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Screening and Characterisation of Keratin-Degrading *Bacillus* sp. from Feather Waste

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Authors' contributions

This work was carried out in collaboration between both authors. Authors MTD and SMW designed the study. Author MTD performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors MTD and SMW managed the analyses of the study. Author MTD managed the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Aim: This study focuses on the screening and characterisation of keratin-degrading *Bacillus* species from feather waste.
Methods: Nine bacteria were isolated from feather waste obtained from a poultry layout at Egbeda local government secretariat, Ibadan, Nigeria. These bacteria were grown in basal medium with feather as primary source of carbon, nitrogen, sulfur and energy. Feather degrading bacteria were screened for both proteolytic activity and keratin degradation on skimmed milk agar and keratin azure medium respectively. They were also screened for their ability to degrade other keratin substrates such as hair and nail.
Results: Three of the isolates with higher feather degradation levels also showed high proteolytic activity and release of azure dye. They were selected and identified phenotypically and genotypically using 16S rRNA sequencing as *Bacillus licheniformis*-K51, *Bacillus subtilis*-K50 and *Bacillus* sp.-K53. The bacteria were capable of degrading other keratin-containing substrates such as nail and hair. *Bacillus subtilis*-K50 and *Bacillus licheniformis*-K51 showed significant difference ($P = .05$) in degradation among the three different keratin sources used yielding higher degradation

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with feather as keratin source with respective optical densities of 0.07 and 0.11 followed by hair and least in nails with optical densities of 0.05 and 0.07 respectively. Highest degradation of all the three keratin substrates was observed in *Bacillus licheniformis*-K51.
Conclusion: The three isolated bacteria possess the ability to degrade keratin and utilize feather as keratin substrate. As a result, these can be considered as potential candidates for degradation and utilization of feather keratin.

Keywords: Keratin-degrading bacteria; feather waste; keratin azure-dye; Bacillus species.

1. INTRODUCTION

Keratins are insoluble, structural fibrous proteins that associate as intermediate filament, they form the integral component of the outermost layer of the skin of animals and humans. They are present in feathers, horns, hooves, hair, nails and other exoskeletal materials and epithelium [1]. They function as structural materials that have protective, connective or supportive roles [2].

There are certain microorganisms that break down keratin proteins; they are referred to as keratinolytic microbes, most of which produce keratinases which are mainly extracellular enzymes used for the biodegradation of keratin [3]. These keratinases have a wide range of activity for temperature and pH [4]. They belong to serine metalloprotease group which are able to liberate the free amino acids from keratinous proteins [5]. Microbial keratinases are mainly extracellular in nature, although intracellular [6] and few that are cell-bound have been reported [7,8].

Keratinolytic microbes are widespread in nature and largely isolated from poultry wastes. They are mostly fungi, bacteria of the genera *Bacillus* and *Streptomyces* [9]. Of these keratinases producing microorganisms, keratinase production is common among certain species of *Bacillus*. Many *Bacillus licheniformis* and *Bacillus subtilis* strains are described as keratinolytic [10,11]. Complete disintegration of feathers has been observed among keratinase producing strains of *Bacillus licheniformis* and the enzyme has also shown a wide range of proteolytic activity [12,13].

Feathers rank one of the most composite of all keratin containing structures obtainable in vertebrates [14]. The average young feather consists of about 90% protein (keratin) and is produced in large amount as waste by poultry processes worldwide. This keratin-containing poultry waste is not easily degradable in nature and as a result, considered hazardous to the environment [15]. Keratin contained in feather can be utilized to induce keratin-degrading enzyme for the degradation of keratinous compounds by keratinolytic bacteria, hence, providing ample use for feather waste. This research work therefore focuses on the screening and characterisation of keratin-degrading *Bacillus* species from feather waste.

2. MATERIALS AND METHODS

2.1 Sample Collection

Samples of soil and decaying feather were obtained from poultry farm waste in Egbeda local government secretariat poultry layout, Ibadan, Nigeria.

2.2 Growth Medium Preparation

The growth medium (feather broth) used in the isolation and cultivation of keratin-degrading bacteria was compounded by a modified method [12] and it contained, per liter, the following constituents: NaCl (0.5 g); K_2HPO_4 (0.3 g); KH_2PO_4 (0.4 g), Yeast extract (5 g) and feather (10 g), pH 7.2. White local chicken feathers were thoroughly washed with clean water, dried in the oven at 40°C for 24 h and milled to powder.

2.3 Isolation of Bacteria

Serial dilutions (up to 10^{-9}) of the samples were prepared by adding 1g of decayed feather and soil to 9 mL of sterile saline solution. Appropriate dilutions were then plated on sterile Nutrient Agar medium followed by incubation at 37^{o}C for 24 h. The colonies that appeared were then sub-cultured onto sterile Nutrient Agar plates. This was repeated until pure cultures were obtained.

