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ISOLATION AND CHARACTERIZATION OF KERATINOLYTIC BACTERIA FROM SOME SELECTED POULTRY SLAUGHTERHOUSES IN KADUNA METROPOLIS, NIGERIA.

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Abstract

Among several agro-waste products, keratinous ones remain a big challenge in terms of degradation, thereby constituting environmental nuisance. This study was focused on isolating keratinolytic bacteria from soils and determining their ability to degrade keratinous materials using chicken feather as substrate. Consequently, the keratinolytic abilities of some bacterial isolates from selected chicken slaughterhouses were evaluated. Six bacteria with keratinase producing ability were isolated from soil samples gotten from different chicken slaughterhouses. Two isolates (ST2 and SA2) showed the highest keratinase production and maximum degradation of feathers and were taken for molecular studies and identification. The isolated bacteria were screened for keratinolytic ability on chicken-feather agar medium and the keratinolytic bacteria were identified through 16s rRNA gene sequence analysis. The keratinase activity was determined with respect to the percentage whole feather degradation and quantification of soluble proteins produced. The two isolates were identified as Bacillus cereus and Bacillus piscicola. The keratinase activity and amount of soluble protein ranged from 8.4 ± 0.07 U/ml – 18.1 ± 0.02 U/ml and 0.014 ± 0.003 mg/ml – 0.26 ± 0.003 mg/ml respectively. Also, the degree of whole feather degradation ranges from 24% - 82% with the isolated Bacillus sp. The pH of the medium increased from 7.2 - 8.6 at 40°C due to the presence of soluble proteins in the medium. The amount of soluble proteins increased with the increase in the percentage feather degradation, which further suggests the possible role of the isolates Bacillus cereus and Bacillus piscicola in the degradation of keratinous wastes. Moreso, keratinolytic potentials of these isolated bacteria species makes them a very useful biotechnological tool for industrial purposes.

Keywords

Keratinolytic, Keratinase, Bacillus sp, Bacteria, Degradation

1. Introduction

1.1 Background of study

Keratin is a versatile bioactive protein that is abundantly distributed in nature. It is the major component of hair, nail, feather, wool, hooves, scales, and horns (Debananda *et al.*, 2018). It is an insoluble macromolecule recalcitrant to degradation by common proteases like pepsin, papain, and trypsin due to its highly supercoiled and tightly packed molecular structure stabilized by hydrogen bonding or hydrophobic interaction (Katarzyna *et al.*, 2011). It can be subdivided into soft and hard keratins based on the sulphur content. Hard keratins have high disulphide bond content that makes them tough and inextensible as found in feathers, hair, hooves, and nails. On the contrary, soft keratins are more pliable due to low content of disulphide bonds as found in skin (Kreplak *et al.*, 2004). Feather biomass is made up of over 90% proteins, which is primarily keratin and insoluble proteins, extensively cross-linked by disulphide bonds (Yadav and Khosla, 2021). The intensive development of human economic activity, including agricultural and animal production has led to the accumulation of large amount of waste from poultry farms, abattoirs, leather industry and hatcheries into the environment (Jayathilakan *et al.*, 2012). Agro-industrial processes have gained increasing attention in recent years and, expectedly so, would continue to increase as the world's increasing population requires sustenance. And such activities have led to the accumulation of agro-wastes with recyclable value and when these agro-waste are dumped into the environment, could constitute an environmental nuisance (Wojciech *et al.*, 2018). Millions of tons of feathers are liberated annually from poultry processing farms as waste products and approximately 90% is β-keratin (Santos *et al.*, 1996). The major amino acids in the structure comprise glycine, alanine, serine, cysteine, and valine with less lysine, methionine, and tryptophan (Wojciech *et al.*, 2018). Keratinous wastes have been traditionally digested using physico-chemical methods like incineration, landfilling and alkaline hydrolysis which have been associated with greenhouse effect and underground water contamination. But such techniques are energy-intensive and technologically demanding and lead to the degradation of some essential amino acids (Rahayu *et al.*, 2015). Therefore, an efficient method that is more environmentally friendly and sustainable is required through the use of microbes to degrade keratin wastes. The aim of this study is to isolate and characterize bacteria with keratinolytic ability from some selected poultry slaughter houses in Kaduna metropolis, Nigeria, and their potential application in the degradation of keratin.

