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Isolation and Molecular Characterization of Chicken Feather-Degrading *Lysinibacillus* spp Strain Kik1

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Abstract
Large quantities of feathers are produced as byproduct in poultry farms. Keratinolytic bacteria utilizing keratinolytic enzymes have the capability of breaking down feathers to reduce waste from the environment. Reweighing after drying was used to determine feather biodegradation and spectrophotometric method for bacterial growth and keratinase activity. Thirteen bacterial isolates were screened in two folds for biodegradation potential and keratinolytic activity using free cells of bacteria. The Primary Screening Phase illustrates four (4) isolates that showed biodegradation potential of 65, 82, 68 and 73% tagged DO2, DO4, DO5 and DO8 respectively and were selected into secondary phase of screening. Free Bacterial Resting Cells were utilized in which isolate DO4 illustrated greater biodegradation capability of 96%. It was chosen as the best bacteria for further research. These bacteria had a 96% similarity with Lysinibacillus spp when identified using the 16s rRNA gene sequence analysis. Lysinibacillus spp Strain KIK1 was registered in the GenBank with accession number MZ701840.

Keywords: Feather, Keratinolytic, Resting cells, Biodegradation, *Lysinibacillus* spp.

Introduction
Chicken is a delicacy that grows in popularity every year as one of the best sources of cheapest protein. Feathers are produced in millions of tones during growing and slaughtering processes of these hens. Korniłłowicz-Kowalska and Bohacz, (2011) found that these feather-wastes are made up of fibrous and structural keratin polymers that are heavily cross-linked by cysteine disulphide bonds, hydrogen bonds, and hydrophobic interactions (Eslahi *et al*., 2014).

Feathers and other keratins have this feature, which makes them resistant to destruction by soil bacteria and proteolytic enzymes like trypsin, pepsin, and papain (Bach *et al*., 2012). Feathers, on the other hand, have a high protein content and can be used as a source of protein

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and critical amino acids in animal feed, fertilizers, and other industrial purposes (Fakhfakh *et al*., 2011). Furthermore, being low-cost agricultural wastes, feathers have a high biosorbent characteristic, making them useful for removing heavy metals from surface water (Al-Asheh and Banat, 2003).

Nevertheless, the frequent usage in the heavy metal bio-removal from sewages of industries and some other chemically contaminated effluent has resulted in the accrual of heavy metal poisoned feathers which is a secondary contaminant that can be poisonous to several feather biodegrading bacteria. Landfilling, incineration, burning, or the use of chemicals to dispose of or convert them to protein hydrolysates are expensive, environmentally unfriendly, and dangerous to living organisms (Sharaf and Alharbi, 2013; Sharma *et al*., 2013), not to mention the loss of desired essential amino acids like methionine, lysine, and cysteine (Farag and Hassan, 2004; Rajput and Gupta, 2013). Microbial degradation of unpolluted feathers (primary feather wastes) has been developed as an alternative to conventional ways, and it is garnering interest since it is turning feather-waste into profit (Sivakumar *et al*., 2002; Sivakumar and Raveendran, 2015; Yusuf *et al*., 2015).

Feather-degrading microorganisms (FDMs) such as bacteria, Streptomyces, and fungi have been isolated from various environmental sources and are available in the literature (Demir *et al*., 2015; Ghasemi *et al*., 2012). Utilizing keratinase substrate, FDMs secreted extracellular enzyme (keratinase) to aid in bioconversion of feathers to protein mass. This enzyme is used in a variety of sectors, including leather, cosmetics, and others (Agasthya *et al*., 2013). The quantity of keratinase enzyme formed by different Feather Degrading Microorganisms differs according to species of the organisms, the substrate and medium environment. Various physical and medium variables, on the other hand, have a significant impact on proliferation of a given feather degrading microorganism in a keratin medium then consequent keratinase synthesis. In this research isolation and screening of the best feather degrading bacteria (*Lysinibacillus* sp strain KIK1) was carried out which showed a high biodegradation capacity compared to other screened bacteria.

Materials and Methods

Feather and Sample Collection

Feathers of chickens were obtained from chicken abattoir in Azare, Bauchi State, Nigeria and was transferred to the laboratory in a sample tool box. All the chemicals and reagents utilized in the research are of analytical grade.

Collection of Samples

Soil was obtained from various feather dumping sites within Azare metropolis. Sample was collected from 2 to 3 cm depth and transferred into a sterile plastic bag using sterile spatula (Jibo and Karamba, 2024). The sample was transferred into an ice box and brought into microbiology research laboratory in Sa'adu Zungur University, Gadau, Bauchi State for further analysis.

