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Characterization of Keratinolytic Fungi and Bacteria Isolated From Soil-Rich Animal Wastes in Ijebu-Igbo Abattoir, South-West, Nigeria

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ABSTRACT

Massive production of keratinous wastes from agriculture and industries constitutes a major source of pollution globally. Hence, the objective of this study was to isolate keratinase producers that can be used for bioconversion of animal-rich wastes in an eco-friendly manner. Soil rich horns, hooves and feathers were collected from abattoir and Moyo poultry farms in Ijebu-Igbo and transported to Microbiology laboratory, Babcock Univetrsity. Fungi and bacteria were isolated using compounded agar medium and nutrient agar respectively. Keratinase activity of the isolates was assessed using skim milk agar. The keratinase gene in bacteria was confirmed by Polymerase Chain Reaction. Fungi and bacteria that produced better zone of inhibition on skim milk agar were sequenced using primers targeting 18S rDNA (fungi) and 16S rRNA (bacteria) genes. Fungi (18) and bacteria (4) showed hydrolytic activities on skim milk agar. However, 6 fungi (*Aspergillus flavus*, *Aspergillus pseudonomius*, *Aspergillus nomius*, *Aspergillus tamarii and Wickerhamomyces anomulus*) and 4 bacteria (*Proteus mirabilis*, *Enterobacter hormaechei*, *Morganella morganii* and *Shimwellia blattae*) were identified. The results revealed that different microbial genera capable of producing keratinases can be economically obtained and harnessed for industrial waste management. Hence, improvement of the keratinolytic organisms for industrial waste management is an asset especially in renewable energy and animal feeds.

Keyword: *Bacteria, Bioconversion, Fungi, Keratinase, keratinous wastes,*

INTRODUCTION

keratinolytic keratinolytic microorganisms are one of the most abundant and predominant organisms found in our environment and can decompose biomass wastes [1]. Keratinophilic fungi are anthropophilic, zoophilic and geophilic [2]. Most keratinophilic fungi belong to fungi imperfecti (*Chrysosporium*, *Penicillium*, *Alternaria*, *Aspergillus*, and *Doratomyces*). Some have been implicated as causative agent in human infections [3]. The distribution of keratinophilic fungi depend on the presence of animal wastes, creatinine level in the soil, pH, as well as geographical location [3]. A number of keratinolytic microorganisms other than fungi, have been reported [4-7]. Different studies have detected keratinases of various types from *B. subtilis*, *S. maltophilia* and *B. licheniformis* [8-10].

Animal wastes include feathers, horns and hooves which are solid wastes that linger for long time on earth due to their keratinous nature. Feathers are almost pure keratin protein found as waste or byproducts obtained from poultry processing plants with an annual turnover of 8×10^9 tons [11]. The cattle horns and hooves contain keratin, amino acids, peptides, lipids, nucleic acids, and microelements. Keratin is the cornified part of the epidermis of vertebrates, which includes quill, hair, nail, horn, fleece hoof and wool [11]. A great deal of substrates rich in keratin are released in the environment after the death of animals or insects [12]. The aggregation of these wastes constitutes an environmental hazard to the general public.

The role of animals in both developing and developed nations is imperative, especially for livelihood and nutrition [13]. The growing demand for animal resources necessitated a corresponding increase in its production in order to mitigate the widespread hunger and malnutrition. This growing demand in cattle meats and poultry birds is posing a significant challenge in management of associated wastes. The annual global waste generation resulting from slaughtering and other related practices are enormous, leading to environmental pollution [14]. Improper management of animal wastes can have severe consequences on the environment such as pollution issues, proliferation of rodents, creepy crawlies, pests, pathogens, groundwater contamination, and alteration of soil topography to mention a few [15]. Besides, uncontrolled application of chemicals in the agriculture and animal industries has compounded the problems with attendant consequences on the health of the population [13]. Hence, this study aimed to isolate and characterize Keratinophilic fungi and bacteria that can be used for industrial and waste management purposes.

MATERIALS AND METHODS

Sample Collection and location

Soil rich cow horns and hooves (500 g) were collected from dumps site at depth of 15 cm from Ijebu-Igbo (6°58'19.13" N 3°59'57.77" E) abattoir, while the feathers were obtained from Moyo poultry farm in Ijebu-Igbo, South-West, Nigeria. The samples were collected in clean black polythene bags and taken to the Microbiology Laboratory of Babcock University.