2.4 Screening for Proteolytic Bacteria

Test for proteolytic activity of the bacterial isolates was carried out on skimmed milk agar plates using a modified method [16]. Pure

bacteria cultures were inoculated on the sterile milk agar plates and incubated for 24 h at 37°C. Isolates that produced clear zones of hydrolysis in this medium were selected and screened for keratin degrading ability.

2.5 Screening for Feather-Degrading Bacteria

Feather-degrading ability of the proteolytic isolates was carried out in feather broth medium as compounded by a modified method [12]. The proteolytic isolates were inoculated into 20 mL of feather broth medium in Erlenmeyer flasks and incubated on an orbital shaker for 7 days at 150 rpm and 37^{o}C. The dissolution of feathers in the flasks were observed visually and measured by taking the optical density of the medium.

2.6 Screening for Keratin-Degrading Bacteria with Keratin Azure

Test tubes containing 10 mL of growth medium (without feathers) with 1% (w/v) finely chopped keratin azure (Sigma-Aldrich corporation, St Louis Missouri, United States) were inoculated with isolates and incubated at 37^{o}C on a rotary shaker at 150 rpm for 7 days. The release and diffusion of azure dye into the uncolored layer of the basal medium inferred degradation of keratin.

2.7 Screening for Keratin-Degrading Bacteria Using Different Keratin Substrates

Selected isolates from above were inoculated into 20 mL of growth medium (containing 1% feather) in 100 mL Erlenmeyer flasks and incubated at 37°C on a rotary shaker at 150 rpm for 7 days. Undigested feathers in the flasks were removed by filtration through Whatman No 1 filter paper and the optical density of the filtrate was determined at 280 nm against an uninoculated control. This was repeated for each isolate replacing feather in the feather growth medium with 1% nail and 1% hair as keratin substrates.

2.8 Characterisation of Isolates

The isolates were characterised using phenotypic and genotypic techniques. The cultural characteristics of the isolates were observed with respect to their appearance, colour, size, elevation, pigmentation, opacity, consistency and shapes on plates and rate of growth. Microscopic tests carried out to demonstrate the cellular characteristics include endospore staining and Gram's reaction test.

2.8.1 Gram's staining technique

The pure culture of the samples was separately stained [17]. A thin smear of each isolate from 18-24 h old culture was made on a glass slide and heat-fixed by passing over a flame. The smears were flooded with two drops of crystal violet for 30 seconds, rinsed with water and Gram's iodine solution was added for 1min. The stain was decolorized by pouring ethanol on the glass slide until the violet colour was no longer observable and then washed with tap water. It was counterstained with two drops of safranin reagent for 10 seconds, rinsed again with ordinary water and dried by blotting using a filter paper. Microscopic observations were carried out using oil immersion objective. Bacteria that were Gram-positive were identified as purple in colour while gram-negative bacteria were identified by their pinkish coloration. The Gram's staining technique was utilized in determining the different shapes and arrangements of the bacteria as well.

2.8.2 Endospore staining technique

A thin smear of each isolate from 18-24 h old culture was made on a glass slide and heat-fixed by flaming. Malachite green solution was poured on the smears and steamed for 5-10 min ensuring that the stain does not dry out, after which it was washed carefully with water. Safranin solution was used to counterstain for 15seconds after which it underwent washing with water, blot dried and viewed under the light microscope using the oil-immersion objective [17]. Spores stained green and vegetative cells stained red.

2.9 Biochemical and Physiological Tests

2.9.1 Catalase test

The method of Fawole and Oso [18] was used. A thick emulsion of each isolate was made on a clean glass slide. Two drops of 3% H_2O_2 was then added and the reaction was observed. Effervescence indicated a catalase positive reaction.

2.9.2 Oxidase test

A few drops of 1% solution of tetramethyl-p-phenylenediamine dihydrochloride is placed onto

a piece of whatman filter paper. The filter paper is then smeared with some bacteria cultures taken with a loop. A purple colour developing within 5 to 10 seconds indicates a positive reaction. Colour development may however take up to 10 to 15 seconds in a weak or delayed positive reaction. This was carried out according to the method of Cowan [19].

2.9.3 Indole test

Each bacterial isolate was inoculated into culture tubes containing tryptone broth and incubated at temperature of 35°C for 48 h, after which two millilitres each of chloroform and Kovac's reagent were added separately to each broth culture in the tubes and shaken gently. The culture tubes were made to stand for 20 min in order to allow the rising of the reagent to the top of the medium. A red colour at the reagent layer is indicative of production of indole by the test sample [18].