Analytical method

The collected feathers were thoroughly washed with tap water and detergent (Meng *et al*., 2013), then defatted in a solution containing 200 ml tap water, methanol, and chloroform in a ratio of 1:1:10. The feathers were dried in the oven at 35°C (Nayaka and Vidyasagar, 2013). Biodegradation was determined by reweighing after drying (Cedrola *et al*., 2012). The DMSO chicken feather solubilize keratin was used for the analytical method to determine the keratinase activity within the medium. The feather hydrolysate was mixed with solubilize

keratin and was determined spectrophotometrically at 280 nm absorbance after centrifugation as describe by Jin *et al*., (2017). Bacterial growth was measured using spectrophotometer at OD_{600nm} (Karamba *et al*., 2020).

Isolation of Feather Biodegrading Bacteria

Media Preparation

Nutrient agar and broth media were prepared in conformity with the instructions of the manufacturer. Feather Meal Broth was prepared using the following composition; Yeast Extract, 0.1g; NaCl, 0.5g; $MgCl.6H_2O$, 0.16g; KH_2PO_4, 0.4g; K_2HPO_4, 0.3g; Feather 1g in 100 ml distilled water. pH was adjusted to 7.5 (Using I N of NaOH in which the feather was the only source of carbon). The medium was autoclaved for 15 minutes at 121°c before use (Yusuf *et al*., 2015).

Handling and Growth of Bacteria Isolates

One gram (1 g) sample of soil was served to sterilized distilled water (10 ml) and mixed properly. It was serially diluted to 10^{-6}. 0.1 ml of sample was transferred into nutrient agar medium from each of the dilution factor. The petri dish plates were inverted clock and anti-clock wise to obtain uniformity. They were incubated at 30 ℃ overnight. The bacteria isolated were sub cultured on nutrient agar medium in order to attain pure isolates.

Preparation of Bacterial Resting Cells

This was accomplished using the method of Ibrahim (2016). Nutrient Broth (8 g/L) and Glucose (8 g/L) was formulated. It was sterilized by autoclaving at 121℃ for 15 minutes then allowed to cool. Pre-cultured Bacteria (2% V/V) were added. Aeration was attained using incubator shaker model LYZ-200B for 48 hours at 150 rpm, followed by centrifugation at 10,000g for 10 minutes at 4℃. The harvested bacterial cells were rinsed two folds with 100 mM phosphate buffer pH 7.0 before being resuspended in same phosphate buffer solution. The OD_{600nm} of the suspended bacteria was adjusted to 1.0 then stored at 8℃ for further studies.

Determination of keratinolytic activity using DMSO- solubilized white chicken feather keratin

After incubation of FMB containing degraded chicken feather, the broth culture medium was taken and centrifuged at 10, 000 rpm for a period of 15 minutes. The feather protein hydrolase (free supernatant) was used to quantify keratinase activity of the bacteria.

One milliliter of chicken feather hydrolase was reacted with 1 ml keratin solution. The mixture was incubated for 10 minutes at 50 ℃ in a shaking water bath. The reaction was halted by the addition of 2 ml 20% trichloroacetic acid. The precipitate was removed by centrifugation at 10,000 rpm for 15 minutes and the optical density of the supernatant was taken at 280nm (Yusuf *et al*., 2015).

Molecular Identification of Bacterial Strain and Phylogenetic Tree Analysis

The most potent bacteria that will be used for further studies was molecularly identified using DNA extraction sequencing analysis by 16srRNA gene sequencing. DNA obtained from the isolate was removed by the use of quick bacteria genomic DNA extraction kit. Manufacturer instructions were followed to produce a final volume of 100 µl. Polymerase Chain reaction (PCR) was carried out under the following conditions: 1 cycle of initial denaturation at 95 ℃ for 5 minutes, 30 cycles (94 ℃ denaturing for 50 seconds, 58 ℃ annealing for 30 seconds, and 72℃ extensions for 1 minute) and 1 cycle of final extension at 72 ℃ for 5 minutes (Karamba

and Mukhtar, 2013, 2012). Thirteen (13) 16s rDNA sequences of related bacteria were obtained from gene bank and aligned using Clustal W by means of mega 6.06 (Karamba *et al*., 2015a).

Screening of Feather Degrading Bacteria

To screen the microorganism for feather degradation, each isolate was inoculated into a separate 500 ml glass flask holding 1 g of fresh feather. It was then incubated in an incubator shaker at 150 rpm for 5 days at room temperature. Feather degradation analysis was carried out to examine the feather biodegradation capacity of the isolates. Primary and Secondary methods were adapted (Karamba *et al*., 2015b).

