Isolation and Characterization of Keratinase Producing Organisms from Fish Scale Dumpsite at Gusau Dam, Gusau, Zamfara State, Nigeria.

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ABSTRACT

The global fishing industry, a vital source of food and economic activity, generates substantial amounts of fish scale waste, a byproduct of fish processing that poses significant environmental challenges. This waste, often discarded in landfills, contributes to environmental pollution and the depletion of valuable resources, particularly keratin, a protein with diverse industrial applications. Microbial bioconversion of the rich keratinous waste abundant in the environment provides a rich source of available protein which lurks in keratin-rich waste and this achievement will lead to a reduction in environmental pollution and improve the economy as the proteins gotten and microorganisms isolated can be utilized in the production of high-value products in both agriculture, pharmaceutical, cosmetics, detergent, textile, fertilizer, and biomedical industries. This study aimed to isolate keratinase-producing organisms from decaying fish scales. The research commenced by enriching decayed fish scales in a basal medium, followed by inoculation onto three different media: Fish Scale Agar (FSA), Starch Casein Agar (SCA), and Nutrient Agar (NA). This resulted in the isolation of 27 bacterial strains, they were subjected to proteolytic enzyme activity analysis on skim milk agar to identify the proteolytic organisms and six (6) isolates namely $SCAA_1$ (2cm), $SCAA_2$ (1.3cm), $SCAA_3$ (4cm), $SCAA_4$ (4.2cm), $FSAA_2$ (2.4cm) and $FSAA_5$ (2.2cm) showed hallow zones of different magnitude on skim milk agar. FSAA2 (68%) and SCAA1 (66%) showed highest levels of degradation of whole fish scales and these two isolates were identified as Alcaligenes faecalis (SCA A1) and Pseudomonas aeruginosa (FSA A2) using a combination of Gram's reaction, biochemical tests, and 16S rRNA gene sequencing.

Keywords Keratin, Microbial bioconversion, Fish scale, Pseudomonas aeruginosa, Alcaligenes faecalis

INTRODUCTION

Keratins are valuable protein sources for animals; however, they are insoluble proteins and cannot be digested by common proteases. They are components of a range of by-products occurring especially abundantly in slaughterhouses and meat and poultry plants: skin remains, bristle, animal hair, horns and hooves, feathers, etc. [1]. The structure of keratin is rich in disulfide bridges and sulphur compounds that make it insoluble and resistant to proteases lysis [2]. Traditional physical and chemical methods for processing keratins (high temperature, high pressure, acid or alkali treatment) result in the loss of important amino acids in keratins, consume a lot of energy and also lead to serious environmental pollution [3].

Keratinases are a subset of proteases with keratinolytic activities, mainly produced by fungi, actinomycetes and bacteria, and decompose keratins into available amino acids and peptides. However, the low yield and the purification difficulties of wildtype strains restrict the industrial application of keratinases. Microbial keratinases; which are member of proteolytic enzymes, is a group predominantly associated with keratin hydrolysis [4, 5].

Keratins are fibrous, structural and insoluble proteins that constitute the epidermis and its appendages, such as

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skin, hair, nails, hooves, horns, scales, claws, and feathers [6, 7]. Keratins are classified as α-keratin and β-keratin, based on the major secondary structural elements of polypeptides, α-helices or pleated β-sheets, respectively [6]. The conformational orientations of the cysteine residues and interactions of hydrophobic groups confer mechanical stability to the polymer against biotic and abiotic factors; thus, a major contributor for the recalcitrance of keratin to decomposition [8].

Agro-industrial wastes, especially those emanating from leather and poultry processing industries are considered to have little or no economic relevance due to their structural stability, which makes valorisation difficult. Nonetheless, several strategies have been employed to harness the-locked up potentials from keratinous wastes, and these strategies have included thermoenergetic processing, acid or alkaline hydrolysis [9]. Keratin waste valorisation with the above-indicated procedures has yielded products which have not been suitable for industrial applications [9]. Keratinases which may bioconvert keratin into peptides and amino acids, on the other hand, have not been a front runner in the valorisation of keratinous waste biomass [10].