2.9.4 Methyl red and voges-proskauer's test

Into culture tubes was dispensed 10 mL of the glucose phosphate as prepared according to the method of Harrigan and McCance [16]. The medium was sterilized and inoculated with the bacteria and incubated for 2-5 days at 37°C. After incubation, five millilitres of the culture was taken into sterile tubes and few drops of methyl red indicator were added and a red colouration was considered positive for methyl red test. To 1 mL of 6% of ∝-naphtol solution and 1 mL of 10% NaOH was added in a sterile tube. Development of red colouration within 5min indicated a positive reaction for Voges-Proskauer's test.

2.9.5 Nitrate reduction test

The nitrate reduction test was carried out by the method of Payne [20]. Five millilitres of broth medium consisting of peptone water and 0.1% KNO_3 was dispensed into culture tubes consisting of an inverted Durham tube each. The tubes were covered with a screw-cap and then sterilized at 121°C for 15 min. The tubes were allowed to cool and then inoculated with bacterial isolates and were incubated at 37°C for 4 days. The addition of 1% suphanilic acid in 5N acetic acid followed by 0.5mL of 0.6% dimethyl naphthylamine ir acid to each tube was done. Red colouration of the medium showed that the test organism was able to reduce nitrate present in the medium and a positive result and the presence of gas in the Durham tube showed that nitrogen gas was produced.

2.9.6 Action of isolates on litmus milk

In 100 mL of dH_2O, 10.5g of dehydrated litmus milk was dissolved. The solution was dispensed in screw cap tubes and autoclaved at 110°C for 10 min. The cooled milk was inoculated with the isolates and incubated for seven days at 37°C. Uninoculated tubes served as control. The tubes were observed daily for coagulation and colour change from purple to pink. This indicates proteolytic activities and acid production respectively [16].

2.9.7 Gelatin hydrolysis

Into screw-cap culture tubes, 9 mL of 10% gelatin broth were dispensed autoclaved to sterilize at a temperature of 121°C for 10 min. The inoculation of the bacteria was done by stabbing onto the medium and incubating at a temperature of 37°C for 5-7 days. The positive gelatin hydrolysis test was indicated if liquefaction occurs when the culture remains fluid after immersion in iced water for 15 min.

2.9.8 Starch hydrolysis

Nutrient agar with the addition of 1% soluble starch was sterilized and poured to set in sterile petri-dish. Plates containing medium were inoculated with 18-24 h old cultures by streaking across the surface. After incubation at 37^oC for 2-3 days, Gram's iodine was poured on the plates and observed for blue-black colouration. The starch that was not hydrolysed starch gave a blue-black colouration on the addition of iodine. Clear zones around the region of growth showed zone of hydrolysis of the starch [21].

2.9.9 Production of ammonia from peptone

Eighteen-hour old culture of isolates was inoculated in tubes containing sterile peptone water and incubated along with a control (containing uninoculated tubes of sterile peptone water) at 37°C for 72 h. One millilitre of culture was added to 1 mL of Nessler's reagent in a clean tube. The development of an orange to brown colour indicated the presence of ammonia. The sterile control tested at the same time turned pale yellow or no colour reaction.

2.9.10 Hydrogen sulphate production

Lead acetate agar was prepared according to the manufacturer's specifications. The medium was dispersed into McCartney bottles and autoclaved

at 110°C for 10 min. It was allowed to cool upright. Agar deeps were then stabbed with 18 h old cultures and incubated at 37°C for 48 h. observation of a black colouration along the line of stab indicated a positive result [16].

2.9.11 Carbohydrate fermentation

The sugars used for fermentation included fructose, maltose, lactose, sucrose, glucose, arabinose, mannose, xylose, raffinose, dextrose, mannitol, sorbitol, galactose and cellubiose. Nutrient broth containing 0.5% of each of the sugars was prepared into which about two drops 0.01% phenol red indicator was added separately. Ten millilitres each of the broth medium was dispensed in glass tubes inserted with inverted Durham tubes. The culture tubes were then sterilized at 110°C for 10 min. After being cooled, each tube containing sugar was inoculated with a loopful of each of the bacterial isolates being tested. The control comprised of a medium that was not inoculated with the test isolate. The tubes were incubated at a temperature of 37^0C for 72 h. A change in colour from red to yellow and the presence of air trapped in the Durham tube shows acid and gas production respectively. This was carried out by a modified method of Fawole and Oso [18].

2.9.12 Growth of Isolates at different NaCl concentrations

Thirty millilitres (30 mL) of nutrient broth containing 4% and 6.5% Sodium Chloride were dispensed separately into screwed-capped tubes and sterilized at 110°C for 10 min. Isolates are inoculated in the nutrient broth containing the salt and incubated at 37°C for 72 h. Increase in turbidity of the broth was recorded as positive for growth. Uninoculated tubes served as control [22].

