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Screening and Identification of Keratin-degrading Microorganisms Isolated from Different Chicken-feather Waste Dumpsite Soils in Ilorin Metropolis, Nigeria

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ABSTRACT: The eradication of keratin waste substrate in environment is of great concern, hence the need to look for an ecofriendly way of getting rid of them. Hence, the objective of this paper was to isolate, screen and identify keratin degrading microorganisms from soil samples collected from different chicken feather dumpsites in Ilorin metropolis, Kwara State, Nigeria using appropriate standard methods. A total of thirteen (13) microorganisms were purified and screened for proteolytic activity, seven (B1, B2, B3, B4, B5, F1 and F3) showed appreciable zone of clearance and were further used in keratinase activity determination over 21days, after which percentage chicken feather weight loss was determined. B1 showed highest keratinase activity of 42.48 U/ml at day 7, B2 40.88 U/ml at day 9, B3 24.32 U/ml at day 11, B4 49.36 U/ml at day 9, B5 19.36 U/ml at day 7, F1 45.52 U/ml at day 11 and F3 45.04 U/ml at day 13. All isolate except B5 had the least activity at day 1. Percentage chicken feather weight loss after 21 days revealed that B1, B2, B3, B4, B5, F1 and F3 had 49, 38, 6, 64, 9, 39 and 57 respectively. Molecular identification of best keratinase producers (B1, B2 B4 F1 and F3) revealed the isolates to be *Pseudomonas aeruginosa*, *Bacillus cereus*, *Alcaligenes faecalis*, *Aspergillus niger* and *Aspergillus aculeatus* respectively. These organisms have shown they are good keratinase producers and should consider in the large-scale degradation of chicken feather pollutant.

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The steady increase in global population has also led to increase in consumption of animal protein, being one of the cheapest and healthiest sources hence resulting in high feather generation in the environment (Das *et al*., 2024). According to de Q. Souza (2023), about 8.5 million tons of chicken feather wastes are generated globally on a yearly basis. Knowing that decomposition of chicken waste is difficult owing to its recalcitrant nature, its accumulation and management in the environment is considered a challenge (Chaturvedi *et al*., 2021). Feathers are made up about 92% of keratin, a protein that includes different amino acids like cystine, glutamine, lysine, proline, serine, threonine, and

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valine (Gupta *et al*., 2023). Different methods such as chemical hydrolysis, incineration and land filling have been used to eliminate feather waste, many of which are not ecofriendly and, in some cases, led to environmental pollution (Maurya and Singh, 2024). The use of biological means to degrade feather has been reported to be the only eco-friendly, cost effective and also with useful byproduct alternative method (Derhab, El-Metwally *et al*., 2022).

A proteolytic enzyme known as keratinase which is produced by microorganisms has been most efficient in degradation of keratin substrates in the environment (Wang *et al*., 2023). Among the microorganisms reportedly producing keratinase includes bacteria of genera Bacillus, Micrococcus, Microbacterium, and Pseudomonas, actinomycetes of genera Streptomyces and Thermoactinomyces, and fungi of genera Alternaria, Aspergillus, Doratomyces, and Rhizomucor (Srivastava *et al*., 2020) among others. Some microbes can simultaneously produce extracellular and intracellular keratinases because they are immobilized on the cell surface and are simple to use in waste treatment, cell-bound keratinases are also identified, and this type of enzyme may be of significant interest for industrial applications as well (Li, 2021).The objective of this paper is to isolate, screen and identify keratin degrading microorganisms from different feather dumpsites in Ilorin metropolis.

MATERIAL AND METHODS

Sampling and Collection of control keratinase producer: Soil and feather samples were aseptically collected from five (5) different feather dumpsites across Ilorin metropolis, Kwara state. A chemically competent *E. coli* (BL21) cell suitable for transformation and protein expression, and in production of keratinase enzyme purchased from Sigma Aldrich, USA was used as control.

Isolation of feather Keratin degrading microorganisms: Microorganisms used in this study was isolated using the method described by Lateef *et al.* (2015) with slight modification. Enrichment was carried out by dissolving 10 grams of soil in 90 mL of minimal basal medium containing feather powder as the sole carbon source. This was in an incubator shaker at 30 ^{o}C for 72 hours. 1 mL from the incubated mixture was 10 folds serially diluted up to 10^{-6}. The 1 mL from the 10^{-3} tube was plated on potato dextrose agar for fungi growth and incubated at room temperature for 72 hours while 10^{-6} was plated on Nutrient agar for bacteria growth and incubated for 24 hours. Growths observed were purified and stored at 4 ^{o}C for further use. Media used were produced by Hi Media, UK and were prepared according to manufacturer's instruction.

