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Isolation, screening, and molecular characterization using 16S rDNA gene of feather-degrading bacteria isolated from poultry soil in Basrah Province, Iraq

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Abstract. *Al-Amery NMA, Alyousif NA. 2024. Isolation, screening, and molecular characterization using 16S rDNA gene of feather-degrading bacteria isolated from poultry soil in Basrah Province, Iraq. Biodiversitas 25: 3217-3226.* Bioremediation of feathers based on the production of keratinase enzyme is one of the most promising methods and has gained increasing interest in biotechnology. This study aimed to isolate, identify, and screen the bacteria that can potentially degrade feathers from the soil in Basrah province, Iraq using 16S rDNA gene. Ten soil samples were collected from various poultry fields and 97 bacterial isolates have been isolated from the samples. Seventy-four protease-producing isolates were found among isolates. The highest number of bacterial isolates that were found to be able to break down feathers were B6, E1, F7, M6, and J9 according to the values of keratinase activity were 42.1 U/mL, 29.1 U/mL, 28.8 U/mL, 28.1 U/mL, and 28.1 U/mL, respectively. The isolate B6 was the most promising isolate, because it was the best isolate with a higher value of 42.1 U/mL. Based on the sequencing of the 16S rDNA gene, these 5 isolates were identified as *Bacillus amyloliquefaciens, B. subtilis, B. licheniformis, B. subtilis*, and *B. pumilus*, respectively. Eleven bacterial isolates have been reported as new strains and recorded in NCBI GenBank. The current study reported several isolates that have the potential to produce keratinase with different capabilities for the first time in the world.

Keywords: Feathers degradation, keratinase, keratin, keratinolytic bacteria, 16S rDNA sequencing

INTRODUCTION

The dramatic rise in the world's population has increased the pressure on food industries to double their production to meet the population's food needs. Poultry fields are an important and diverse component of the food sector, where poultry products, including eggs, chicken, and turkey meat, are an inexpensive and important source of protein in most people's diets (Srivastava et al. 2019). Feather waste is a by-product of growing commercial chicken processing and is being thrown in massive quantities. Fibrous protein known as keratin, is mostly found in more than 90% of feathers, hair, scales, hooves, noses, horns, claws, and other structures (Shen et al. 2022).

Poultry farms produce 8-8.5 billion tons of feathers annually, and these quantities of feathers produced cause environmental pollution and waste a large source of protein (Sah et al. 2015). In addition, the poultry industry produces many by-products, representing an enormous amount of feather waste that must be managed properly to avoid environmental damage and loss of raw materials important to resources and the feed industry (Ungureanu et al. 2022). Feathers are un-degradable by common protein enzymes and insoluble in diluted acids, alkaline reagents, water, and organic solvents due to the high degree of interconnectedness in the structure of keratin by disulfide bonds, hydrogen bonds, and hydroponic interactions (Tork et al. 2010).

Keratin is the third uncontrollable core protein after cellulose and chitin. It is present in large quantities in nature and insoluble in any solvent, whether it is water or any other organic solvent, thus accumulating in the environment and remaining for decades (Qian et al. 2018).

The low economic importance of feathers and their resistance to biodegradation in the environment, cause disposed of randomly as most poultry farm owners adopt traditional methods of disposing of poultry feathers, such as burning and burial (Bagewadi et al. 2018). When feathers are buried, they will remain in the environment for a long time without degradation due to their strong structural composition (Tamreihao et al. 2019). Burning to eliminate feathers will significantly contribute to greenhouse gas emissions and cause ash disposal problems (Cheong et al. 2018; Emran et al. 2020).

Biotechnology methods for recycling and converting feather waste into highly nutritious soluble substances have evolved using several microorganisms capable of producing keratinase enzymes, which is an effective enzyme capable of analyzing insoluble keratin, where this method is environmentally friendly and cost-effective (Tork et al. 2010). Keratinase enzyme is a class of protease enzymes with keratinizing properties, categorized into the serine or metalloprotease families. Keratinase enzymes from various sources exhibit distinct features, including ideal pH, temperature, and resistance to metal ions and chemical agents (Moridshahi et al. 2020).

Many keratinase-producing bacteria can be isolated from soils contaminated with poultry feathers, such as *Bacillus licheniformis* (Sabri and Aldeen 2014), *Sphingomonas*

paucimobilis, Brevibacillus brevis, B. cereus, and *Aeromonas hydrophila* (Almahasheer et al. 2022), *Rhodococcus erythropolis, Geobacillus, Pseudomonas* sp., and *B. pumili* (Akhter et al. 2020; Alahyaribeik et al. 2020).) Currently, the 16S rDNA gene amplification by polymerase chain reaction and sequencing analysis are successfully used to identify the bacteria isolated from different sources. It is considered the best tool for identifying bacterial isolates due to its sensitivity, dependability, and fastness (Foysal and Lisa 2018; Mohammad and Alyousif 2022). This current study aimed to isolate and screen a feather-degrading bacteria and determine the prevalence of feather-degrading bacteria from various contaminated soils in Basrah province in Iraq. Identification of feather-degrading bacteria by 16S rDNA sequencing technique and determining their potential to degrade the feathers.