2. Materials and methods

2.1 Collection of soil and feather samples and preparations/pre-treatment Soil samples were collected from three different poultry slaughterhouses in Kaduna metropolis: Sabo market in Chikun LGA, Station market in Kaduna North LGA, and Kakuri market in Kaduna south LGA. The soil samples were taken at a depth of 30 cm and transferred into a polythene bag labeled SA, ST, and KA according to their respective locations and were taken to the laboratory for further studies (Nnolim *et. al.*, 2020). The feather sample was collected from Olam Hatchery Limited, Km 25 Abuja-Kaduna Expressway

Chikiri village, Kaduna, Nigeria. The collected feather sample were properly washed for 3-4 times with tap water and sun dried for 24hrs. The dried feathers were suspended in chloroform: methanol (1:1) for 2 days for degreasing followed by rinsing in deionized water and then sun dried and partly grinded into fine powder for subsequent use (Paxton *et al.,* 2020).

2.2 Isolation of bacteria

For the isolation of bacteria, 1 g each of the soil samples collected from the three different slaughterhouses were incubated separately in 50 ml of nutrient broth medium at 37°C and 80-100 rpm. After 5 days, 0.1 ml each of the enriched samples were spread on feather meal medium (10 g ground pre-treated feathers, 0.5 g NaCl (Sodium chloride), 0.3 g K_2HPO_4 (di-potassium hydrogen phosphate), 0.4 g KH_2PO_4 (potassium di-hydrogen phosphate) and 15.0 g agar powder per liter of distilled water) and were incubated at 37°C for 48 hours. The bacterial colonies obtained were further subcultured on the same medium to get the pure isolates (Morris *et al.,* 2012).

*2.3 Screening for keratinolytic ability*0.5g of the pre-treated poultry feathers were shredded into pieces about 1 cm long. And 500 ml of basal medium; (0.5 g) ammonium chloride (NH_4Cl, (0.5 g) sodium chloride NaCl, (0.4g) Potassium dihydrogen phosphate KH_2PO_4, (0.3g) di-potassium K_2HPO_4, (0.1g) Magnesium chloride $MgCl_2$, /L, pH 7.5. was prepared and sterilized. 1 ml of 18 h old culture suspension of each isolate was added to 50 ml of sterile basal salt medium amended with shredded feathers in 250 ml Erlenmeyer flask as described by Aly *et al.* (2019) with some modifications. The flasks were incubated at 37°C and 80-100 rpm on shaker incubator. After 72 hours the feather degradation was observed visually by the medium's viscosity, and as well as by measurement of percentage reduction in dry weight of feathers as compared to that of control (un-inoculated medium). The feathers were separated from culture medium by filtration using whatman's no.1 filter paper, and the culture supernatants were assayed for changes in pH, soluble protein content and extracellular keratinase activity at 37°C and 40°C.

2.4 Determination of percentage feather degradation The residual feathers were washed, dried at room temperature and weighed to determine the percentage of degradation using the equation below. Degradation (%) = (TF− RF) × 100 ÷ TF Where, TF = the total feather weight added to the medium. RF = the residual feather weight on the filter paper. (Nnolim, *et. al.,* 2020). The six bacterial isolates were subjected to biochemical tests and the two isolates (ST2 and SA2) showing highest keratinase production and maximum degradation of feathers were taken for molecular studies and identification.

2.5 Keratinase Assay

Keratinase activity was carried out according to Gradisar *et al.* (2014) with slight modification, where 1ml of the crude enzymes from different filtrates were diluted in 2ml Tris HCl buffer (0.05M, pH8) and then 1g of chicken feather powder was added as the keratin substrate. The mixtures were incubated at 37°C for 1h in a water bath. The enzymatic activity was stopped by the addition of 2ml of 20% tricloroacetic acid (TCA) and centrifuged at 10,000rpm for 10 min, the absorbance of the obtained supernatants was

recorded at 280 nm and compared to the control mixture (20% TCA and the crude enzyme), where 0.01 increase of corrected absorbance at 280 nm with the control was considered as 1 unit (U/ml) of keratinase activity under the experimental conditions.

2.6 Soluble protein estimation

The soluble protein content of the fermentation media was determined in the culture supernatant using bovine serum albumin as standard (Lowry *et al.,* 1951). To 1ml of the supernatants (0.1:10 diluted), 2.5ml of alkaline cupric sulphate reagent (98ml of 2% Na_2CO_3 in 0.1 N NaOH mixed with 1ml of 0.5% $CuSO_4.5H_2O$ in distilled water and 1% potassium sodium tartrate in distilled water) was added and incubated at room temperature for 10 min. To this, 0.25ml of freshly prepared Folin Ciocalteau's reagent (prepared afresh by diluting with distilled water in the ratio 1:1) was added and incubated for 30 minutes in the dark. Absorbance was measured at 660nm against the control as blank (Nnolim, *et. al.,* 2020).

2.7 Characterization of bacteria isolates

The bacterial strains were identified by microscopic examination, biochemical tests and molecular identification.

