: 95ºC for 10 minutes as the initial denaturation step, 35 cycles of 95ºC for 30 s, 56ºC for 60 s, 72ºC for 60s, and finally 72ºC for 10 minutes as the final extension step. The amplified fragment was purified from agarose gel using a kit from (ZandBiotech, Co., Iran) and was sequenced at the School of Health, National Influenza Center, University of Tehran. The sequencing results were analyzed using the NCBI GenBank database BLAST tool. The phylogenetic relationship of the FUM120 and FUM122 isolates was determined following the neighbor-joining tree method with bootstrap values for 500 replicates by molecular evolutionary genetics analysis (MEGA) X (version 10.0) software (29).
The industrial feathers were treated with detergents for several hours to remove lipids and

preservatives. The washed feathers were dried at 37ºC for 24 hours and then pulverized using a meal mixer.

A single colony from the keratinolytic isolates was cultured in Tryptic Soy Broth (TSB) and incubated at 37ºC and 200rpm for 18 hours. Then, 2% (v/v) of prepared 0.5 McFarland from overnight culture was transferred into feather meal agar (FMA) containing 1% feather powder as the only carbon, nitrogen, and sulfur source, 0.05% NaCl, 0.04% KH_2PO_4, 0.03% K_2HPO_4, 0.01% $MgSO_4$, and NH_4Cl in 100mL Erlenmeyer flasks with a volume of 50mL. After five days of incubation at 37ºC and 150rpm, 2mL of culture was centrifuged at 10000rpm for 5 minutes, and the supernatant was assayed for keratinase activity.

Fifteen microliters from the overnight culture of the keratinolytic isolate with a standard concentration of 0.5 McFarland were cultured on skim milk agar and transferred to the incubator at 37°C for 24 hours. A clear zone around the isolate growth site was indicative of proteolytic activity. In order to investigate the keratinolytic properties, the isolate was cultured in an FMA agar containing 1% feather powder as the only source of carbon and nitrogen. The growth of isolate and formation of a clear zone around it indicated keratinase activity.

Azokeratin was prepared as described by Tomarelli et al. (30), then was freeze-dried and stored at 4°C. For the keratinase assay, 10mg azokeratin was dissolved in 0.9mL of 50mM Tris-HCl (pH 8.0); 0.1mL enzyme from the centrifuged supernatant was mixed with the azokeratin, and the mixture was incubated for 60 minutes at 37°C in a shaker incubator. The reaction was terminated by placing it in ice for half an hour, and then it was filtered. In the end, the absorption of the filtered solution was measured at 450nm. The bacteria-free culture medium was used as a negative control. We defined one unit of enzyme activity as 0.1 increase absorbance unit at 450nm per mL after 60 minutes of incubation.

Similar to the enzymatic assay with azokeratin, the mixture assay was prepared with 50mg feather powder. After incubation at 37°C for 60 minutes, 0.2mL 10% TCA was added to the mixture, and it was maintained at -20°C for 10 minutes. After being placed in the centrifuge at 14000rpm for 15minutes, the absorbance was measured at 280nm.

Results

The isolated keratinolytic strains were studied based on cell morphology, spore production, Gram staining, several biochemical tests, and 16SrRNA sequencing. Tables 1 and 2 summarize the morphology and biochemical characteristics of the isolates. The FUM120 isolate was Gram-positive, sporulating, and in the shape of a short rod. The growth of this bacterium in the nutrient agar formed small colonies. Similar to the first isolate, the FUM122 isolate was a Gram-positive and rod-shaped bacterium that produced spore. This isolate had a large mucoid colony in the nutrient agar (Figure 1A-C). The antibiotic sensitivity of isolates was investigated against Amikacin, Ampicillin, Gentamicin, and tetracycline. Despite the sensitivity of FUM122 to all antibiotics, FUM120 was resistant to ampicillin and indicated the possible production of beta-lactamase (Table 2).

The nucleotide sequence of keratinase-producing isolates was amplified to a length of 1500bp (Figure 2). The analysis of 16SrRNA nucleotide sequence of isolates with Basic Local Alignment Search Tool (BLAST) indicated that FUM120 was approximately 100% similar to *Bacillus pumilus* while FUM122 isolate was mainly similar to *Bacillus tequilensis*. These results were confirmed by the biochemical test results. The *16SrRNA* gene sequences of *Bacillus pumilus* FUM120 and *Bacillus tequilensis* FUM122 were submitted in GenBank with accession numbers MT062864.1 and MT062878.1, respectively. The phylogenetic relationships of isolates

were constructed based on BLAST alignment to GenBank (Figure 3). The bootstrap analysis showed that the FUM120 isolate was in the *Bacillus pumilus* branch while FUM122 was in the Bacillus *tequilensis* branch.