MATERIALS AND METHODS

Samples collection

Soil samples containing poultry feathers were collected from several different areas in Basrah province, Iraq (Table 1 and Figure 1), where 10 soil samples were collected from poultry fields, following the method Gupta and Tuohy (2012). The soil samples were collected by removing the top soil 5-15 cm deep and then taking 100 g of soil by a sterile laboratory spatula and placing in sterile clean plastic containers and transporting to the laboratory in the Department of Ecology, College of Science, University of Basrah and kept in the refrigerator at 4°C even conducting laboratory analysis on it.

Table 1. The sites of collected sample

Number of samples	Site of samples	Latitude (N)	Longitude (E)	Samples code
2	Al-Dair	30˚32'46.32"	47˚44'57.48"	A
		30˚48'27.936"	47˚34'16.068"	B
4	Al-Kabasi Al-Kabir	30˚33'8.352"	47˚49'25.824"	C
		30˚33'51.084"	47˚49'7.356"	D
		30˚34'46.956"	47˚48'23.868"	E
		30˚35'11.94"	47˚47'18.528"	M
2	Al-Kabasi Al-Sagher	30˚36'12.636"	47˚47'48.516"	G
		30˚35'56.904"	47˚48'20.34"	H
1	Qarmat Ali	30˚34'44.184"	47˚45'51.516"	J
1	Al-Jazeera	30˚35'24.18"	47˚48'11.628"	F

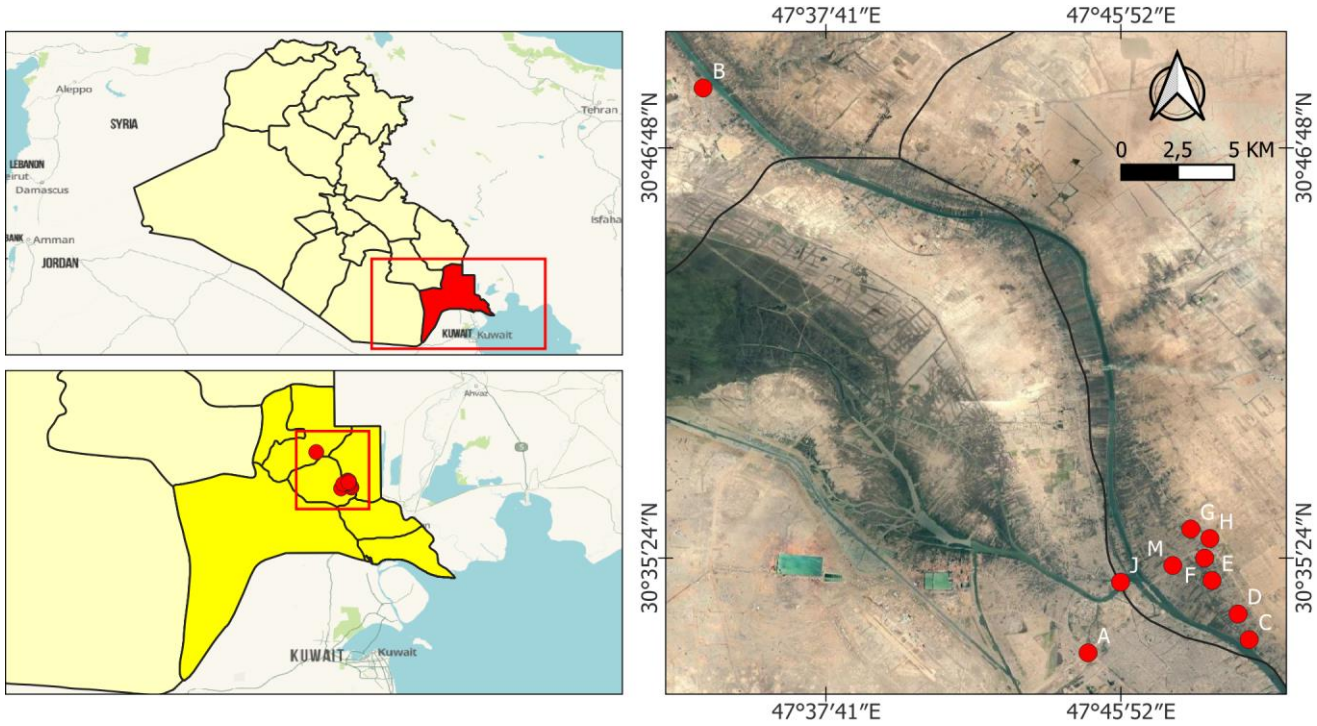

Figure 1. Map of study sites in Al-Basrah Province, Iraq

Procedures

Preparation of native chicken feathers

The chicken feathers were washed several times with tap water. They were then defatted by soaking in a mixture of chloroform: methanol (1:1 v/v) for 2 days, followed by chloroform: acetone: methanol (4:1:3 v/v/v) for 2 days. The solvent was replaced every day. Finally, the feathers were washed several times with tap water to eliminate the solvent residual and dried at 50°C for 3 days (Tork et al. 2010).