Results and Discussion

Isolation of Feather Biodegrading Bacteria

Previous report indicated that bacteria that degrade feathers have been isolated from feather disposal sites (Sutoyo *et al*., 2019). However, because the bacteria that degrades feathers was obtained from recalcitrant feather dumping locations, our study found comparable results. The bacteria were able to thrive in a medium that included solely feather as a carbon source (Yusuf *et al*., 2015). It could be because the bacteria were able to efficiently synthesize the enzymes needed to digest feather. It just needs a small amount of nitrogen source, which aids in the bacteria's high percentage breakdown.

Screening of Feather Degrading Bacteria

Primary Screening

Initially, the sampled soil was placed on a nutrient agar media to permit any live bacteria to proliferate. All microorganisms that might breakdown feather were screened using feather meal broth medium. (13) Isolates were acquired by culturing on the same nutrient agar medium in order to obtain pure bacterial colonies; one (1) colony was injected into feather meal broth medium. Individual bacterial isolates were cultivated and incubated for five days at 120 rpm in an incubator shaker. Four (4) of the thirteen isolates studied were shown to have the capacity to degrade feathers. This is intriguing because it showed that not all bacteria isolated from a feather dumping site can metabolize feather. Furthermore, this finding shows that while diverse bacterial species can grow on media containing feather, not all of them can degrade it. In reality, only 4 of the 13 bacterial isolates i.e. 30.7% demonstrated strong proliferation ability on feather-containing media and have biodegradation capacity (Figure 1). Isolates DO4 and DO8 have a biodegradation capacity of more than 70%, while isolates DO2 and DO5 have a biodegradation capability of more than 60%, respectively. Although all isolates grew at almost the same pace since they were all above 60% degradation, some showed a greater potential for biodegradation than others. As a result, isolates DO4, DO5, and DO8 were chosen for further analysis in order to find the optimum feather biodegradation bacteria.

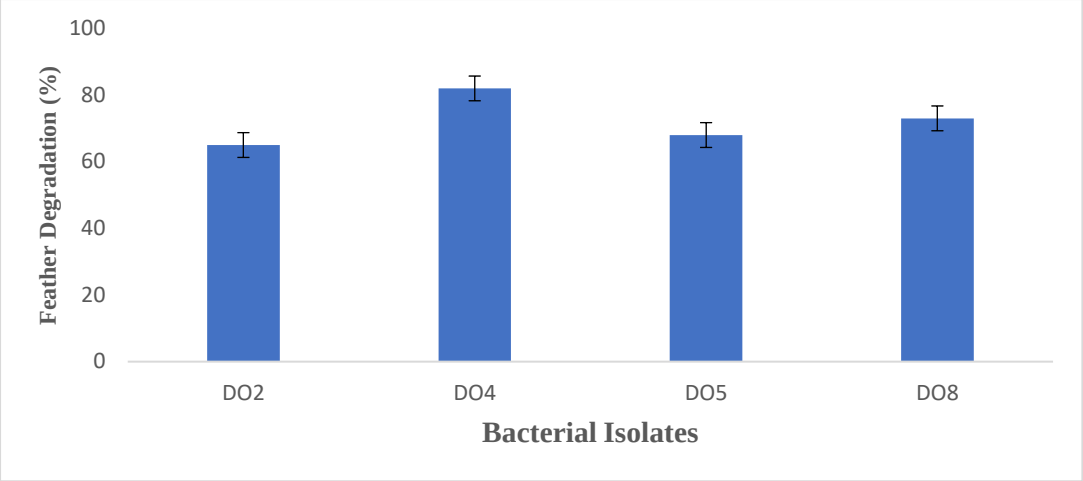

Figure 1: Primary screening of four bacteria after 96 hours of incubation. Data represent mean ± STDEV with N=3

Secondary Screening

The resting cells of the bacteria were prepared as described above and utilized during the secondary phase of screening using the same culture conditions. The biodegradation capacity of the three isolates was found to be satisfactory (Figure 2). Isolate DO4 have the highest biodegradation capacity of 92% followed by Isolates DO2, DO8 and DO5 with 69%, 75% and 70% respectively. Free resting cells have greater capacity for biodegradation compared to the preceding inoculums used during the primary phase of screening, as evidenced by the reduction in biodegradation time from 4 days to 2 days. The justification could be in the preparation of resting cells, where cells were harvested at early stationary phase after 48 hours of incubation, and are thought to have higher biodegradation capability than bacterial cells cultivated at exponential phase of growth, given that their capacity to withstand environmental stress at that phase may be higher (Karamba *et al*., 2020). As a result, isolate DO4 was chosen for further study since it has a better ability for degrading feather in the least time period.