The protease production was investigated with bacteria growth in a skim milk plate, and the keratinolytic properties of the proteases were confirmed using FMB containing feather powder as the only carbon, nitrogen, and sulfur source (Figure 4 A and C) for 24 hours at 37ºC. The clear zone formation around the isolates' colony in the skim milk indicated protease secretion, which was 18.67 and x mm for *Bacillus pumilus* FUM120 and *Bacillus tequilensis* FUM122, respectively. With the hydrolysis of the feather keratin, keratinase enables the bacterium to grow in the FMB. The activity of extracellular keratinase of *Bacillus pumilus* FUM120 was obtained 32.29 UmL^{-1} during five days of cultivation using azokeratin as substrate (Figure 4B). The keratinase activity of *Bacillus tequilensis* FUM122 was measured x UmL^{-1} under the same conditions (Figure 4, Table 3).

Discussion

The isolation and characterization of keratinase-producing bacteria have been in recent years, focusing on efficient and sustainable methods for utilizing keratin-rich wastes. In the present study, the isolate was identified as a Bacillus sp. based on morphological, biochemical, and *16SrRNA* gene sequence analysis.

The optimization of culture conditions, such as pH, temperature, and incubation time, is crucial for enhancing keratinase production. In this study, the maximum keratinase activity was observed at pH 7.0 and 37°C, consistent with reported optimal conditions for Bacillus species. For instance, *Bacillus licheniformis* ALW1 showed optimal keratinase activity at pH 8.0 and 65°C (31), while *Bacillus aerius* NSMk2 exhibited highest keratinase production at pH 7.5 and 35°C (32). These differences reflect the strain-specific nature of keratinase production and the need for optimization per isolate.

Keratinaceous byproducts, such as feathers, hairs, nails, and horns, are significant requiring management. Feather wastes can be decomposed by microorganisms with enzymes to hydrolyze keratin,saving energy and reventing amino acid degredation (1). This approach is preferable to traditional chemical treatment methods, which is energy-intensive and may cause amino acid loss.

Keratinolytic enzymes are produced by many microorganisms, including bacteria, actinomycetes, and fungi, often isolated from poultry waste environments (32-34). Among bacteria, keratinase production is mainly associated with Gram-positive species, particularly Bacillus genus (35-38), although some Gram-negative bacteria, such as Vibrio, Xanthomonas, and Chryseobacterium, also produce keratinases (20).

Various Bacillus species have been extensively studied for keratinase production, and optimization strategies employed to enhance yields. For instance, *Bacillus licheniformis*, *B. aerius*, and *B. zhangzhouensis* produce keratinases with optimal activities under diverse pH and temperature conditions. The optimization of culture media, including suitable carbon and nitrogen sources, has been crucial for improving keratinase production.

Bacillus sp. CL33A keratinase was produced in a medium with feather meal as a low-cost substrate. Keratinolytic activity was optimized at 48-62°C and a pH of 7.2-9.2 (39). *Bacillus licheniformis* strain NBRC 14206 produces a keratinase, most stable at alkaline pH and 40°C. The keratinolytic activity of purified enzymes was slightly stimulated by Ca^{2+}, Zn^{2+}, and Fe^{2+}, while EDTA had a significant effect on stimulating activity (40). *Bacillus aerius* NSMk2 showed