Isolation of keratinase-producing bacteria

About 1 g of feathers-contaminated soil was added into a tube containing 9 mL of distilled water and vortexed. A series of dilutions was performed from 10^{-1} to 10^{-6}; 0.1 mL of each dilution was taken with a micropipette and spread over a petri dish containing a skim milk agar medium using L- shape spreader, then dried and incubated at 35°C for 24 h. The bacterial colonies that exhibited the clear zone formation on the agar medium around colonies were recorded as positive (Barman et al. 2017).

Identification of bacteria by 16S rDNA

The isolated bacteria were identified according to sequence and analysis of the 16S rDNA gene. The PrestoTM Mini g DNA bacteria kit (Geneaid, Taiwan) was used to extract the bacterial DNA. The 16S rDNA gene was amplified using universal primers 27F (5-AGAGTTTGATCCTGGCTCAG-3) and 1492R (5-GGTTACCTTGTTACGACTT-3) (Miyoshi et al. 2005). The PCR mixture was formed in a volume of 25 µL containing 12.5 µL of master mix (Promega, USA), 1 µL of template DNA, 1 µL each of forward and reverse primers, and 9.5 µL of nuclease-free water. The PCR program for amplifying the 16S rDNA gene was an initial denaturation of 96°C for 3 min, followed by 27 cycles involving 96°C for 30s, primer annealing at 56ºC for 25s, extension temperature at 72°C for 15s, and final extension at 72°C for 10 min.

Sequencing the products of amplified 16S rDNA gene

The products of the amplified 16S rDNA gene were sent to Macrogen company (South Korea) for accomplishing the purification and sequencing of PCR results. The obtained 16S rDNA gene sequences were proofread using chromas and aligned with nucleotide sequences databases of NCBI utilizing BLAST tools http://www.ncbi.nlm.nih.gov to identify and assess sequence homology of bacterial isolates.

Screening of keratinase-producing bacteria

The positive isolates were purified on petri dishes containing a nutrient agar medium and then activated in 5 mL of the nutrient broth and incubated for 24 h, 1 mL of which was added to 20 mL of modified mineral salt media (MSM) made of NaCl (0.5 g/L), KH_2PO_4 (0.7g/L), K_2HPO_4 (1.4g/L), $MgSO_4.7H_2O$ (0.1g/L), feather (2.5g/L) and incubated for 72 h (Barman et al. 2017).

Keratinase assay

The keratinase activity was estimated using 20 mg feather, 4 mL of pH 10 buffer (50 mM Glycine-NaOH buffer), and 1 mL of properly diluted keratinase enzyme. The reaction mixture was incubated in a water bath at 60°C for 60 min, followed by the reaction was discontinued by adding 4 mL of (% 5 TCA). The control sample was prepared without adding the properly diluted keratinase enzyme, and the reaction was stopped by adding 1 mL of (% 5 TCA). The mixture was then incubated at room temperature and centrifugation at 4,000 rpm for 20 min; the absorbance of the supernatant was then measured in the UV-VIS spectrophotometer at 280 nm, where 0.01 of the absorption value is equivalent to 1 U (U enzyme unit) (Tiwary and Gupta 2012).

Data analysis

The keratinase assays were done in duplicate the average of duplicate determinations was used to represent the result of keratinase assay for all bacterial isolates.

RESULTS AND DISCUSSION

Isolation of keratinase-producing bacteria

Ninety-seven bacterial isolates have been isolated to find the best bacterial isolates that produce keratinase enzymes. The occurrence and distribution of bacteria in each sample were reported as 6 bacterial isolates from sample A, 9 isolates from sample B, 2 isolates from sample C, 9 isolates from sample D, 3 isolates from sample E, 7 isolates from sample F, 9 isolates from sample G, 31 isolates from sample H, 6 isolates from sample M, and 15 isolates from sample J (Table 2). Furthermore, 74 protease-producing bacteria were found among bacterial isolates depending on their formation of transparent zones around their colonies on the skim milk agar media as a preliminary screening of feather-degrading bacteria (Figure 2, Table 2).