2.10 Molecular identification of Isolates

2.10.1 DNA Extraction

The DNA extraction method was carried out using modified extraction protocols [23].

2.10.2 Polymerase chain reaction amplification

Amplification of the bacterial genome was done following modified PCR amplification protocols [24] using Universal primers. The PCR was performed in a thin walled PCR tube containing the following:15µL dH_2O, 2.5µL 10×PCR buffer, 5µL DNA solution, 0.5µL dNTP mix, 1.25µL of the forward primer (12.5pmol), 1.25 µL of the reverse primer (12.5pmol), 0.125 µL *Taq*DNA polymerase (5U/µL). The PCR conditions were; denaturing at 94^oC for 4min, followed by cycles 92 ^{o}C, 55^oC, 68^oC two cycles 92^oC, 53^oC, 68^oC; two cycles 92^oC, 51^oC, 68^oC; two cycles 92^oC, 49^oC, 68^oC; and 92^oC, 47^oC, 68^oC. The reaction was followed by a final extension of 68^oC for 10min.

2.10.3 Purification of PCR products

Purification of the PCR amplicon to remove free primers and unincorporated dNTP was done using modified PCR purification protocols [24].The product of PCR reaction was transferred into a 1.5 mL tube and 3 volumes sodium iodide was added and mixed thoroughly. To this reaction mixture, 5 µL glassmilk was added, mixed and the DNA was allowed to bind for 5min. The tube containing the reaction solution was spanned down a V_{max} for 5 sec, removed and the supernatant kept separately. The DNA/glassmilk pellet was washed two times with 500µL of the New Wash solution using a pipette. The solution was again span down at maximum velocity for 5sec and the supernatant decanted. The resultant pellet was dissolved in 20-30 µL H_2O (weak-strong PCR product) for 5min and again span down at 30sec then the supernatant (clean PCR product) transferred into a new tube and frozen until used for sequencing.

2.10.4 Sequencing

Automated sequencing of the purified Polymerase Chain Reaction amplicon was carried out utilizing the ABI Bigdye 3.1 cycler sequencing kit (Applied Biosystems, California, USA) on ABI 3730XL and the nucleotide sequence was determined by automated sequencer by Laragen Inc., Culver City California. The sequence of the bacterial isolates underwent alignment and analysis using the Basic Local Alignment Search Tool (BLAST) program available at http://www.ncbi.nlm.nih.gov.

3. RESULTS AND DISCUSSION

3.1 Isolation and Screening of Keratinase-Producing Bacteria

A total of nine bacteria out of eighteen bacteria isolates obtained showed observable feather degradation and proteolytic activity. Isolates K50, K51 and K53 showed high feather degradation and proteolytic activities as shown in Table 1. The proteolytic activity was highest in isolate K51

with clear zone of hydrolysis of 25 mm, followed by isolates K53 and K50 with hydrolysis zones of 24 mm and 13 mm respectively (Plate 1). There was no zone of hydrolysis observed in isolates K7, K13 and K16. Highest dissociation of azure dye from keratin-azure was recorded in isolates K51, K50 and K53 and was indicated by deeper azure colouration in the medium; followed by isolates K18, K59 and K1 which showed a less deep azure coloration, while isolate K13 showed no release of azure dye into the medium (Plate 2).

3.2 Screening of Isolates for keratin-degrading Ability Using Different Keratin Substrates

The keratin-degrading ability of the isolates using different keratin substrates is as shown in Table 2. From the table, highest degradation of all the three keratin sources was observed with isolate K51 followed by isolates K53 and K50 and least degradation was observed with isolate K18. All the isolates, except for isolates K50 and K51 showed no significant difference ($P = .05$) in degradation among the three different keratin sources used. Isolates K50 and K51 showed higher degradation with feather as keratin source with respective optical densities of 0.07 and 0.11 followed by hair and least in nails with optical densities of 0.05 and 0.07 respectively.

3.3 Identification of keratin-degrading bacteria

The biochemical and physiological characteristics of the bacteria showing highest keratin-degradation are shown in Table 3. Microscopic observations of the isolates show single straight Gram-positive rods with positive endospore formation. All the isolates were motile, catalase positive, and negative to indole production, they all hydrolyse casein, gelatin, and starch, reduced nitrate and grew well at 4% NaCl concentration. Isolates K51 and K53 showed positive urease activity and hydrogen sulphide (H_2S) production while Isolate K50 was negative for both urease activity and hydrogen sulphide production. Only isolate K51 was negative for acetoin production in Voges Proskauer's test.