2.7.1 Microscopic examination Gram staining

Carefully, 3 drops of normal saline were added on a clean slide to make a smear with different isolates. The smear was covered with crystal violet for 1 minute and was rinsed with clean water. Lugol's iodine was added and allowed for 1 minute and then rinsed with clean water. Alcohol was then added in drops and allowed for 30 seconds, then was rinsed with clean water. Then the smear was covered with neutral red and allowed for 1 minute, and then rinsed with clean water. The slides were allowed to air dry, then were viewed under a clean microscope and results recorded (Bio. Lab. Manual, 2019). Biochemical tests Motility, Oxidase, Indole, Urease, Catalase and Methyl red (MR) tests were carried out according to the instruction in the BioLab Manual (2019).

2.7.2 Molecular identification

Isolates with the highest keratinolytic ability were further subjected to molecular identification through 16S rRNA gene sequence analysis. The genomic DNA of the bacterial isolates were extracted using the GenElute bacterial genomic DNA kit. The 16S rRNA gene were amplified using the ribose primers (789bp), Ribose-1: GGACTACAGGGTATCTAAT-16S Primer Forward and Ribose-2: AGAGTTTGATCCTGG-16S Primer reverse. For reaction set-up, DNA templates, specific primers and water were added to the premix to make a 20ul reaction. The amplified DNAs were run on 1.3% agarose gel electrophoresis for 1hour. The resultant DNA bands were visualized using UV light box (Biorad). The pure DNA extracts were sequenced to obtain the specific gene sequence of the isolated keratinolytic organisms. The phylogeny analysis of the 16S rRNA sequence was performed using NCBI (National Centre for Biotechnology Information) BLAST (Basic Local Alignment Search Tool) similarity search tool (Edgar, *et al.,* 2004).

2.8 Statistical analysis All experiments were conducted in triplicates and mean values and standard deviation was calculated and recorded for changes in feather degradation, keratinase activity,

soluble protein content in the culture supernatant and change in pH of the medium.

3. Results

Table 1: Degradation of feathers (% weight loss), keratinase activity (U/ml), protein content (mg/ml) by different bacteria isolates at 37°C and their resultant pH after 72 hours.

Day 3 (72 hours)				
Isolates	pH	(% weight loss)	Keratinase (U/ml)	Protein content (mg/ml)
Control	6.0	2	2.801	0.012
KA1	7.3	32	16.3 ± 0.03	0.016 ± 0.005
KA2	7.6	24	10.5 ± 0.13	0.018 ± 0.005
SA1	7.8	36	09.6 ± 0.07	0.014 ± 0.003
SA2	7.7	52	17.9 ± 0.01	0.062 ± 0.067
ST1	7.2	42	17.2 ± 0.01	0.021 ± 0.003
ST2	7.9	64	18.1 ± 0.02	0.049 ± 0.001

SA: Sabo; ST: Station; KA: Kakuri

Table 2: Degradation of feathers (% weight loss), keratinase activity (U/ml) and protein content (mg/ml) by different bacteria isolates at 40°C and their resultant pH.

Day 5 (120 hours)				
Isolates	pH	(% weight loss)	(U/ml)	(mg/ml)
Control	7.1	05	2.853	0.021
KA1	8.3	58	10.9 ± 0.02	0.057 ± 0.003
KA2	8.6	46	8.4 ± 0.07	0.26 ± 0.003
SA1	8.4	56	11.6 ± 0.01	0.041 ± 0.00
SA2	8.2	74	14.8 ± 0.01	0.093 ± 0.008
ST1	8.5	60	13.6 ± 0.01	0.048 ± 0.002
ST2	8.4	82	16.3 ± 0.01	0.059 ± 0.001

SA: Sabo; ST: Station; KA: Kakuri

Table 3: Gram stain of the isolates

Isolates	Shapes	Color
SA1	Short rod (chain)	Purple (+)
SA2	Rod	Purple (+)
ST1	Short rod (chain)	Purple (+)
ST2	Short rods (chain)	Purple (+)
KA1	Rods (chain)	Purple (+)
KA2	Short rods	Red (-)

SA: Sabo; ST: Station; KA: Kakuri

Table 4. Biochemical test results of various isolates

Isolates	Oxidase	Motility	Citrates	Urease	MR	Indole	Catalase
SA1	+	+	-	-	+	-	+
SA2	+	+	+	-	+	-	+
ST1	+	-	+	-	+	-	+
ST2	+	+	+	-	+	-	+
KA1	+	+	+	-	+	-	+
KA2	+	+	+	-	+	-	+