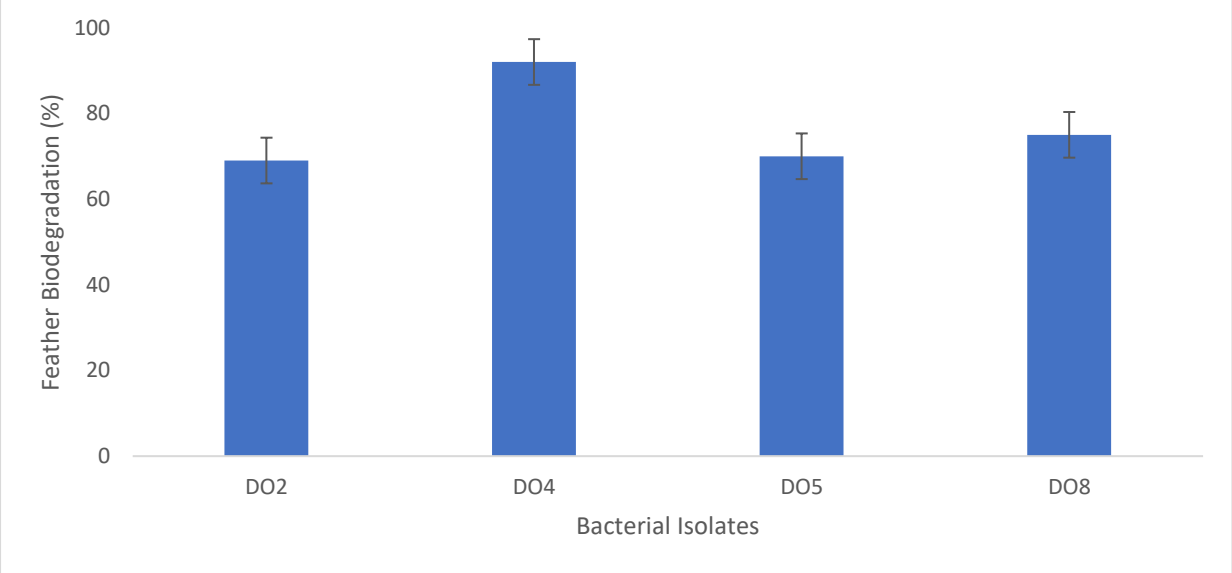

Figure 2: Secondary phase of screening by four bacteria after 96 hours of incubation. Data represent mean ± STDEV with N=3

Genomic extraction

Gram stain smear and microscopic examination illustrates that isolate DO4 was gram-negative rod-shaped bacteria. Gram-negative bacteria have been reported to have feather removal ability (Dhiva *et al*., 2020).

Using Quick bacteria Genomic DNA extraction set, the Genomic DNA extracts of isolate DO4 was acquired successfully. In order to achieve enough gene amplification, it is very important to obtain complete fragment of the DNA during extraction. Bands with the highest intensity showed high concentration of DNA samples (Zeng *et al*., 2008).

Polymerase Chain Reaction (PCR)

Isolate DO4 genomic DNA amplified was utilized as the prototype for 16s rRNA gene amplification. The primers used are most commonly used primers, which are highly conserved for prokaryotes as they amplified the complete regions of the 16s rRNA gene equivalent to 1500 bp (Acinas *et al*., 1997). The amplified gene of isolate DO4 resulted to a fragment that has an anticipated size of 1500 bp (Figure 3).

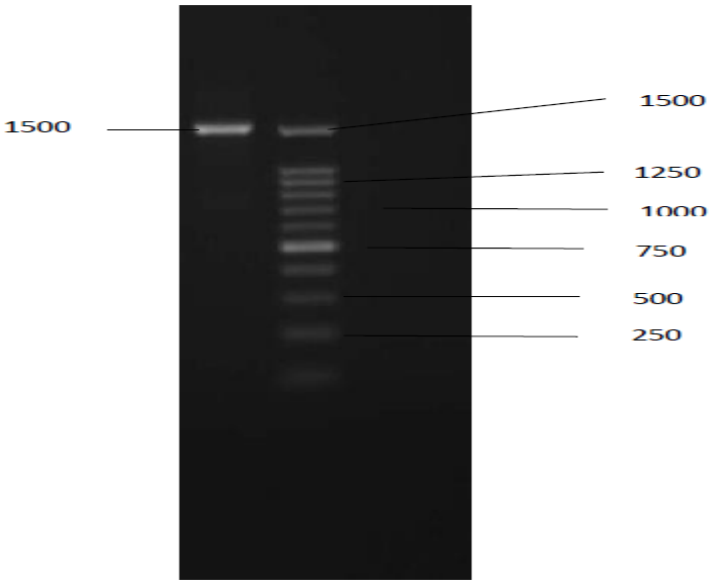

Figure 3: Agarose gel electrophoresis for PCR product of isolate DO4 amplified gene.