Screening of keratinase-producing bacteria

The 74 isolates that exhibited positive results in the preliminary screening were screened to investigate their ability to produce the keratinase enzyme using MSM, in which feathers are utilized as the sole carbon and nitrogen source (Figure 3). These isolates showed varying degrees of keratinase enzyme activity when the enzyme activity was tested using 50 mM Glycine-NaOH buffer ranging between 42.1 U/mL for isolate B6 and 3.9 U/mL for isolate H31. The results showed that 5 promising bacterial isolates including B6, E1, F7, J9, and M6 have reported the highest values of keratinase activity as 42.1 U/mL, 29.1 U/mL, 28.8 U/mL, 28.1 U/mL and 28.1 U/mL respectively among the 74 bacterial isolates that gave the positive results for keratinase enzyme activity. The B6 bacterial isolate was considered the best promising keratinase-producing bacterial isolate according the value of keratinase activity which was 42.1 U/mL, whereas H31 bacterial isolate exhibited the lowest value of keratinase activity at 3.9 U/mL (Table 2).

Figure 2. The transparent zones around bacterial colonies on the skim milk agar media

A **B**

Figure 3. Screening of bacterial isolates for the production of keratinase enzyme: A. Flask containing MSM without bacterial isolate as control; B. Flask containing MSM with bacterial isolate

Table 2. Screening of bacterial isolates for the production of keratinase enzyme

Samples	Isolates code	Isolation and primary screening by skim milk agar	Keratinase assay (U/mL) *
A	A1	+	27.2
	A2	+	22.7
	A3	-	-
	A4	+	19.7
	A5	+	12
	A6	+	23.1
B	B1	+	19.5
	B2	+	16.1
	B3	-	-
	B4	+	14.5
	B5	-	-
	B6	+	42.1
	B7	+	18.3
	B8	+	12.3
	B9	+	19.6
C	C1	+	4.2
	C2	-	-
D	D1	+	11.1
	D2	+	14.1
	D3	-	-
	D4	-	-
	D5	-	-
	D6	-	-
	D7	+	7.1
	D8	-	-
	D9	-	-
E	E1	+	29.1
	E2	+	14
	E3	+	17.7
F	F1	+	16.8
	F2	+	25.4
	F3	+	17.8
	F4	+	21.4
	F5	+	17.2
	F6	+	23.2
	F7	+	28.8
G	G1	+	24.1
	G2	+	14.7
	G3	+	10
	G4	+	15.5
	G5	-	-
	G6	+	14
	G7	+	24.7
	G8	-	-
	G9	-	-
H	H1	+	19.5
	H2	+	13.7
	H3	+	19
	H4	+	12.4
	H5	+	13.8
	H6	+	15.8
	H7	+	19.5
	H8	+	21.1
	H9	+	13.5
	H10	-	-
	H11	+	14.9
	H12	-	-
	H13	+	15.5
	H14	+	24.1
	H15	+	20.9
	H16	+	25.5
	H17	+	19
	H18	+	16
	H19	+	6.6
	H20	+	17.5
	H21	-	-
	H22	-	-
	H23	-	-
	H24	-	-
	H25	-	-
	H26	-	-
	H27	-	-
	H28	+	16.3
	H29	+	15.3
	H30	-	-
	H31	+	3.9
M	M1	+	22.1
	M2	+	22.6
	M3	+	19.5
	M4	+	20.2
	M5	+	27.4
	M6	+	28.1
J	J1	+	25.4
	J2	+	27.8
	J3	+	6.2
	J4	+	21.1
	J5	+	10.5
	J6	+	16.3
	J7	+	13.6
	J8	+	25.7
	J9	+	28.1
	J10	+	27.2
	J11	+	26.6
	J12	+	24.4
	J13	+	22.3
	J14	+	18.9
	J15	+	23.3

Note: *Mean n=2

Identification of bacteria by 16S rDNA

The 16S rDNA gene was amplified for all bacterial isolates using the PCR technique, and the results were seen by electrophoresis under a UV transilluminator (Figure 4). The bacterial isolates were identified by 16S rDNA gene sequencing and analysis.

The molecular characterization and sequencing of the 16S rDNA gene were analyzed using the NCBI BLAST tool to characterize the feather-degrading bacteria by comparing the obtained sequences with sequences of the NCBI database. The habitats' most frequent and distributed species were *Bacillus subtilis*, *Paenibacillus lautus*, and *Rummeliibacillus stabekisii*, respectively. Most feather-degrading bacteria in the current study belong to bacilli, Gram-positive, and spore-forming bacteria.

The results exhibited that bacterial isolates at the level of the genus, indicated that 43 isolates belong to *Bacillus*, 14 isolates belong to *Paenibacillus*, 10 isolates belong to *Rummeliibacillus*, 6 isolates belong to *Enterobacter*, 4 isolates belong to *Glutamicibacter*, 4 isolates belong to *Brevibacillus*, 3 isolates belong to *Escherichia*, 2 isolates belong to *Metabacillus*, 2 isolates belong to *Kurthia*, 1 isolate belong to *Lysinibacillus*, *Exiguobacterium*, *Klebsiella*, *Shigella*, *Rhodococcus*, *Pseudomonas*, *Staphylococcus*, *Cronobacter*, *Enterococcus* and *Planococcus* (Table 3 and Figure 5).