SA: Sabo; ST: Station; KA: Kakuri; MR: Methyl Red

SA2

TTCGCCACTGGTGTTCTCCAATTCTACGATTTCACCGCTACACTCGGAGTTCACTTCCTCTTTGTACTCTGT
TGCCAGTTTCAAGTCCAGGTTGAGCCTGGGATTTCACTCACTTAACACCCCTCGCGCCTTTACGCCCATAA
TTCCGAAAACGCTGCCCCCTACGTATTACCGCGGCTGCTGGCACGAGTTAGCCGGGGCTTTTCTCAGGTA
CCGTCATTGTGCACTATGAAACTATTATTCGATCCTAACAAATTCATCATTCACGACCTGAATGCCTCAGC
TTCCCCATTGGCAACATTCCCAATTGCTGCCTCTGGAAAAATCTGCACGGGTGCCACCCCCAGGGGGGGC
CTGCTCTAACCAGGAGGAGACCCTTGAGGGCGTTACCTCTCCAACTCTTGGAGAGCCGTTACCCCGCCAA
TAGGTAATGATCTTTCCCCCTCCGTAACGACAGCTTAGCACCCTTTCCTTGAGAAATATGGAACAAAAAG

GACATCCCACTTAACTCACCCGCCCGCCACAAACTACAATATGGAGAACTCACTGGGTACTCACTCGCCC
CGTAATTCAAACGAGCTCAACTACATTCGCTCGACTTGCATGTATTACGCACGCCGCACGATCATCCTGA
CAGGATCAAAACTCT