Sugar fermentation test for the bacteria isolates revealed that all the isolates fermented sucrose, maltose, glucose, lactose, mannose, xylose, raffinose, fructose, galactose and cellobiose. None of the isolates was able to ferment dextrose. Arabinose and mannitol were not fermented by isolate K51, Isolates K50 and K53 were not able to ferment sorbitol and mannitol respectively, and all the isolates produced gas during glucose fermentation. Hence, based on the results of the microscopic, biochemical and physiological characteristics, as well as sugar fermentation pattern, the three isolates were identified as *Bacillus subtilis*-K50*, Bacillus licheniformis*-K51 *and Bacillus* sp.-K53.

The Molecular identification of the bacterial isolates is shown in Table 4. The nucleotide sequences obtained and analysed from the 16S rRNA of isolates K50, K51 and K53 yielded strong homologies of up to 99%, 99% and 100% similarities respectively with those of several cultivated strains of *Bacillus* and were confirmed to be *Bacillus subtilis*-K50*, Bacillus licheniformis*-K51 *and Bacillus* sp.-K53 with accession numbers of KX857467.1, KX857466.1 and KX880450.1 respectively.

Table 1. Proteolytic and feather degrading levels of isolates

Isolate codes	Feather digestion level	Diameter of zone of hydrolysis on skimmed milk agar (mm)	Release of keratin azure dye in medium
K1	+	8	2
K7	+	0	1
K13	-	0	0
K16	+	0	1
K18	++	12	3
K50	+++	13	4
K51	++++	25	4
K53	++++	24	4
K59	++	14	2

Key: +++++ Complete degradation ;5- complete dye release; ++++ High degradation; 4- High dye release; +++ Moderate degradation; 3-Moderate dye release; ++ Low degradation; 2- low dye release; + Very low degradation; 1- very low dye release; - No degradation; 0- No dye release

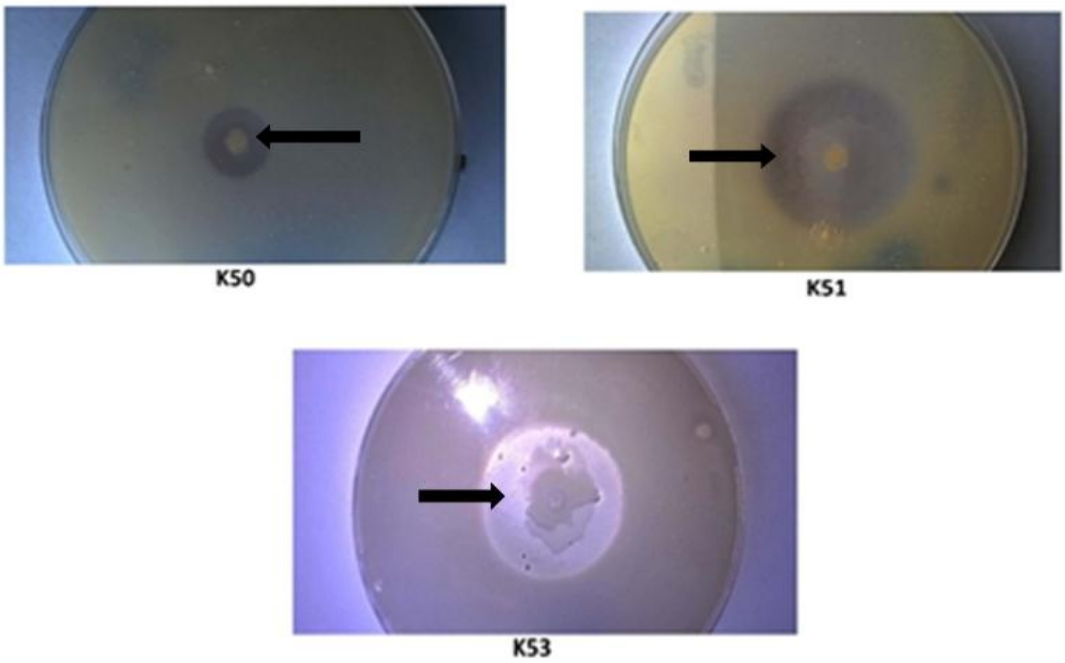

Plate 1. Proteolytic activity of isolates on skimmed milk agar medium
(Arrows indicating zone of hydrolysis) Legend: K50= Isolate K50, K51=Isolate K51, K53= Isolate K53