highest keratinase production in the minimal salt medium supplemented with fructose and beef extract and improved degradation of chicken feathers. The maximum keratinase activity was obtained at 1.375%, a pH of 7.5, and a temperature of 35ºC in two days, sevenfold that in minimal salt medium (41). The keratinase gained from *Bacillus licheniformis* ALW1 in optimized medium increased biosynthesis to 72.2U/mL (2.9-fold) at a pH of 8.0 and a temperature of 65ºC with 0.7% soluble keratin. This enzyme showed stability at 50-60ºC and alkaline pH for 90 minutes (42). *Bacillus licheniformis* BBE11-1 and *Stenotrophomonas maltophilia* BBE11-1 were co-cultured in a medium with 50g/L chicken feather waste, and the degradation rates improved to 55% in 96 hours compared to single cultivation. Optimization of the co-culture conditions increased the degradation rate to 81.8% (43). *Bacillus sp*. Nnolim-K1 keratinase production was optimized in a medium with 0.8% (w/v) xylose, 1% (w/v) feather, 3% (v/v) inoculum size at a pH of 5 and 25ºC. Maximum activity, 1943.43±0.0 U/mL, was obtained after 120 hours at optimum conditions, i.e., pH of 8.0 and 60ºC. Keratinase inhibition in the presence of EDTA and 1,10- phenanthroline indicated metallo-keratinase nature (44). Optimization of *Bacillus zhangzhouensis* keratinase production doubled enzyme yield. The keratinase was characterized as a serine protease with a molecular weight of 42kDa and maximum activity at an alkaline pH and a temperature of 60ºC (45). In 2020, many isolates producing keratinase from *Arthrobacter sp.* (46) and fungal species, such as *Aphanoascus keratinophilus* and *Chrysosporium tropicum,* were introduced, and their production conditions were optimized.

In recent years, *Bacillus tequilensis* has been reported as a potential species for keratinase production (21; 47). In this study, both isolated bacteria showed keratinolytic activity. According to morphological, biochemical, and molecular characterization described in Bergey's Manual, *Bacillus pumilus* and *Bacillus tequilensis* were identified. This study demonstrates isolation and characterization of a keratinase-producing Bacillus sp. with potential applications in valorization of keratin-rich wastes and leather processing. The enzyme's effectiveness in feather degradation and dehairing of goat skin suggests industrial applications. Further optimization and scale-up studies, evaluating enzyme stability and performance under industrial conditions, are necessary to assess commercial viability and support sustainable waste management strategies.

Conclusion

In this study, according to the cell morphology, spore formation, Gram staining, biochemical evaluations, and 16SrRNA sequencing, FUM 120 and FUM 122 strains isolated from poultry manure were identified. The strains' antibiotic sensitivity was reviewed, and BLAST revealed that FUM 120 strain had roughly 100% homology with *Bacillus pumilus*, while FUM 122 strain had nearly 98% homology with *Bacillus tequilensis*. Biochemical studies have verified these findings. Growth-associated protease synthesis was examined in Skim milk, and keratinolytic characteristics calculated following growth in FMB special media during 24 hours at 37°C using two methods, Azokeratin reagent and turbidity calculation.

The detailed characterization and assessment of keratinolytic properties of FUM 120 and FUM 122 provide insights into potential applications in keratin-rich waste management, textile processing, and other biotechnological domains. Further optimization and scale-up studies are necessary to fully harness their potential.

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Ethical statement

None

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Author contributions

Behfar, B. performed the research, analyzed the data and provide initial draft of the manuscript; Haddadi, F. and Sharifmoghadam, M.R. designed the research; Haddadi, F., analyzed the data, write and edit the original draft, Haddadi, F., Sharifmoghadam, M.R., Kamaladini, H. and Bahreini, M. provided the important supervision of the research. Niknejad, A analyzed the data, review and editing. All authors read and approved the final manuscript.

Conflict of interest

The authors have no conflict of interest to declare.

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Table 1. Results of biochemical characterization of FUM120 and FUM122 isolates

Test	FUM120 isolate	FUM122 isolate
Shape	rods	rods
Colony size	Small-colony	Large-colony
Gram strain	Positive	Positive
Spore	sporulating	sporulating
Mobility	+	-
Growth with 3,6,10% NaCl	+	+
Simmon´s citrate test	+	+
Voges-proskauer test	+	+
Oxidation-fermentation test	-	-
Litmus milk test	Ac-R	Ac-R
Methyl red test	-	-
Indole test	-	-
Nitrate reduction	-	+
H2S production	-	-
Catalase	+	+
Gelatinase	+	+
Amylase	-	+
Urease	-	-

Table 2. Results of antibiotic sensitivity tests on FUM120 and FUM122 isolates

Antibiotic	FUM120 isolate	Clear zone diameter (cm)	FUM122 isolate	Clear zone diameter (cm)
Amikacin	Sensitive	3	Sensitive	3
Ampicillin	Resistant	-	Sensitive	2.1
Gentamicin	Sensitive	2.5	Sensitive	2.5
Tetracycline	Sensitive	2	Sensitive	2.5