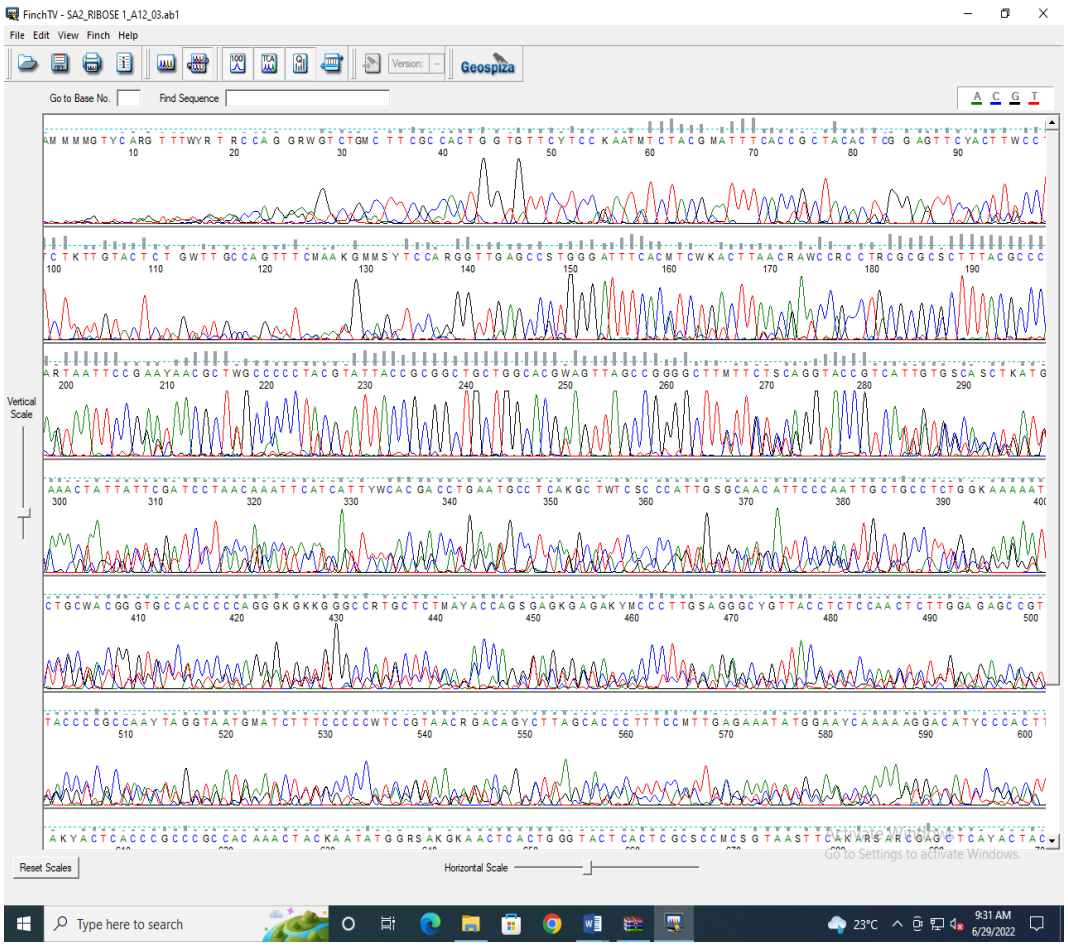

Figure 1. 16s rRNA sequence result of SA2 isolate

SA: Sabo

ST2

AAAGTCGCCTTCGCCACTGGTGTTCCTCCATATCTCTCGCATTTCACCGCTTGGAATTCCGGTTGAGCCGT
GGGCTTTCACATCAGACTTAAAAACCACCTGCGCGCGCTTTACGCCCAATAATTCCGGATAACGCTTGCC
ACCTACGTATTACCGCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCC
AGCTTATTCAACTAGCACTTGTTCTTCCCTAACAACAGAGTTTTACGACCCGAAAGCCTTCATCACTCACG
CGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGC
CGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGTGAGCCGTTA
CCTCACCAACTAGCTAATGCGACGCGGGTCCATCCATAAGTGACAGCCAAGCCGCCTTTCAATTTCGAAC
CATGCGGTTCAAAATGTTATCCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCAGTCTTATGGGCAGGTTA
CCCACGTGTTACTCACCCGTCCGCCGCTAACTTCATAAGAGCAAGCTCTTAATCCATTCGCTCGACTTGCA
TGTATTAGGCACGCCGCCAGCGTTCATCCTGAGCCAGGTCAAACTCTAAA