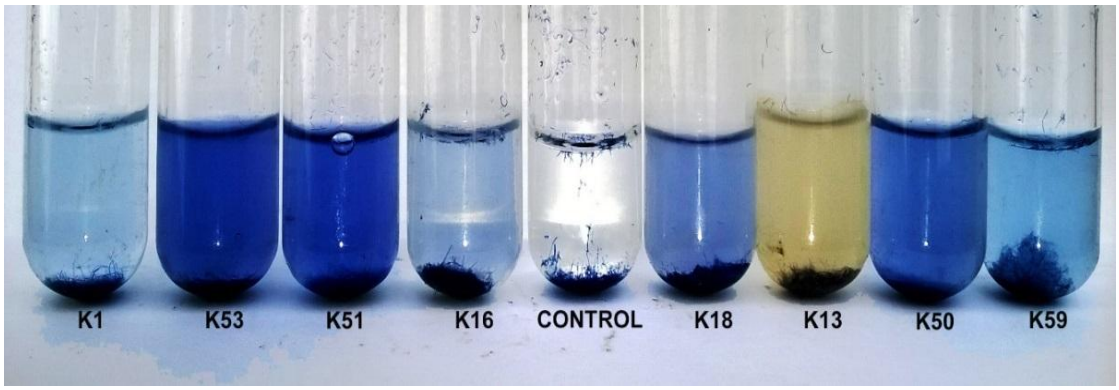

Plate 2. Reaction Characteristics of inoculated keratin azure tubes after 7 days incubation period
(K1, K53, K51, K16, K18, K50 and K59 showing positive reaction indicated by azure dye release into basal medium; K13 showing negative reaction)

Phylogenetic relationship between the keratin-degrading isolates and nearest *Bacillus* strain identified by BLAST analysis is shown in Fig. 1. The result shows that *Bacillus licheniformis*-K51 formed a cluster with *Bacillus licheniformis* strain Sp1, *Bacillus* sp. strain Bacl and *Bacillus licheniformis* strain N3. *Bacillus subtilis*-K50 formed another cluster with *Bacillus subtilis* strain BCRC-10255, *Bacillus tequilensis* strain FJAT-47773, *Bacillus amyloliquefaciens* AMHSBL104 strain and *Bacillus subtilis* strain BD73, while *Bacillus* sp strain K53. and *Bacillus* sp strain BGSC-W9A86 formed a separate cluster. *Escherichia coli* strain ATCC, which was arbitrarily chosen, served as an out-group for the analysis and did not form any cluster.

Table 2. Degradation of keratin using different substrates

	Isolate code/ Optical density								
Substrate	**K1**	**K7**	**K13**	**K16**	**K18**	**K50**	**K51**	**K53**	**K59**
Feather	0.04±0.00^{a}	0.06±0.00^{a}	0.05±0.01^{a}	0.04±0.01^{a}	0.03±0.00^{a}	0.07±0.00^{b}	0.11±0.02^{b}	0.08±0.01^{a}	0.03±0.00^{a}
Nail	0.03±0.00^{a}	0.45±0.00^{a}	0.05±0.00^{a}	0.03±0.00^{a}	0.02±0.12^{a}	0.05±0.00^{a}	0.07±0.00^{a}	0.08±0.04^{a}	0.03±0.00^{a}
Hair	0.04±0.00^{a}	0.06±0.00^{a}	0.05±0.00^{a}	0.04±0.01^{a}	0.03±0.10^{a}	0.06±0.01^{ab}	0.09±0.00^{ab}	0.08±0.01^{a}	0.03±0.00^{a}

Values are in means ± standard deviation; means with different / similar superscripts along the same column are significantly different / not significantly different from one another