Isolation of Potential Organisms

One gram (1.0 g) of the scrape samples of soil-rich hooves, feathers and horns were obtained and suspended into 9.0 mL of distilled water. The samples were serially diluted up to 10^8 cfu/ml and 0.2 mL of each sample was plated unto compounded basal salt medium (g/L: $NaNO_3$, 2; NaCl, 2; K_2HPO_4, 2; $MgSO_4$, 0.5; $FeSO_4$, 0.01; $CaCO_3$, 0.1; agar-agar, 2: feather, horn or hooves powder, 2) by spread plate technique [16]. The plates were incubated for three to five days at 37°C. However, bacteria were isolated using nutrient agar (Oxoid, LTD, Basingstoke, Hamshire, England). Distinct colonies were selected and sub-cultured on nutrient agar and potato dextrose agar plates and

incubated for growth at 37°C for 24 hours and 5 days for bacteria and fungi respectively. The pure cultures were obtained and stored on agar slants at 4°C until needed.

Screening for Keratinase

Milk agar medium was used for the screening of keratinolytic organisms as described previously [17]. The medium composed of (g/L) peptone, 5.0; yeast extract, 3.0; dextrose,1.0; skim milk powder, 10.0; agar-agar 20.0, and maintained at pH at 7.2. All the ingredients of the milk agar medium were sterilized in autoclave except milk powder. The sterile milk powder was added separately after the medium has been cooled to 41°C. The medium was then poured into sterile Petri dishes. The isolates were then inoculated unto milk agar plates. The plates were incubated at 37°C. A clear zone formation on the milk agar plates after 5-7 days was observed. Only keratinolytic organisms showing clear zone formation around their growth on the milk agar medium were picked for identification.

Confirmation of Keratinase Gene by PCR

Keratinase in bacteria was screened by polymerase chain reaction. Briefly, genomic DNA was obtained by Zymo bacterial/fungal extraction kit following manufacturer's instructions. PCR mixture (25 µl) made of 12.5 µl solution of the master mix (New England Biolabs), 9.5 µl H_20, 0.5 µg 10 mM of each keratinase primers:

KerF 5'-GGTACCGAATTCGAGTCTCTACGGAAATAGC-3'

KerR5-CTAGAGGATCCGAGAATGCGCCGGAACATCAGG-3' and 2.0 µg of DNA template. Amplification was achieved using miniPCR (USA) using cycling profile: initial denaturation 94ºC for 3 min, denaturation 94ºC for 30s, annealing 55 ºC for 30s and extension 68ºC for 30s and a final extension at 68ºC for 5 min for 30 cycles. Amplicons were analyzed by agarose gel electrophoresis.

DNA Sequencing

Genomic fungal DNA obtained above was used for PCR reaction. Universal 16S rRNA and ITS (ITS 1 and 4) primers were used to amplify the conserved regions in bacteria and fungi respectively in PCR according to the protocol described above. After PCR amplification, 10.0µL products were resolved by 1.5% agarose gel electrophoresis. The amplicons were sent for sequencing on commercial basis. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [18]. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and then selecting the topology with superior log likelihood value. Evolutionary analyses were conducted using Mega7 software as described by Kumar et al. [19].

RESULTS AND DISCUSSION

Fifty (50) bacteria and 18 fungi were isolated from the samples. Of the 50 bacteria, only 4 (*Proteus mirabilis*, *Enterobacter hormaechei*, *Morganella morganii* and S*himwellia blattae*) showed keratinase activity with skimmed milk agar while 6 fungi (*Aspergillus flavus*, *Aspergillus pseudonomius*, *Aspergillus nomius*, *Aspergillus tamarii and Wickerhamomyces anomulus*) with better activity on skimmed milk agar were identified (Table 1). Diameter of inhibition zone ranged from 3 to 15 mm for isolates. Figure 1 showed the electropherogram of keratinase gene product (bacteria) on gel electrophoresis. The sequenced data of the organisms have been deposited in GenBank (accession numbers: MT431379-MT431384 and MT428324-MT428327) and their phylogenetic trees with close relatives were constructed (Fig 2 and 3). The phylogenetic analysis indicated that the bacterial isolates fell within one cluster whereas the fungal isolates clustered into three groups. The 16S rRNA of *Escherichia coli* strain LAGA3 (MH843165.1) and 28S rRNA of *Saccharomyces paradoxus* (NG_055028.1) were used as outgroup in fungal and bacterial trees respectively. A review by Janda and Abbott in 2007 [20] suggest that 16S rRNA gene sequencing provides genus identification in most cases (>90%) but less so with regard to species (65 to 83%). A more stringent boundary for species delineation was proposed to increase the accuracy of identification [21]. Pairwise nucleotide similarity values for the species (bacteria and fungi) in this study were within the acceptable range for species identification. The phylogenetic trees showed that all the bacteria and fungi obtained were similar (≥98%) to their respective species in GenBank, thus, stressing the importance of sequencing method in microbial taxonomy.