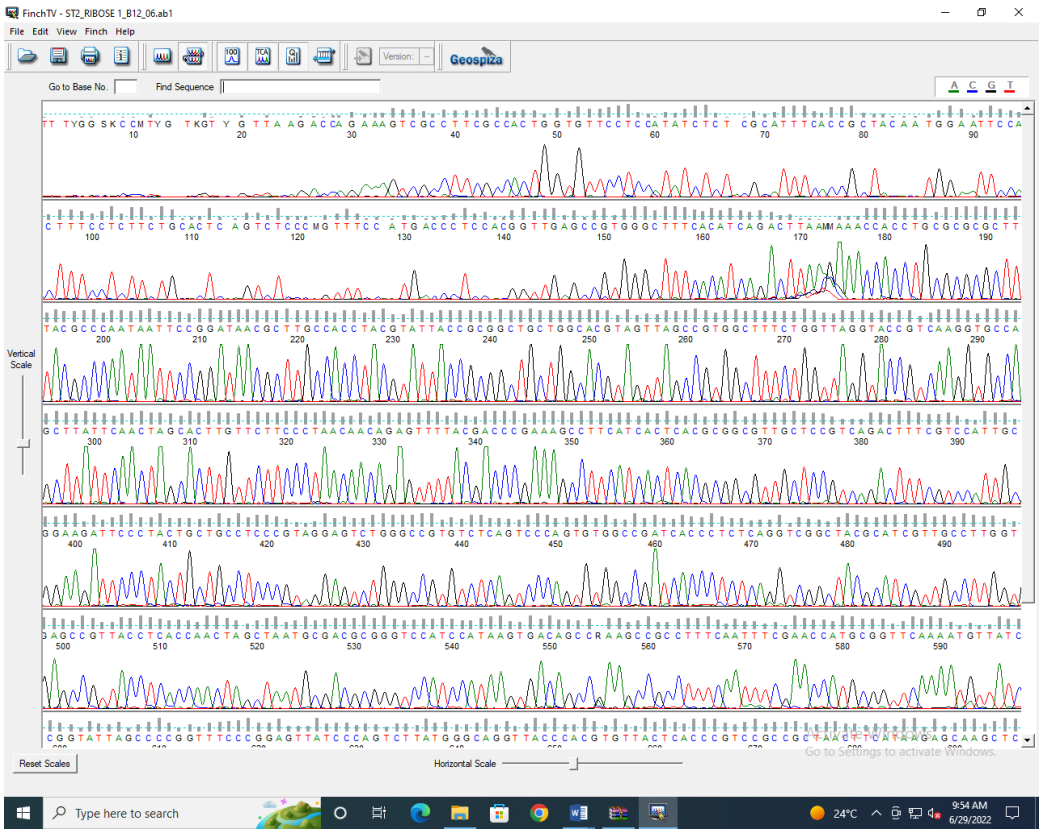

Figure 2. 16 s rRNA sequence result of ST2 isolate

ST: Station

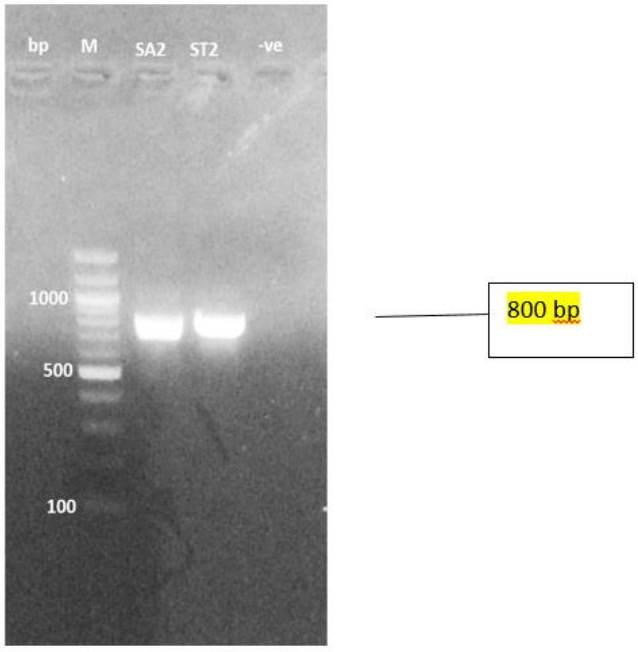

Figure 3. Agarose gel (1.3 %) electrophoresis of the PCR product of the 16S rRNA gene of the bacterial isolatse SA2 and ST2.

SA: SABO; ST: STATION

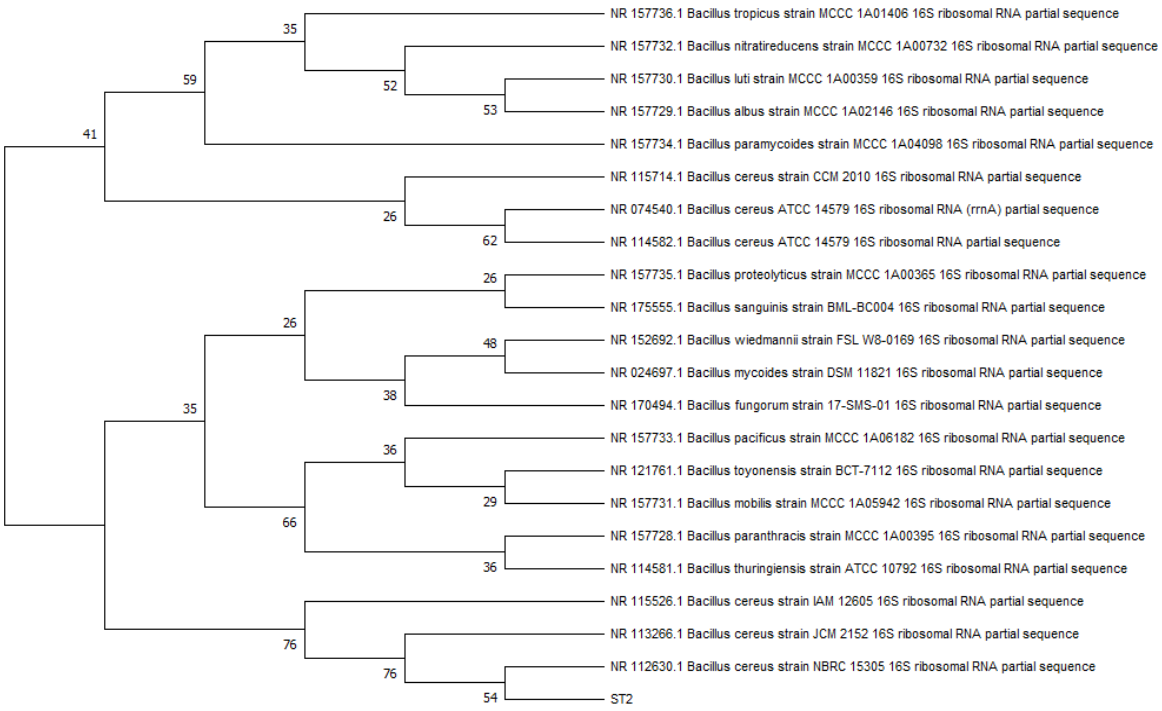

Figure 4. Phylogenetic reconstruction of the 16S rRNA gene sequence of keratinolytic bacterial isolate ST2 with MEGA software using the maximum likelihood algorithm method and 1000 replicate bootstraps.
ST: Station