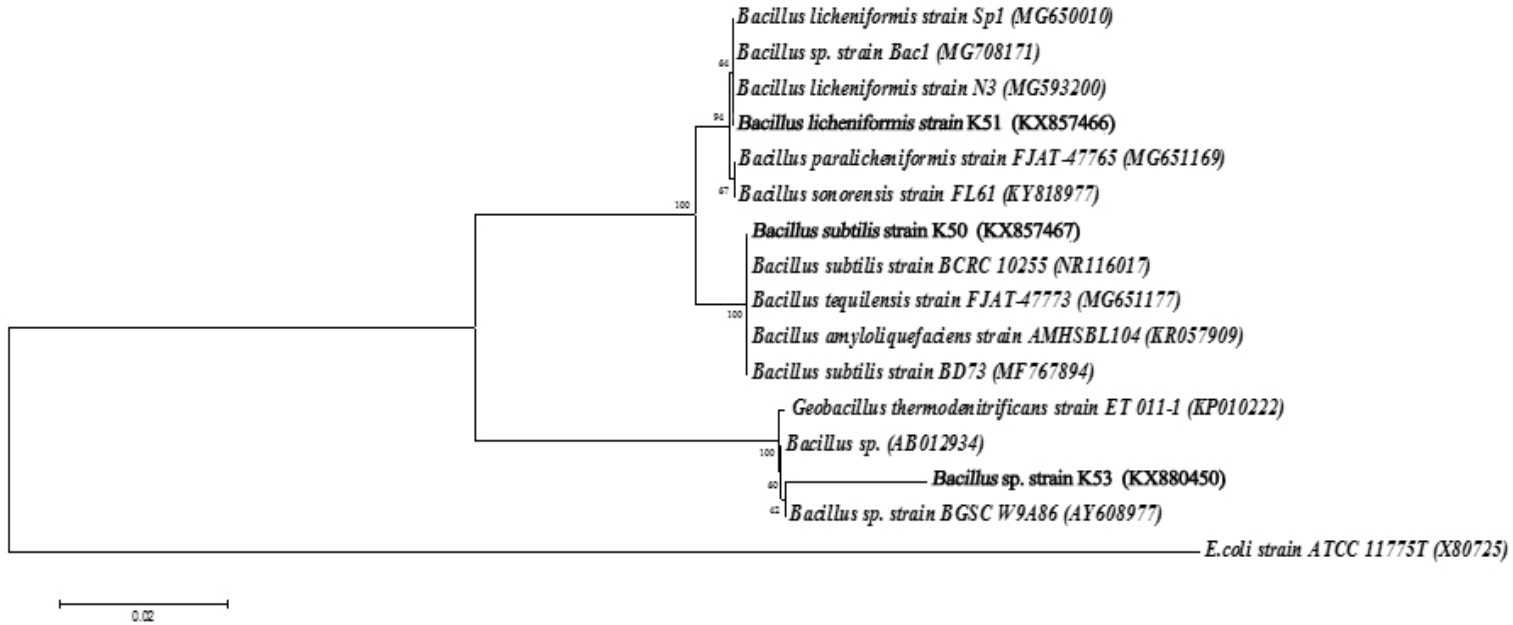

Fig. 1. Phylogenetic tree of *Bacillus subtilis*-K50, *Bacillus licheniformis*-K51 and *Bacillus* sp.-K53. with Reference type strains. (sequence accession numbers are given in parentheses)

Table 3. Biochemical and sugar fermentation pattern of selected isolate

	Isolate codes		
Test	**K50**	**K51**	**K53**
Gram reaction	+	+	+
Shape	Single rods	Single rods	Single rods
Endospore formation	+	+	+
Catalase activity	+	+	+
Starch hydrolysis	+	+	+
Gelatin hydrolysis	+	+	+
Casein hydrolysis	+	+	+
Peptonisation	+	+	+
Motility	+	+	+
Indole production	-	-	-
Nitrate reduction	+	+	+
Methyl red test	-	-	-
Voges Proskauer's test	+	-	+
H_2S Production	-	+	+
Urease activity	-	+	+
Oxidase activity	-	-	-
Growth in 4% NaCl	+	+	+
Growth in 6.5% NaCl	-	-	-
Anaerobic growth	+	+	+
Sucrose	+	+	+
Maltose	+	+	+
Glucose	+g	+g	+g
Lactose	+	+	+
Arabinose	+	-	+
Mannose	+	+	+
Xylose	+	+	+
Raffinose	+	+	+
Fructose	+	+	+
Dextrose	-	-	-
Mannitol	+	-	-
Sorbitol	-	+	+
Galactose	+	+	+
Cellubiose	+	+	+
Probable Identity	*Bacillus subtilis*	*Bacillus licheniformis*	*Bacillus* sp

Key – Negative reaction, + Positive reaction w: weak growth; g:gas production

Table 4. Identity of the bacterial isolates using NCBI database

Isolate Codes	Length of nucleotide sequence	Accession number of nearest homology	Percentage similarity (%)	Name of Organism
K50	1189	KX857467.1	99	*Bacillus subtilis*-K50
K51	1027	KX857466.1	99	*Bacillus licheniformis*-K51
K53	1111	KX880450.1	100	*Bacillus* sp.-K53

4. DISCUSSION

Nine bacteria isolated from poultry waste in this study have been shown to degrade feather keratin. The presence of these keratin-degrading bacteria in poultry waste may be as a result of indigeneity of the bacteria species to the gut of the chicken, nevertheless, it is also probable that they were indigenous to the environment in which poultry faeces are obtained. Over time, isolates might have adapted to utilize feather keratin as substrate, and being the main structural component of feather, the breakdown of keratin will lead to the degradation of feather. The cause of feather decay in nature may be associated with microorganisms and the main

habitat of endospore forming *Bacillus* is the soil, hence, the choice of decaying feathers and the associated soil for isolation in this study. Similarly, in a report [4], keratin-degrading bacterial strains were isolated from different feather dumping sites. In another report [11] isolates were obtained from rotten feathers and soils from poultry farm waste site.