The keratinase activity of feather-degrading bacteria was reported to belong to the genus *Bacillus* at 39 isolates, followed by 10 isolates belonging to *Rummeliibacillus* in which the values of keratinase to varying degrees, and 6 isolates to *Paenibacillus* have low values of keratinase activity, 4 isolates belong to *Glutamicibacter*, 3 isolates belong to *Brevibacillus*, 2 isolates belong to *Metabacillus*, and 1 isolate belong to genera *Exiguobacterium*, *Klebsiella*, *Shigella*, *Rhodococcus*, *Pseudomonas*, *Cronobacterus*, and *Enterococcus* with varying values of keratinase activity. While isolates belong to bacterial genera *Escherichia*, *Staphylococcus*, *Lysinibacillus*, and *Planococcus* did not exhibit the potential to produce keratinase enzyme.

Recording of new bacterial strains

The bacterial isolates of the present study were 100% similar according to the 16S rDNA sequence, except 11 bacterial isolates, namely A3, D2, F7, H1, H2, H11, H21, H22, H30, M1, and J12 were identified as a new bacterial strain. Their sequences were deposited at NCBI with accession numbers, as shown in Table 4.

Discussion

Keratinase enzymes are produced from insects, fungi, bacteria, and some Actinomycetes. These organisms are found in areas or environments where keratinous substances are collected (Sypka et al. 2021). All keratinase-producing organisms use keratinous substances, such as hair, wool, bone, and feathers as carbon sources and the basis for keratinase production. However, some non-keratin proteins, such as skimmed milk, casein, gelatin, soybean meal, or soy flour, can stimulate keratinase production in these organisms (Tantamacharik et al. 2022). Microbial keratinase production is influenced by pH, temperature, carbon and nitrogen sources, medium ventilation, and medium components. The production of keratinase can be increased by finding the most suitable factors for the microorganisms (Devi et al. 2018). Bohacz and Korniłłowicz-Kowalska (2019) reported that microbial keratinase production occurs if no other nutrient source is available to the microorganisms in the environment. Keratinase production offers the potential to use many proteins as an energy source for microorganisms, thus promoting the recycling of organic materials in various ecosystems. Mukhtar et al. (2019) indicated that three species of *Bacillus* are isolated from the soil of poultry farms and feather processing areas near Kasur and Lahore, Pakistan, has the potential to produce keratinase to varying degrees. Almahasheer et al. (2022) stated that 42 bacterial isolates belonging to several species from poultry farm waste in the Eastern Province of Saudi Arabia determined 5 species that were more feather-degrading bacteria.

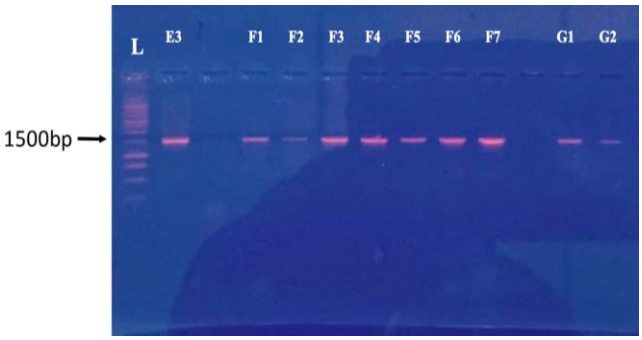

Figure 4. Gel electrophoresis of amplified 16S rDNA gene (1500 bp) by PCR for bacterial isolates. Lane L: 100 bp DNA, Lane E3-G2: PCR products of 16S rDNA gene of some bacterial isolates

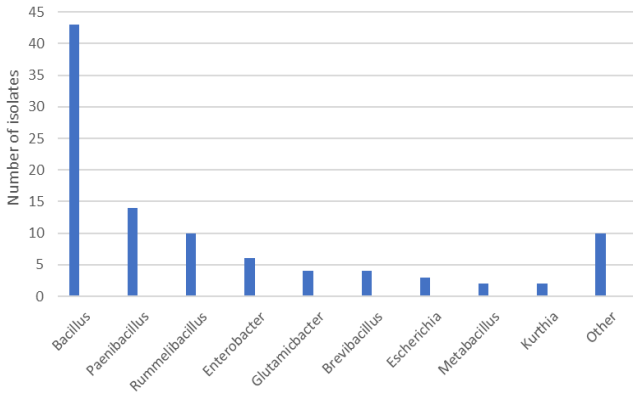

Figure 5. The number of total bacterial isolates at the level of genus (n=97)

Table 3. Bacterial identification by 16S rDNA gene sequence isolates code and the identical to the type strains of NCBI