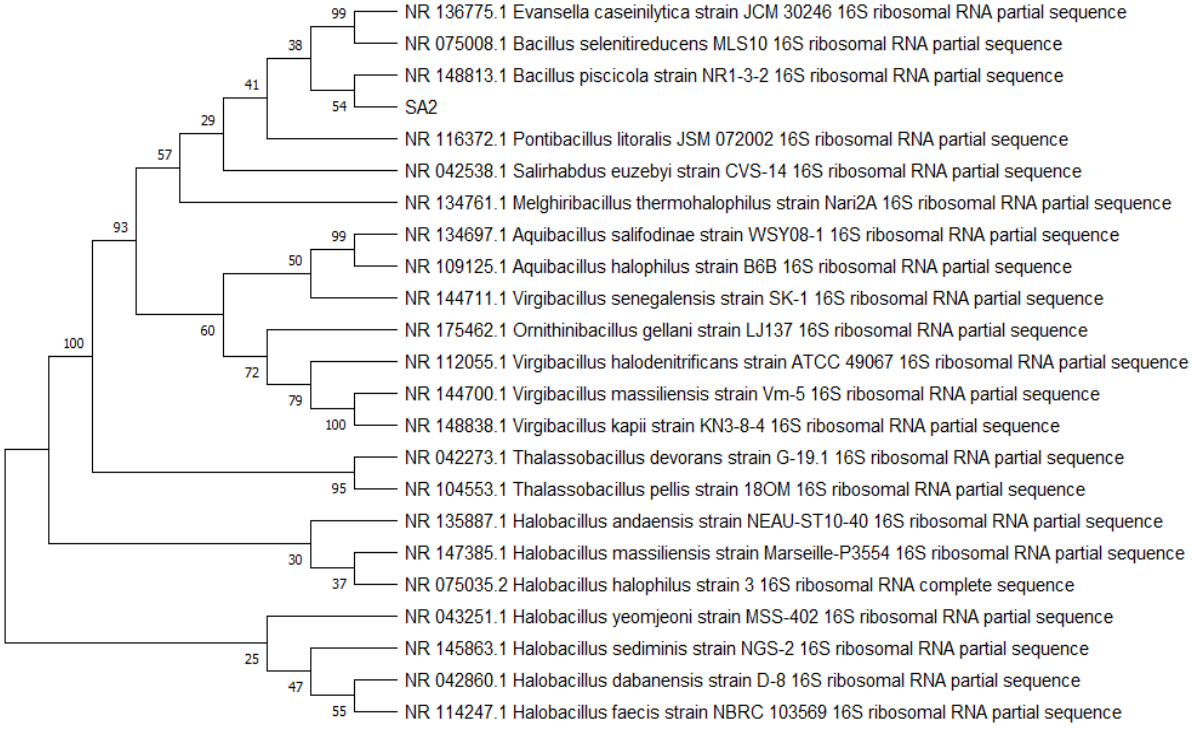

Figure 5. Phylogenetic reconstruction of the 16S rRNA gene sequence of keratinolytic bacterial isolate SA2 with MEGA software using the maximum likelihood algorithm method and 1000 replicate bootstraps.

SA: Sabo

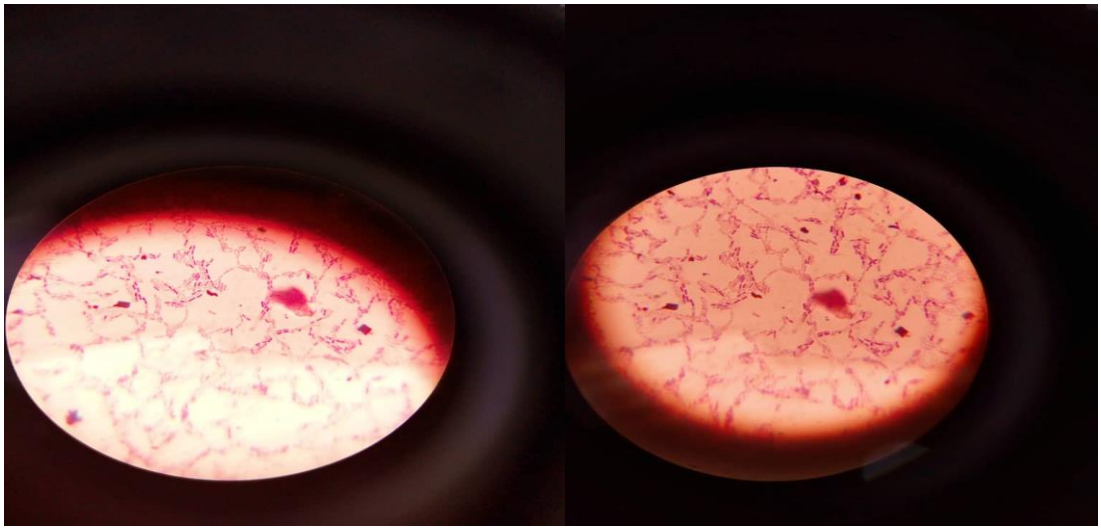

Figure 6: Micrograph of isolates SA2 and ST2
SA: Sabo; ST: Station

Figure 7: Feather before and after degradation by isolates

4. Discussion

Feathers are a rich source of keratin, an insoluble macromolecule recalcitrant to degradation by common proteases like pepsin, papain, and trypsin due to its highly supercoiled and tightly packed molecular structure stabilized by hydrogen bonding or hydrophobic interaction (Katarzyna *et al*., 2011). The global demand of poultry products has resulted in the growth of the poultry industry, and this expansion comes with the environmental