The bioconversion of keratinous wastes into amino acids or peptides with functional values would be an attractive endeavour for several applications including animal feed formulations. The valorisation approach would represent a potentially sustainable strategy for the proper management of keratin-rich agro wastes [11] and keratinolytic microorganisms and enzymes would be beneficial from the biotechnological and industrial viewpoint. Consequently, this study evaluates the keratinolytic potentials of bacterial isolated from degrading scales of fish and also production of keratinase enzymes from the isolated organisms.

MATERIALS AND METHODS

Sample Collection

Soil samples containing decayed fish scales were collected from two dump sites at Gusau, Zamfara State whereas Mackerel Fish scale samples were collected from Iced fish sellers in Gusau, Zamfara State for keratin substrate preparation. The samples were aseptically transported to the laboratory and subsequently processed.

Keratin – Substrate Preparation

The keratin substrate was prepared from Fish scales obtained from Iced fish sellers. The scales were thoroughly washed and rinsed with distilled water, and subsequently, dried at 60 ^{0}C for 48 hrs. The dried scales were milled into fine powder with a pulveriser fitted with a 2 mm mesh and stored in an airtight container at room temperature.

Isolation of Keratinolytic Microorganism

Approximately 10 g of the soil samples containing the fish scale were inoculated in 250 mL Erlenmeyer flask containing 90 mL each of sterile basal medium with the following constituents (g/L): K_2HPO_4, 0.3; KH_2PO_4, 0.4; $MgCl_2$, 0.2; $CaCl_2$, 0.22; NH_4Cl, 0.5; Fish scale powder (FSP),10 [12] and initial pH of 6.0. The flask was incubated in a rotary shaker for 3 days at 30 ^{0}C for enrichment. For bacterial isolates, the enriched culture broth (0.1 mL) was spread plated after serial dilutions on an FSP agar plate (with the following constituents (g/L): K_2HPO_4, 0.3; KH_2PO_4, 0.4; $MgCl_2$, 0.2; $CaCl_2$, 0.22; NH_4Cl, 0.5; FSP, 10; and bacteriological agar, 15) supplemented with nystatin (50 mg/L) to inhibit fungal growth. After 24 h of incubation at 37 ^{0}C, distinct colonies were picked and subcultured. The isolates of bacteria were compared and identified according to Bergey's Manual of Systematic Bacteriology [13]. Pure isolates was maintained on Tryptone – yeast extract Broth and stored at 4˚C.

Preliminary Screening of Isolates for Proteolytic Activity

Initial screening of isolates was done by proteolytic activity assay using Milk Agar plates (with the following

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constituents in g/L, Casein enzymic hydrolysate,5; dextrose, 1; yeast extract, 2.5; sterile Skimmed milk powder, 28 and agar, 15). Bacteria were inoculated onto plates and incubated for 24hrs. Isolates with hallow zones of clearing are protease positive and were used for the succeeding steps.

Gelatinase Assay

Pre-screened isolates were stab inoculated into 10 mL Nutrient Gelatin (NG) which contained 13 g/L dehydrated Nutrient Broth and 120 g/L gelatin. Uninoculated NG was used as negative control. Tubes were incubated at room temperature for 48 hours. Upon incubation, tubes were placed in the freezer for 15 minutes and allowed to solidify. Liquefaction of gelatin was indicative of a positive result for gelatinase enzyme [14].

Catalase Test

Presence of catalase enzyme was tested on pre-screened isolates by teasing loopfuls of colonies into separate glass slides. Hydrogen peroxide was poured unto the cells using Pasteur pipette. Presence of bubbles was indicative of catalase enzyme's presence in the cells [15].

Keratinolytic Activity Screening

Microbial strains with proteolytic activity on skimmed milk agar plates were grown on a basal medium containing (g/L): K_2HPO_4, 0.3; KH_2PO_4, 0.4; $MgCl_2$, 0.2; $CaCl_2$, 0.22; and whole fish scale as the only source of carbon and nitrogen [16]. Equal amount of fish scales was added to the broth and then sterilized. Loopful of each isolate was inoculated into each of the separate fish scale broth. The submerged fermentation was carried out in triplicate using 250 mL Erlenmeyer flasks containing 100 mL working medium for 96 h at 30^0C in an orbital shaker (130rpm). Cultures were monitored for the presence of fish scale degradation. After incubation, flasks with complete or considerable decomposition of intact Fish scale were selected for further studies. The cultures were filtered, and the residual fish scale was used to determine percentage degradation. Microbial strains that displayed high keratin degrading capacity were maintained on FSP agar slants at 4 ^{0}C for the preparation of fresh inoculum. Isolates that exhibited signs of fish scale utilization would be selected and subjected also to DNA isolation, polymerase chain reaction and sequencing.