Using feathers as a primary source of energy, carbon and nitrogen in the isolation medium used in this study, the organisms were able to grow utilizing the feather as a carbon source, therefore, the formulation allowed for the elimination of extraneous organic carbon and nitrogen sources which might interfere with the assay for keratin utilization. This is in line with a similar report [25] where keratinolytic bacteria were also isolated using medium containing feather as a primary source of energy.

During keratin degradation in keratin-azure medium, a direct relationship between dye release and keratin degradation could be inferred because the azure dye becomes dissociated from the keratin upon the breakdown of the keratin by the isolates. Similarly, azure-based culture media assays have been employed in assessing keratinase production in microorganisms and it has been regarded as a quick and reliable technique [26].

From this study, *Bacillus subtilis*-K50, *Bacillus licheniformis*-K51and *Bacillus* sp.-K53 which showed highest feather degradation levels also showed highest release of azure dye from the keratin-azure medium and subsequently, largest zones of hydrolysis on skimmed milk agar. In the same pattern, the isolates with the lowest feather degradation levels showed lowest release of azure dye in keratin-azure medium and subsequently smallest zones of hydrolysis on skimmed milk agar. A direct relationship may be inferred in the abilities of the feather degrading bacteria to produce enzyme for the breaking down of keratin in keratin-azure medium and also hydrolyse skimmed milk in skimmed milk agar. This was also observed for keratinase from *Bacillus species* [27]. Keratinases do not only hydrolyse keratin but have the ability to hydrolyse a wide range of soluble and insoluble protein substrates, serving as a basis for the proteolytic hydrolysis of skimmed milk by the feather-degrading bacteria in this study. However, this direct relationship is not always the case as a study [11] shows that *Pseudomonas putida*-B2 with lowest proteolytic activity among the isolates in the study recorded a higher keratinolytic activity than those of other isolated bacteria. This observation may be due to the ability of *Bacillus* to produce one or more other broadly specific bacterial proteases [28]. In such a case, the proteolytic activity test will not only show the proteolytic ability of the keratinase from the isolates but might have included the activities of other proteolytic enzymes synthesized alongside with keratinase.

From this work, hair and nail were able to serve as keratin source along with feathers. This observation is consistent with the fact that keratin forms the major insoluble structural components of hair and nail [29], and since in most cases the presence of keratin induces the production and release of keratinases from microbes, it can be said that the keratin present in the hair and nails utilized in this study is likely to have also induced the production and release of keratinase in the bacterial isolates**.** It was also observed that among the three keratin-containing substrates, feather was mostly utilized followed by hair and then nail. In a similar report [30], higher degradation level was recorded using feathers as substrate compared with other keratinous substrates utilized in their study. Keratins differ in resistance towards enzymes and in sulfur content, and keratin types vary in different substrates which differ in amounts of alpha and beta keratins [31]. This may serve as a factor that determined the varying keratinolytic activity produced by the different keratin protein substrates. The three bacteria with highest feather-degrading ability focused on in this study have been identified to be *Bacillus subtilis*-K50, *Bacillus licheniformis*-K51and *Bacillus* sp.-K53. *Bacillus* species are the main producers of extracellular proteases and a major proportion of keratin-degrading bacteria are Gram positive and mainly belong to the genus *Bacillus*. This is most likely because they are abundant in soil and are able to exist in dormant spore forms to resist harsh environments or in a vegetative state when conditions are good. In related finding [32], keratin degrading isolates were observed to be predominantly *Bacillus* species. A similar report on the isolation of keratin-degrading strains of *Bacillus* from decomposing feather has also been made [33].

The phylogenetic analysis of the bacteria isolated in this study suggested close relatedness amongst the keratin-degrading bacteria with those of several cultivated *Bacillus* strains. This is as a result of the strong genetic sequence homologies which exist between them. Similarly,

in another report [26], the evolutionary relationship of the keratin-degrading *Bacillus* species confirmed detailed evolutionary closeness with *Bacillus* strains having closest gene sequence identity.

5. CONCLUSION

Three keratin-degrading bacteria; *Bacillus subtilis*-K50*, Bacillus licheniformis*-K51 *and Bacillus* sp.-K53 were isolated from poultry feather waste and were able to grow on medium containing feather as the major source of carbon, nitrogen and energy and they are predominantly proteolytic. Keratin-azure was vital in providing an indication of keratin degradation by the bacteria. The bacteria are capable of utilizing feather as keratin substrates more easily than hair and nails. The bacteria obtained from this study possess keratinolytic capabilities and can be involved in processes that require degradation of keratins.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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