Table 1. The organisms and inhibition zone produced

S/n	Sample Code	Radius of zone (mm)	Organism	Species name	GenBank accession number
1	B3a1	15	Fungi	*Aspergillus flavus*	MT431379.1
2	B3a2	7	Fungi	*Aspergillus pseudonomius*	MT431380.1
3	5	7	Fungi	*Aspergillus tamarii*	MT431383.1
4	9	6	Fungi	*Wickerhamomyces anomalus*	MT431381.1
5	10	6	Fungi	*Aspergillus nomius*	MT431382.1
6	6	10	Fungi	*Aspergillus nomius*	MT431384.1
7	4	5	Fungi	NS	-
8	11	7	Fungi	NS	-
9	12	8	Fungi	NS	-
10	13	7	Fungi	NS	-
11	A3b1	4	Fungi	NS	-
12	B3b1	5	Fungi	NS	-
13	B3b2	4	Fungi	NS	-
14	8	4	Fungi	NS	-
15	15	5	Fungi	NS	-
16	A3a1	4	Fungi	NS	-
17	14	3	Fungi	NS	-
18	7	4	Fungi	NS	-
19	SB3	4	Bacteria	*Enterobacter hormaechei*	MT428327.1
20	SA5	4	Bacteria	*Proteus mirabilis*	MT428326.1
21	GB5	5	Bacteria	*Shimwellia blattae*	MT428325.1
22	GA3	4	Bacteria	*Morganella morganii*	MT428324.1

The inherent growth potentials of microorganisms within short period and amenability to genetic manipulation make them an ideal source for protease production. The most commercially available source of alkaline proteases is from *Bacillus* species [22]. Due to environmental pollution occasioned by human activities, there is need to source for proteases from other potential environmental microbes. Major environmental issues are created by the slaughterhouses (abattoirs) and poultry farmhouses that generates loads of waste which are accumulated in the ecosystem. This negative impact results in eutrophication, decreased species diversity and acidification of the soil [23]. Hence, the importance of keratinolytic organisms for waste removal [24].

In this study, bacterial species isolated from soil rich animal wastes where reported for the first time to our knowledge as keratinase producers; *P. mirabilis*, *E. hormaechei*, *M. morganii* and S. *blattae*. Reyes at al. [25] isolated only *Bacillus* species,

which had been known to exhibit unique physiological traits when exposed to environmental stresses such as nutrient limitation. Riffel and Brandelli [17] isolated Gram-negative bacteria (*Burkholderia*, *Chrysobacterium* and *Pseudomonas*) and a gram-positive bacterium (*Microbacterium* sp.) with kerantinase activity. Similar studies have reported potentials of some bacteria in the production of keratinases [26-27]. The phylum Proteobacteria are the prominent keratinase producers and has been reported as largest and most widespread due to their diverse metabolic activities that offer them the ability to thrive in different environments [28-30]. There is huge and untapped potentials in bacteria for industrial and waste management especially when these organisms are genetically improved for better activity. Many microbial enzymes have been used in feed supplements, fertilizers, antioxidant, bioremediation, brewing, detergent, fuel, food, leather, pharmaceutical, paper, and textile industries [11].

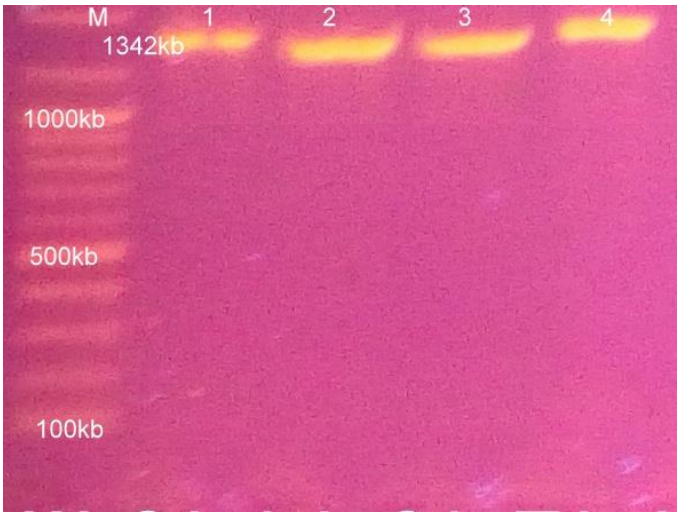

Figure 1: Electropherogram of keratinase gene product
Lane M 100bp ladder, lane 1, 2, 3 & 4: keratinase positive isolates