Determination of Percentage Fish Scale Degradation

Fish scale degradation by isolated microbial strains were estimated using scale weight loss approach [17]. Unutilized scales were recovered by filtering the culture broth through Whatman no. 1 filter paper. Afterwards, the residues were washed with distilled water to remove microbial cells; then dried in an oven at 60 ^{0}C for 24 h to achieve a constant weight. The dry weight of the residual fish scale was determined, and the percentage of degradation was calculated with the equation shown below.

$$\text{Percentage scale degradation (\%)} = \left(\frac{\text{1- RF}}{\text{WF}} \right) \text{X } 100$$

Where RF = dry weight of residual scales after fermentation; WF = dry weight of intact scales before fermentation.

DNA Isolation

Genomic DNA was extracted from the cultures received using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The 16S target region was amplified using OneTaq® Quick-Load® 2X Master Mix (NEB, Catalogue No. M0486) with the primers presented in Table 1. The PCR

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products were run on a gel and cleaned up enzymatically using the EXOSAP method. The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analysed on the ABI 3500xl Genetic Analyzer (Applied Biosystems, ThermoFisher Scientific) for each reaction for every sample. BioEdit Sequence Alignment Editor version 7.2.5 was used to analyse the. ab1 files generated by the ABI 3500XL Genetic Analyzer and results were obtained by a BLAST search (NCBI) [18].

Figure 1. Soil sample with degraded fish scales

RESULTS

Media	Number of isolates
SCAA	5
SCAB	3
FSAA	6
FSAB	6
NAA	4
NAB	3

Where SCA represent Starch casein agar FSA represent Fish scale agar NA represent Nutrient agar.

Figure 2. Proteolytic activity on skim milk agar of SCAA1 and FSAA2

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Table 2. Preliminary Screening of Isolates for Proteolytic Activity on Skimmed Milk

Organism	Proteolytic Activity	Zone of proteolysis (cm)
SCAA1	+	2
SCAA2	+	1.3
SCAA3	+	4
SCAA4	+	4.2
SCAA5	-	0
SCAB1	-	0
SCAB2	-	0
SCAB3	-	0
SCAB4	-	0
FSAA1	-	0
FSAA2	+	2.4
FSAA3	-	0
FSAA4	-	0
FSAA5	+	2.2
FSAA6	-	0
FSAB1	-	0
FSAB2	-	0
FSAB3	-	0
FSAB4	-	0
FSAB5	-	0
FSAB6	-	0
NAA1	-	0
NAA2	-	0
NAA3	-	0
NAA4	-	0
NAB1	-	0

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NAB2	-	0
NAB3	-	0

Where SCA represent Starch casein agar isolates

FSA represent Fish scale agar isolates

NA represent Nutrient agar isolates

\+ (positive) results indicates presence of hallow zone on Skimmed Milk Agar.

\- (negative) result indicates absence of hallow zone on Skimmed Milk Agar.

Table 3: Percentage fish scale degradation

Isolates	**WF(g)**	**RF(g)**	**% Degradation**
SCAA1	0.5	0.17	66
SCAA2	0.5	0.18	64
SCAA3	0.5	0.22	56
SCAA4	0.5	0.20	60
FSAA2	0.5	0.16	68
FSAA5	0.5	0.20	60

Where SCA represent Starch casein agar isolates

FSA represent Fish scale agar isolates

NA represent Nutrient agar isolates

RF = dry weight of residual scales after fermentation;

WF = dry weight of intact scales before fermentation.

Table 4: Grams Reaction and Biochemical Test

Test performed	**SCAA1**	**FSAA2**
Grams reaction	- rods	- rods
Gelatinase	-	+
Catalase	+	+
Spore test	-	-
Hydrogen sulphide	-	-

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Indole	-	-
Oxidase	+	+
Methyl Red	-	-
Voges – Proskauer	+	-
Citrate utilization	+	+
Urease	-	-
Glucose	-	-
Sucrose	-	-
Lactose	-	-
Motile	+	+
P/O	Alcaligenes spp	Pseudomonas spp

Where: + = indicates positive results.