Isolate DO4 was chosen for identification using the 16s rRNA sequencing based on the data obtained. Using NCBI BLAST, the 1210 nucleotide sequences was equated to the Gene Bank data base. According to 16s rRNA gene sequencing, it shares 96.79% of its DNA with *Lysinibacillus* spp. *Lysinibasillus* strain KIK1 had its 16s rRNA gene sequence submitted to the NCBI GenBank and assigned accession number MZ701840. It showed significant alignment with thirteen sequences with *Lysinibacillus* spp in NCBI blast.

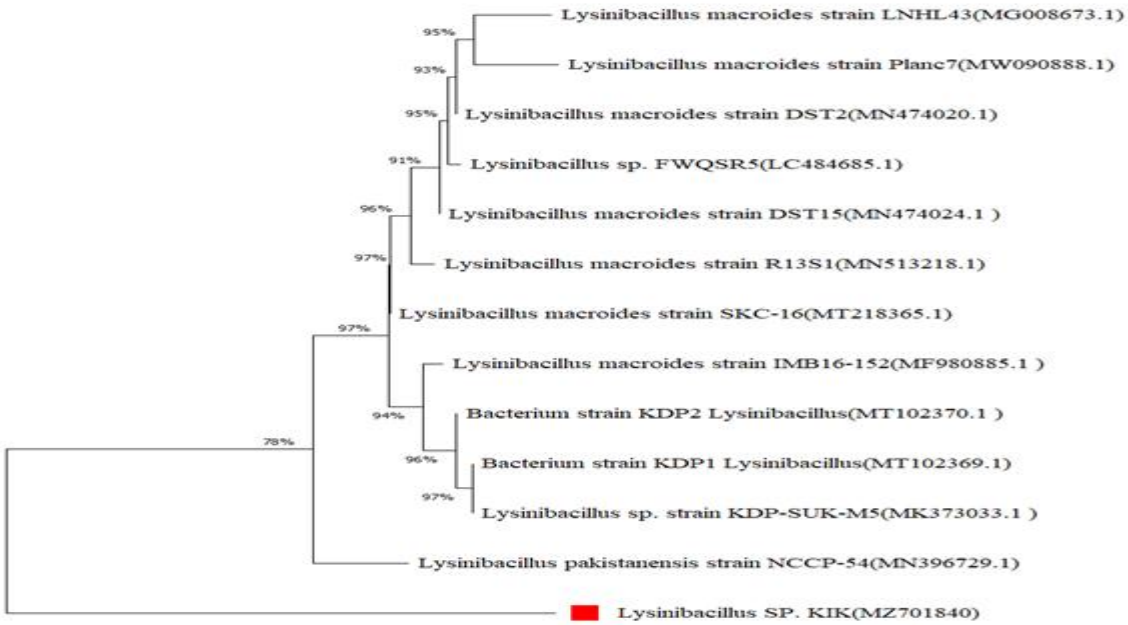

Figure 2: Cladogram Neighbour Joining Method showing the Phylogenetic Relationship among strain KIK1 and some other interrelated analogous bacteria on the 16s rRNA gene sequencing.

Figure 4 shows the evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The tree is depicted to scale, the distance of the division is in similar units like that of the evolutionary distances utilized to conclude the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) and are in the units of the number of base substitutions per site. The number of sites where at best one explicit base is existing in at least one sequence for every progeny clade is illustrated next to each inner bulge in the tree. This study includes 13 nucleotide sequences. The codon positions comprised $1^{st}+2^{nd}+3^{rd}$+Noncoding. Entirely ambiguous locations were deleted for each pair of sequence (pairwise deletion option). There were sum total of 1210 locations in the closing data set. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018).

Conclusion

Several bacteria were isolates from the soil obtained from chicken abattoir in Azare, Bauchi State, Nigeria. This is an environment where it is laden with feather loving microorganisms. From the various isolates screened for feather biodegradation using chicken feather waste, a newly isolated bacteria *Lysinibacillus* spp strain KIK1which was registered in NCBI GenBank and assigned accession number MZ701840 was discovered to be the most active biodegradation bacteria. Ten percent of the free resting cells of bacteria was able to degrade 96% of 3 g of feather within 48 hours of incubation

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