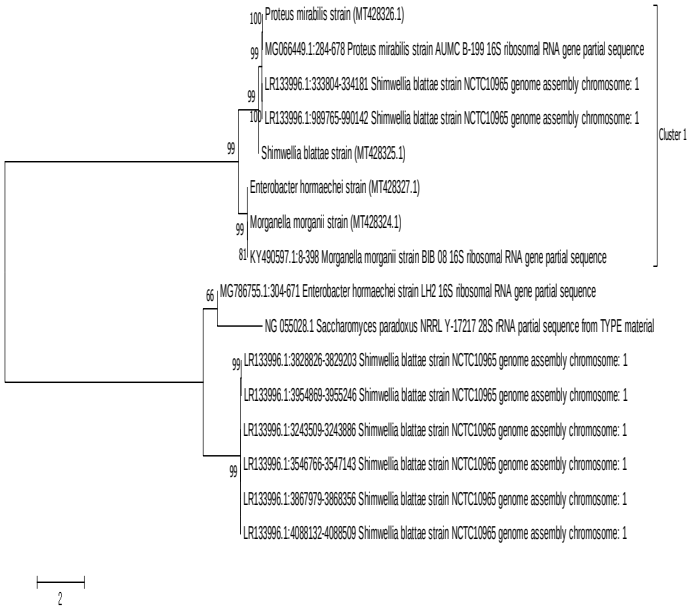

Figure 2: Phylogenic trees of the bacterial isolates showing their closest relatives in Genbank. The evolutionary history was inferred using the Neighbor-Joining method and distances were computed using the Jukes-Cantor method. Cluster 1: All the isolates were within one cluster.

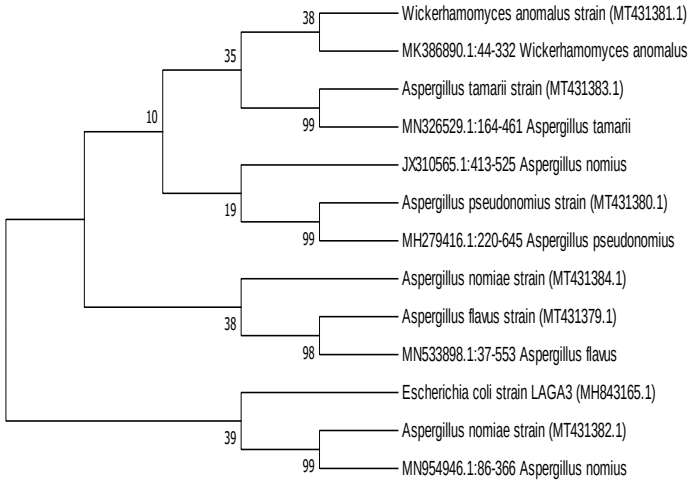

Figure 3: Phylogenic trees of the fungal isolates showing their closest relatives in Genbank
The evolutionary history was inferred using the Neighbor-Joining method and distances were computed using the Jukes-Cantor method. All the fungi were distributed with three clusters. Cluster 1: *W. anomalus*, *A. tamarii*, and *A. pseudonomius*; Cluster 2: *A. nomiae* and *A. flavus*; Cluster 3: *A. nomiae* (MT431382.1).

Fungi isolated in this study with keratinase activity were *A. flavus, A. pseudonomius, A. nomius, A. tamarii and W. anomulus* differed from the fungi obtained earlier [11] of which *Microsporum gypseum*, *Microsporum fulvum*, *Chrysosporium indicum*, *Chrysosporium tropicum* and *Sepedonium* sp were reported. However, Yazdanparast and coworkers [31] isolated *A. niger* and other fungi in parks of municipality District of Tehran and was similar to the results obtained in this study. Several reports in literature have identified similar species of fungi while some different fungi [32-36]. This variation may be due to geographical locations, sample techniques, study interest and sample size.

CONCLUSION

The results revealed that bacteria and fungi capable of producing keratinases can be economically obtained and harnessed for industrial waste management. Hence, improvement of the keratinolytic organisms for industrial waste management is an asset especially in renewable energy and animal feeds.

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