- = indicates negative result which shows absence of hallow zone on Skimmed Milk Agar.

P/O = presumptive organisms.

Annotation of ladder used in gel

Fig 3: A photographic image of an agarose gel indicating the amplification of 16S target region.

Table 5: BLAST prediction

S/no	**Sample ID**	**Organism**	**Sequence length (bp)**	**% identity**	**Accession no of BLAST hit**	**E-value**	**Alignment score**	**Highest query coverage (%)**
1	FSAA2	Pseudomonas aeruginosa	1508	98.14%	JF513146.1	0.0	≥200	100%
2	SCAA1	Alcaligenes faecalis	1494	97.00%	AY548384.1	0.0	≥200	100%

>FSAA2

TAGAGTTGAGTTTCAATCATGGCTCAGATTGAACGCTGGCGGCAGACCACACACATGCAAGTCGAT
CGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGTCTAGGAATCTGCCT
GATAGAGGGGGATAACGTCCGGAAACGGTCGCTAATACCGCATACGTCCTGAGGGAGAAAGTGGG
GGATCTTCGGACCTCACGCCATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGTAAAGGCC

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TACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGT
CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCAT
GCCGCGTGTGTGAAGAAGGCCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGT
TAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCG
GTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGC
AAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTGGCAAGCTAGAGTAC
GGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTG
GCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGAT
TAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCGGTGGGATCCTTGAGATCTTTGTG
GCGCAGCTAACGCGATAAGTCCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAA
TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC
CTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCAGACACAGGT
GCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCT
TGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAA
GGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCG
GTACAAAGGGATGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGC
AGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTRAATCAGAATGYYACGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAGTAGCTA
GTCTAACCGCAAGGGGGACGGTACCCACGGAGTGATTCATGACTGGGGTGAAGTCGTAACAAGGT
AACCGTAA

>SCAA1

GAGTTTGATTATGGCTCAGATTGAACGCTAGCGGGATGCTTTACACATGCAAGTCGAACGGCAGCG
CGAGAGAGCTTGCTCTCTTGGCGGCGAGGGGCGGACGGGTGAGTAATATATCGGAACGTGCCCAG
TAGAGGGGGATAACTACTGGAAAGAGTGGCTAATCCCGCATACGCCCTACGGGGGAAAGGGGGGG
ATCGCAAGCCCTCTCACTATTGGAGCGCCCGATATCGGATTAGTTAGTTGGTGGGGTAAAGGCTCA
CCAAGGCAACGATCCGTAGCTGGTTTGAGAGGACGACCAGCCCCACTGGGACTGAGACACGGCC
CAGACTCCTACGGGAGGCAGCAGGGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATC
CCGCGTGTATGATGAAGGCCTTCGGGTTGTAAAGTACTTTTGGCAGAGAAGAAAAGGTATCTCCTA
ATACGACATACTGCTGACGGTATCTGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGT
AATACGTGGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGTCTAGGCGGTTCGGAAA
GAAAGATGTGAAATCCCAAGGCTCACCCTTGGAACTGCATTTTTAACCCGAGCTAGAGTAGTCAG
AGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGGCGAA
GGCAGCCCCCTGGGATAATACTGACGCTCAGACACGAAAGCGTGGGGAGCAAACAGGATTAGATA
CCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGCTGTTGGGGCCGTTRRGSCTTAGTATCGCAG
CTAWCGCGTGAAGTTGACCGCCTGGGGAAACCGGCCCAAGAGTAAAAATCAAAGGAATTGACGG
GGACCCCCACAAGCGGTGGATGATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTT
GACATGTCTGGAAAGTCGAAGAGATTTGGTCGTGCTCTCAAGAGAACCGGAACACAGGTGCTGCA
TGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCAT
TAGTTGCTACGCAAGAGCACTCTAATGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GACAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGCCATACAATGGTCGGGACAGAGGGTC
GCCAACCCGCCAGGGGGAGCCAATCTCAGAAACCCGATCGTATTCCGGATCGCAATCTGCAACTC
AACTGCGTGAAGTCGGAATCGATAGTAATCGCGGATCAGAATGTCGCGGTGAATACGATCCCGGGT
CTTGTACACACCGCCCGTCCCACCTGGGAGTGGGTTTCACCAGAAGTAGGTAGCCTAACCGTAAC
GAGGGCGCTTACCACGGTGGGATTCATGACTGGGGTGAAGTCGAAACAAGGTAACCGA