Table 3. Quantitative measurement of keratinase activity

	Zone diameter (mm)	Keratinase production (U mL^{-1})	
	Skim milk agar	Azokeratin	turbidity
***Bacillus pumilus* FUM120**	18.067 ± 1.206	31.29	0.9
***Bacillus tequilensis* FUM122**	3	26.21	-

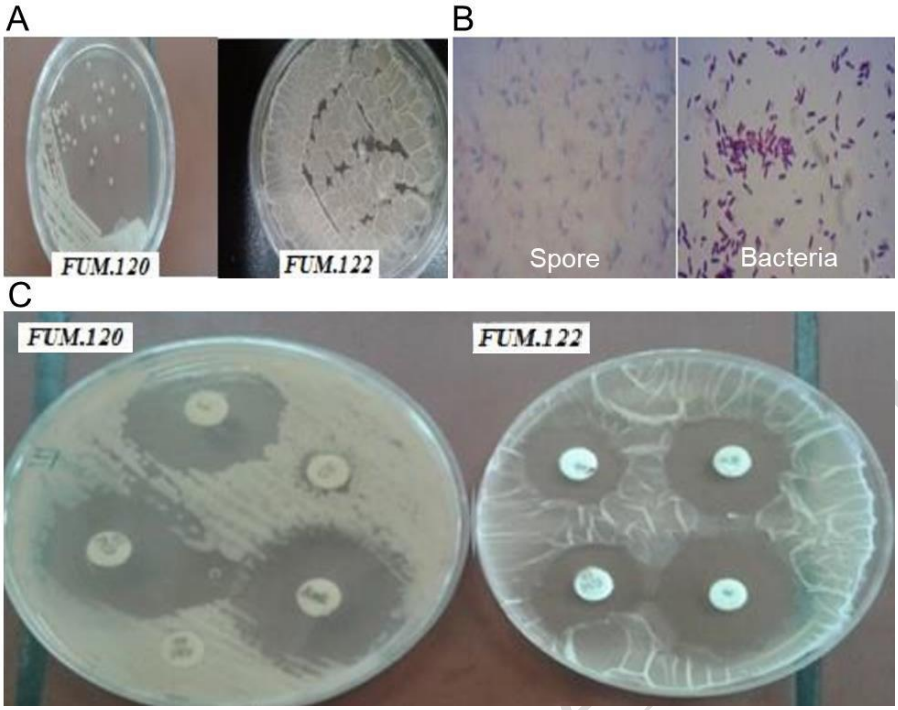

Figure 1. The bacterial identification: (A) Colony morphology in the nutrient agar, (B) Bacterial morphology under microscope, (C) Results of antibiotic sensitivity tests on FUM120 and FUM122 isolates

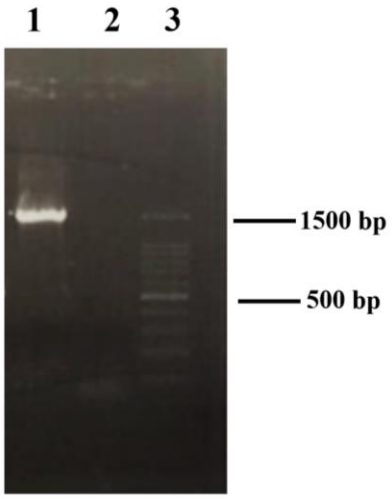

Figure 2. The nucleotide sequence of keratinase-producing isolates was amplified to a length of 1500 bp (1), Negative control (2), Ladder (3).