impact of accumulation of poultry wastes. The conventional methods of treating these wastes such as incineration, landfilling and alkaline hydrolysis contribute immensely to greenhouse effect, underground water contamination and degradation of some essential amino acids (Rahayu *et al*., 2015). Biotechnological approaches such as the use of microbes in the treatment of these wastes is eco-friendly and cost effective. In line with this, some bacteria strains were isolated from soil samples gotten from some selected chicken slaughter houses in Kaduna metropolis. Basal medium amended with grounded chicken feather as the source of carbon and nitrogen was used for microbial enrichment. Previous research has revealed many keratinolytic bacteria from soil samples gotten from keratinous waste dumpsites (Jaouadi *et al*, 2013). Two pure isolates each, were isolated from each soil sample from the selected locations. The bacteria isolate from soils gotten from chicken slaughter houses were screened for their keratinolytic abilities by observing their growth on chicken feather meal agar plates (CFM) containing grounded chicken feather as the only source of carbon and nitrogen. Subsequently, the isolates were subjected to whole feather degradation using basal salt medium with the initial pH of 6.0 at $37^{\circ c}$ and subsequently at $40^{\circ c}$. These bacteria were able to degrade whole chicken feather at different rates as was observed by the percentage reduction in weight of the initial whole feather as compared with the control. Also, the viscosity of the fermentation medium changed, this could be as a result of protein degradation which equally increased the pH of the fermentation medium. These observations were in line with similar research on keratinolytic bacteria from different ecological niche as reported by Tatineni *et al.* (2008) and also, Kshetri *et al.* (2016) has a similar report. Consequently, two isolates SA2 and ST2 were found to have the highest whole feather degradation with relatively higher keratinase activity. These two organisms were identified as *Bacillus piscicola* (SA2) and *Bacillus cereus* (ST2) through their 16s rRNA sequence analysis. The varied degree in the keratinolytic activity shown by the isolated bacteria could be as a result of their different optimal fermentation process conditions. And, the keratinase activity of the various isolates was based on their ability to degrade whole chicken feather with the exception of two isolates KA1 and SA1. Isolate KA1 has a relatively lower whole feather percentage degradation of 32 with keratinase activity of 16.3 ± 0.03, as compared to isolate SA1 which has a higher whole feather percentage degradation of 36 with a relatively lower keratinous activity of 9.6 ± 0.07 at 37°C as shown in table 1, however, at 40°C, isolate SA1 showed a relatively higher keratinase activity and lower whole feather percentage degradation as compared to isolate KA1. These observations could be as a result of the slight increase in temperature as previously reported by Bhange *et al* (2015). Meanwhile, the soluble protein content of the fermentation medium increased at higher temperature of 40°C across all isolates. These observed disparities could be as a result of either, the influence of the fermentation medium and environmental conditions on the keratinase production (Yusuf *et al*, 2016) or differences in the utilization of degradation products by microbes as their source of nutrient (Taskin and Kurbanoglu,

2011). It could also be that the enzyme specificity towards keratin degradation was higher in strain showing higher percentage degradation (Nnolim *et al,* 2020). The importance of optimization of the fermentation variables like pH and temperature for better output, cannot be over emphasized and the isolated bacteria showed excellent keratinolytic activities under a moderate pH and temperature conditions. This observation suggests that the isolated bacteria could be cost effective at the pilot scale production. It was also observed that the pH of the fermentation medium increased across all isolates with respect to temperature and age of the medium. This observed increase in the pH of the fermentation medium could be as a result of chicken feather degradation, which produces various proteinous molecules and/or associated metabolic by-products (Tiwary *et al.,* 2010). Moreover, it has been reported that acidic pH is more suitable for microbial keratinase production (Abdel-Fattah *et al.,* 2018). Hence, the pH changes to alkaline could be necessary for effective keratinolysis. This is because an alkaline pH has also been reported among feather degrading bacteria by Nnolim *et al* (2021) and also by He *et al* (2018).

5. Conclusion

The presence of soluble proteins in the fermentation medium indicated the effective degradation of whole chicken feather by the isolated bacteria. The ability of the bacteria isolates to effectively degrade whole chicken feather into digestible proteins showed their potentials as industrially important organisms. Moreso, the efficient conversion of the recalcitrant keratinous agro-waste biomass (Chicken feather) into digestible proteins through microbial-based technology encourages sustainability while maintaining ecological friendliness. And the bacteria specie reported in this study have shown these potentials. However, the specific gene(s) coding for the keratinase would be more useful when considering cloning and expression into vectors for larger-scale industrial keratinase production, which is beyond the scope of this study.

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