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DISCUSSION

This study aimed to isolate and characterize keratinolytic organisms from fish scale dumpsites at Gusau Dam, Gusau, Zamfara State. The results demonstrate the successful isolation and identification of keratinolytic bacteria from this environment, highlighting their potential for bioremediation of keratinous waste.

The initial step involved the isolation of bacteria from soil samples collected from the fish scale dumpsites. The use of various media, including Starch Casein Agar (SCA), Fish Scale Agar (FSA), and Nutrient Agar (NA), allowed for the cultivation of a diverse range of bacterial species. It was observed in the first table that higher number of isolates were obtained on SCA and FSA compared to NA and this suggests that the fish scale environment supports the growth of bacteria capable of utilizing keratin and other complex organic substrates. This is consistent with the findings of other studies, such as those by Reyes [19] and Nnolim [20], who also reported the successful isolation of keratinolytic bacteria from poultry waste dumping sites and agro-waste dumpsites, respectively.

The isolated strains were screened for proteolytic activity using Skimmed Milk Agar which identified bacteria capable of producing proteases, enzymes that break down proteins. The results in Table 2 indicated that a significant number of isolates exhibited proteolytic activity, as evidenced by the formation of clear zones around the bacterial colonies as shown in Figure 2. This finding is in line with the observations of other researchers, such as Wibowo et al., [21], who also employed Skimmed Milk Agar for the screening of keratinolytic bacteria from puffer fish skin waste.

Further evaluation of the proteolytic activity was conducted by measuring the percentage of fish scale degradation by the selected isolates. The results in table 3 showed that isolates SCAA1, SCAA2, SCAA3, SCAA4, FSAA2, and FSAA5 exhibited significant fish scale degradation ranging from 56% to 68%. This suggests that these isolates have potential for breaking down keratinous waste, which could be beneficial for bioremediation efforts. These findings are comparable to those reported by Abdel-Fattah et al., and Nnolim et al., [22,23], who documented feather degradation rates of up to 63% and 85%, respectively, using keratinolytic bacteria.

The selected isolates were subjected to biochemical tests to determine their physiological characteristics. The results revealed that the isolates SCAA1 and FSAA2 displayed similar biochemical profiles, indicating their potential taxonomic similarity. The identification of these isolates as Alcaligenes spp. and Pseudomonas spp., respectively, further confirmed through 16S rRNA sequencing in table 5. This approach provides a reliable and accurate method for bacterial identification, as demonstrated in other studies;[19 and 20].

The findings of this study align with previous research on keratinolytic bacteria. Similar to other studies [22,17,23], the current research highlights the presence of keratinolytic bacteria in various environmental niches, particularly those enriched with keratinous waste. The use of similar techniques, such as the screening of isolates on Skimmed Milk Agar and the assessment of keratin degradation, underscores the consistency of these methods for identifying and evaluating keratinolytic potential.

This study contributes valuable information regarding the diversity and potential of keratinolytic bacteria in fish scale dumpsites. The identification of Alcaligenes spp. and Pseudomonas spp. as efficient keratin degraders highlights their potential for bioremediation applications. The demonstrated ability of these bacteria to break down keratinous waste highlights their potential to significantly reduce environmental pollution and promote sustainable waste management practices. Furthermore, the study's findings can be utilized for the development of novel biotechnological processes, such as the production of keratinase enzymes for industrial applications, including leather processing, food processing, and bioremediation.

CONCLUSION

This study successfully isolated and characterized two keratinolytic bacteria Pseudomonas aeruginosa and Alcaligenes faecalis from fish scale dumpsites, demonstrating their potential for bioremediation and biotechnological applications. The findings contribute to the understanding of microbial diversity in waste environments and highlight the potential of these keratinolytic bacteria for sustainable waste management and the development of novel biotechnological processes.

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