Isolates code	Closest species	Accession number	Identity (%)
A1	*Bacillus subtilis* strain MK736112.1	MT111083.1	100
A2	*Bacillus cereus* strain BBS15	MK956956.1	100
A3	*Bacillus cereus* strain yasmun8	OK632087.1	99.79
A4	*Bacillus cereus* strain NIBSM_OsG2	KY930333.1	100
A5	*Bacillus cereus* strain B105	KP966475.1	100
A6	*Bacillus albus* strain ROA098	MZ026474.1	100
B1	*Bacillus cereus* strain D85	MT256066.1	100
B2	*Rhodococcus pyridinivorans* strain ZZ47	KU234681.1	100
B3	*Bacillus safensis* strain 36-1	PP325787.1	100
B4	*Shigella flexneri* strain FDAARGOS_689	CP054913.1	100
B5	*Escherichia coli* strain 148-c pink	MN208184.1	100
B6	*Bacillus amyloliquefaciens* strain 19E2	FJ705346.1	100
B7	*Bacillus licheniformis* strain MS-1	PP789724.1	100
B8	*Bacillus subtilis* strain 197WMS3	MK713732.1	100
B9	*Bacillus licheniformis* strain QT338	MT043735.1	100
C1	*Klebsiella pneumoniae* strain A187	ON329113.1	100
C2	*Glutamicibacter creatinolyticus* strain PS6	KY814694.1	100
D1	*Kurthia gibsonii* strain KH2	MN453416.1	100
D2	*Kurthia gibsonii* strain kgi-B1	ON385944.1	99.86
D3	*Enterobacter cloacae* strain S25	MN062623.1	100
D4	*Escherichia coli strain* TR_AVFP-2	CP110096.1	100
D5	*Escherichia coli* strain SX20220601	PP494230.1	100
D6	*Bacillus cereus* strain BF2	KU955350.1	100
D7	*Enterobacter ludwigii* strain AA1	MT613360.1	100
D8	*Enterobacter* cloacae strain CICC10011	MK780068.1	100
D9	*Enterobacter cloacae* strain IIPRAJCP-2	MT436392.1	100
E1	*Bacillus subtilis* subsp. stercoris strain EGI18	MN704394.1	100
E2	*Rummeliibacillus stabekisii* strain 811	MT658588.1	100
E3	*Rummeliibacillus stabekisii* strain PP9	CP014806.1	100
F1	*Rummeliibacillus stabekisii* strain PP9	CP014806.1	100
F2	*Bacillus siamensis* strain TSS18	MF620076.1	100
F3	*Rummeliibacillus stabekisii* strain PP9	CP014806.1	100
F4	*Bacillus subtilis* strain OTG013	MN216298.1	100
F5	*Bacillus pumilus* strain ES-21	KX426046.1	100
F6	*Bacillus licheniformis* strain CJ-G-NA7	HM584297.1	100
F7	*Bacillus licheniformis* strain PP1	OP531847.1	99.93
G1	*Pseudomonas aeruginosa* strain CUVET23-830	CP130957.1	100
G2	*Brevibacillus borstelensis* strain Gp-1	MT292327.1	100
G3	*Glutamicibacter creatinolyticus* strain XM13	MT023390.1	100
G4	*Rummeliibacillus stabekisii* strain PP9	CP014806.1	100
G5	*Staphylococcus carnosus* strain HSP-S16	MG669651.1	100
G6	*Cronobacter sakazakii* strain BW1904	MT476368.1	100
G7	*Enterococcus faecalis* strain KR 101	JQ388687.1	100
G8	*Planococcus rifietoensis* strain NF4	MT263532.1	100
G9	*Enterobacter ludwigii* strain JCR-38	KU714599.1	100
H1	*Paenibacillus lautus strain BG81*	OR195936.1	99.36
H2	*Exiguobacterium* mexicanum strain A-EM	CP040676.1	99.93
H3	*Paenibacillus lautus* strain B174/17	OL413662.1	100
H4	*Bacillus subtilis* strain S38	OQ504786.1	100
H5	*Rummeliibacillus stabekisii* strain PP9	CP014806.1	100
H6	*Bacillus cereus* strain 165PP	KM349191.1	100
H7	*Glutamicibacter creatinolyticus* strain PS6	KY814694.1	100
H8	*Paenibacillus lautus* strain B682/17	OL604447.1	100
H9	*Rummeliibacillus stabekisii* strain PP9	CP014806.1	100
H10	*Lysinibacillus macroides* strain LJB15	KX027335.1	100
H11	*Paenibacillus lautus* strain M1HC19	MK256307.1	99.45
H12	*Paenibacillus lautus strain XAS3-14*	JF496308.1	100
H13	*Rummeliibacillus stabekisii* strain PP9	CP014806.1	100
H14	*Bacillus siamensis* strain TSS18	MF620076.1	100
H15	*Rummeliibacillus stabekisii* strain VITNJ7	KM047492.1	100
H16	*Paenibacillus lautus* strain XAS3-14	CP017659.1	100
H17	*Bacillus subtilis* strain MML5328	MF688046.1	100
H18	*Rummeliibacillus stabekisii* strain PP9	CP014806.1	100