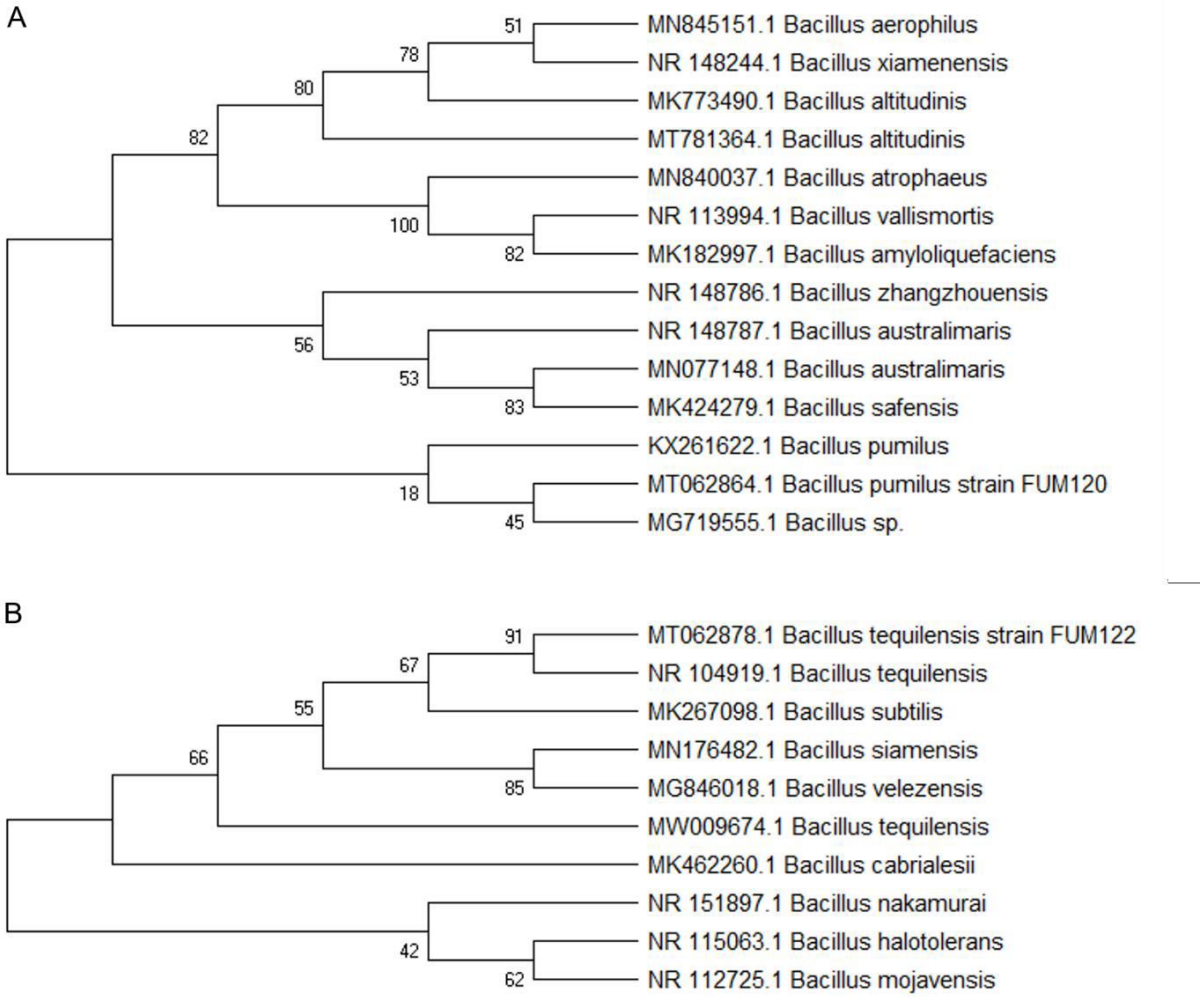

Figure 3. Neighbor-joining phylogenetic relationships of *Bacillus pumilus* FUM120 (A) and *Bacillus tequilensis* FUM122 (B) By bootstrap value for 500 replicates based on 16S rRNA gene sequencing to GenBank

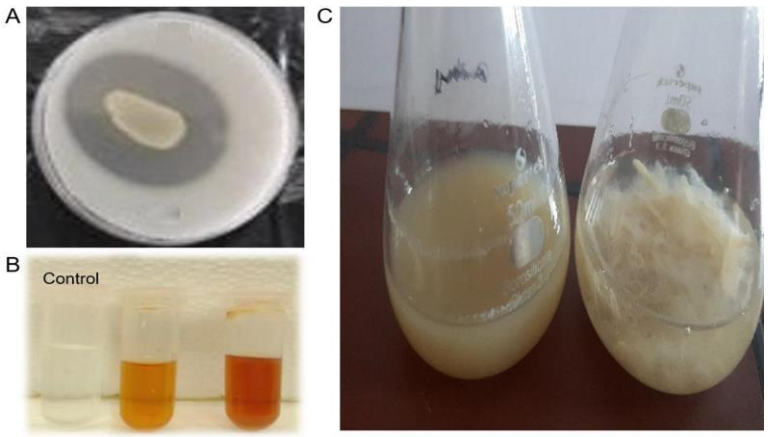

Figure 4. Investigation of enzyme activity with three methods A) The formation of the clear zone in skim milk medium, B) Azokeratin reagent, C) Hydrolysis of the feather in FMB