H19	*Glutamicibacter creatinolyticus* strain XM13	MT023390.1	100
H20	*Paenibacillus lautus* strain B682/17	OL604447.1	100
H21	*Paenibacillus lautus* strain N3-6	JX094159.1	99.59
H22	*Paenibacillus lautus* strain N3-6	JX094159.1	99.58
H23	*Paenibacillus lautus* J49TS8	LC588626.1	100
H24	*Brevibacillus borstelensis* strain UTM105	KF952566.1	100
H25	*Paenibacillus lautus* strain B682/17	OL604447.1	100
H26	*Bacillus subtilis* strain T1Z35	OQ472445.1	100
H27	*Paenibacillus lautus* strain DS19	EU834247.1	100
H28	*Bacillus subtilis subsp. subtilis* strain FJAT-1748	PP236932.1	100
H29	*Bacillus subtilis* strain AU04	MF590152.1	100
H30	*Paenibacillus lautus* J49TS8	LC588626.1	99.86
H31	*Paenibacillus lautus* strain C-M4-1	MT790713.1	100
M1	*Metabacillus endolithicus* strain IMCC1017	PP478157.1	99.93
M2	*Bacillus subtilis* strain AU04	MF590152.1	100
M3	*Bacillus wiedmannii* strain B16-2	MT256061.1	100
M4	*Bacillus subtilis* strain S38	OQ504786.1	100
M5	*Metabacillus halosaccharovorans* strain NS982R	MH050345.1	100
M6	*Bacillus subtilis* strain MML5328	MF688046.1	100
J1	*Bacillus licheniformis strain I85*	KU922420.1	100
J2	*Bacillus sonorensis strain 1779*	MT597627.1	100
J3	*Bacillus licheniformis* strain MR-1	MG597490.1	100
J4	*Bacillus licheniformis* strain ATCC 14580	ON597434.1	100
J5	*Brevibacillus borstelensis* strain UTM105	KF952566.1	100
J6	*Bacillus sonorensis* strain NECC10260	OL690590.1	100
J7	*Brevibacillus borstelensis* strain UTM105	KF952566.1	100
J8	*Bacillus licheniformis* strain E2-10-2-2	MK063874.1	100
J9	*Bacillus pumilus* strain LX11	KP192031.1	100
J10	*Bacillus subtilis* subsp. spizizenii strain JS-4	MN443608.1	100
J11	*Bacillus vallismortis* strain LZH-L2	MZ267282.1	100
J12	*Bacillus cereus* strain HBU30207	MW805748.1	99.92
J13	*Bacillus amyloliquefaciens* strain SRG15	MK743994.1	100
J14	*Bacillus paralicheniformis* strain KLK27	MT634493.1	100
J15	*Bacillus siamensis* strain TSS18	MF620076.1	100

Table 4. The bacterial isolates were recorded as new bacterial strains

Samples	Isolates code	New bacterial strains	Sequence identity (%)	Accession number
A	A3	*Bacillus cereus* strain NOORBSRA3	99.79	PP911483
D	D2	*Kurthia gibsonii* strain NOORBSRD2	99.86	PP911484
F	F7	*Bacillus licheniformis* strain NOORBSRF7	99.93	PP911485
H	H1	*Paenibacillus lautus* strain NOORBSRH1	99.36	PP911486
	H2	*Exiguobacterium mexicanum* strain NOORBSRH2	99.93	PP911487
	H11	*Paenibacillus lautus* strain NOORBSRH11	99.45	PP911488
	H21	*Paenibacillus lautus* strain NOORBSRH21	99.59	PP911489
	H22	*Paenibacillus lautus* strain NOORBSRH22	99.58	PP911490
	H30	*Paenibacillus lautus* strain NOORBSRH30	99.86	PP911491
M	M1	*Metabacillus endolithicus* strain NOORBSRM1	99.93	PP911492
J	J12	*Bacillus cereus* strain NOORBSRJ12	99.92	PP911493

In the current study, the molecular characterization of bacterial isolates based on the 16s rDNA gene sequencing showed that the most feather-degrading bacteria belonged to the genera *Bacillus, Rummeliibacillus, Paenibacillus, Glutamicibacter, Brevibacillus, Klebsiella, Metabacillus, Exiguobacterium, Shigella, Rhodococcus, Pseudomonas,* and *Cronobacterus*. Species belonging to the genus *Bacillus* are prevalent in sampling sites where they appear in 8 out of 10 sites because they can withstand harsh environments such as dry and hot environments and areas where solar radiation is severe. After all, these species belonging to *Bacillus* can form a thick external spore and are endospore resistant (Alyousif 2022), adding to their ability to produce keratinase enzyme, which gives it the ability to be present in areas contaminated with keratinous substances. Four species of *Bacillus* were obtained in the current study, including *B. subtilis, B. cereus, B. siamensis,* and *B. amyloliquefaciens* have the potential to produce

keratinase enzymes with good quantity according to keratinase activity test, and this agrees with previous studies reported by Bose et al. (2014), Ahmadpour et al. (2016), de Paiva et al. (2019), Tanruean et al. (2019), Alahyaribeik et al. (2020), and Alshehri et al. (2021). In the present study, 4 bacterial species including *Bacillus licheniformis*, *B. pumilus*, *B. paralicheniformis*, and *B. sonorensis* reported their ability to produce keratinase enzyme. These species has reported their ability to produce keratinase enzyme in earlier studies, such as Mehta et al. (2014), Alahyaribeik et al. (2020), Dada and Wakil (2021), and Sharma et al. (2022). *Bacillus wiedmannii* has not been previously isolated from soils containing feathers or poultry soil. However, Petek et al. (2024) isolated from wool and reported the potential to produce keratinase enzyme and wool analysis. Also, *Bacillus vallismortis* has not been isolated from the soil of poultry farms. Yue et al. (2013) isolated the bacterium from animal feces from the Beijing Zoo in China and indicated its ability to produce keratinase enzyme to degrade chicken feathers in the laboratory. The bacterial species associated with *Bacillus* are an important group of microorganisms in many soils. Many of these species of bacteria are important in practical applications, because they produce enzymes and other industrially useful products and play an important role in nutrient recycling, nitrogen stabilization, and soil enrichment with minerals (Yahya et al. 2021).

Rummeliibacillus stabekisii was not reported for their production of keratinase enzyme in previous studies. The species was isolated for the first time from the Payload Hazardous Servicing Facility at the Kennedy Space Center, FL, USA, in 2009 (Vaishampayan et al. 2009). In the current study, 40 bacterial isolates of *Bacillus*, 10 bacterial isolates of *Rummeliibacillus*, 9 bacterial isolates of *Paenibacillus*, 5 bacterial isolates of *Enterobacter*, 4 bacterial isolates of *Glutamicibacter*, and 3 bacterial isolates of *Brevibacillus* species from poultry soil reported their ability to degrade the feathers, some of them are reported for the first time globally (Table 3). This study was supported by Zilda (2021), who indicated that the bacterial isolates from some hot springs belonging to the *Brevibacillus borstelensis* species can produce thermostable proteolytic active enzymes. One isolate of *Pseudomonas aeruginosa* bacterium was obtained from the present study and proved effective in degrading feathers as reported by Tork et al. (2010).

The current study reported nine isolates that have the potential to produce keratinase enzymes with different capabilities for the first time in the world belonging 9 species, including *Glutamicibacter creatinolyticus, Klebsiella pneumonia, Exiguobacterium mexicanum, Shigella flexneri, Metabacillus endolithicus, Metabacillus halosaccharovorans, Rhodococcus pyridinivorans*, *Cronobacter sakazakii*, and *Enterococcus faecalis*. *Glutamicibacter* creatinolyticus was isolated for the first time from diffuse subcutaneous nodules adherent to muscular tissues from a mare in Italy in 2016 (Busse 2016). Gurav et al. (2016) indicated that species belonging to the genus *Klebsiella* could degrade chicken feathers, but did not indicate *K. pneumonia* among the isolates. Otherwise, *K. pneumoniae* was recorded ability to produce keratinase enzyme with good values for the first time in the world.

In the present study, 11 bacterial isolates were diagnosed as new strains with differences in 16S rDNA gene sequences, and their sequences were deposited at the NCBI. The generation of new strains due to bacteria exposed to changed environments leads to the loss of the ability to repair DNA damage and becomes hereditary (Al Khafaji et al. 2023). Microbial enzymes occupy a prominent position in bioeconomics and proteinase enzymes account for about 60% of all microbial enzymes. The bacterial keratinase belongs to the versatile protease group that is gaining increasing interest in biotechnology. The bacterial keratinase enzymes are widely used in the medical, food, basic biology, animal feed, and detergent industries. They can be applied to biodegradation of keratin waste, such as feathers, hair, and wool (Nnolim et al. 2020).

In conclusion, feather degradation by feather-degrading bacteria is more favorable than conventional and chemical procedures since it is cost-effective, ecologically friendly, and recovers important amino acids. Moreover, 97 bacteria were isolated from poultry soil and 74 bacteria isolates could produce keratinase with different activity values. The highest number of bacterial isolates that were found to be able to break down feathers were B6, E1, F7, M6, and J9 according to the values of keratinase activity were 42.1 U/mL, 29.1 U/mL, 28.8 U/mL, 28.1 U/mL, and 28.1 U/mL, respectively. The isolate B6 was the most promising isolate, because it was the best isolate with a higher value of 42.1 U/mL. Based on the sequencing of the 16S rDNA gene, these 5 isolates were identified as *Bacillus amyloliquefaciens*, *B. subtilis*, *B. licheniformis*, *B. subtilis*, and *B. pumilus*, respectively. In addition, eleven bacterial isolates were recorded as new strains in NCBI GenBank.

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