Screening of isolates for Proteolytic activity: This was carried out using skim milk agar (SMA) medium containing in g/L; skim milk powder (28), Tryptone (5), Yeast extract (2.5), Glucose (1), Agar (15) and final pH (at 25 °C) 7.0±0.2. bacteria and fungi isolated previously were inoculated onto a freshly prepared SMA by spot inoculation. Plates were incubated at 37 ^{o}C for 24 hours for bacteria and at room temperature for 72 hours for fungi. The appearance of clear zone around the growth indicates protease production and tendency to degrade keratin substrate (Hassanein, 2022).

Biochemical and molecular identification of Isolates: For bacterial isolates, morphological characterization, Gram staining, motility, indole catalase, methyl red, Voges Proskauer, citrate, urease, oxidase and sugar fermentation tests were all carried out (Agbabiaka *et al*., 2020). For fungal isolates, cultural and microscopic examinations of isolates were carried out. This was done to avoid duplication of isolates in subsequent analysis. Molecular identification was carried out on isolates with best keratinase activities.

*Keratinase enzyme production:*Enzyme production was carried out as described by Ramalingum *et al*. (2022) with slight modification. Standardized inoculum (0.5 MacFarland) of different isolates were inoculated into flask containing 100ml of minimal basal medium (g/L) containing: Feathers (10), $MgCl_2.6H_2O$ (0.1), NaCl (0.5), NH_4Cl (0.5), K_2HPO_4 (0.4), The above flasks were then incubated at 37 °C for 72 hours (bacteria) and 25 °C for 168 hours (fungi) on rotary shaker. All the above incubated media were then centrifuged at 5000 rpm for 15 minutes. The supernatants were then used for crude enzyme preparation.

Keratinase activity: Keratinase activity was determined using the method of Cheng *et al*. (1995) with modification. About 1 mL of crude enzyme was reacted with 1ml keratin solution in water bath at 50 °C with shaking for 10 minutes. The control experiment consisted of consisted of the keratin solution only. The reaction was then stopped by the addition of an equal volume of 20% trichloroacetic acid. The reaction mixture was centrifuged at 5 000 rpm for 15 minutes to remove the precipitate keratin and the optical density of the supernatant was measured at 280 nm. One unit of keratinase activity was defined as an increase in the corrected absorbance (A_{280}) by 0.01 units with respect to

control, per ml per minute under the condition described above.
It was calculated as: $U = 4 \times n \times A_{280} / (0.01 \times t)$

Where n is the dilution rate; 4 is the final reaction volume (ml); t is the incubation time (min).

Feather degradation rate: The feather degradation rate was evaluated by weight loss method previously described by Jain and Agrawal (1980). One gram of feather was added to 100 mL of degradation medium (minimal basal medium) as the sole carbon source. After incubation, medium was filtered through filter papers, and the feather residue was thoroughly washed with distilled water and dried and weighed to calculate weight loss. Results were expressed as percentage weight loss relative to the initial dry feather weigh

Percentage (%) weight loss = {(initial weight – final weight)/initial weight} x 100

RESULTS AND DISCUSSION

Enumeration and Screening of Isolates for Proteolytic activity: The total bacterial count (TBC) of the five (5) different dumpsite soil samples A, B, C, D, E ranges from 61.00 ± 11.8 to 154.67 ± 16.0 ($x10^6$ CFU/g). The total fungal count (TFC) ranges from 9.33 ± 3.2 to 24.00 ± 2.6 (x104 CFU/g) as shown in Table 1. A total of 13 isolates (8 bacteria and 5 fungi) were screened, 7 (5 bacteria and 2 fungi) were observed to have pronounced clear zones around them indicating proteolytic activity (Table 2) with no significant difference from the proteolytic activity displayed by control *E. coli* BL21, which according to Lai *et al*. (2023) is a great potential for hydrolyzing keratin due to the fact that keratinases are a class of proteolytic enzymes, although not all proteolytic enzymes will hydrolyze keratin.

Table 1: enumeration of microorganism

Dumpsite	TBC ($x10^6$ CFU/g) Mean ± SD	TFC ($x10^4$ CFU/g) Mean ± SD
A	92.67 ± 8.6	20.33 ± 9.5
B	117.33 ± 20.0	16.67 ± 5.7
C	104.67 ± 23.9	24.00 ± 2.6
D	61.00 ± 11.8	9.33 ± 3.2
E	154.67 ± 16.0	18.67 ± 6.0

Characterization and Identification of Bacterial Isolates: A result of five (5) bacterial isolates characterized and identified based on their morphology, biochemical characterization and Gram staining is presented in Table 3 while the cellular identification of fungi is presented in Table 4.

Table 2: screening for proteolytic activity

Isolate	Mean Zone of Clearance ± SD (mm)
B1	11.1 ± 2.1^{c}
B2	11.9 ± 1.1^{c}
B3	6.6 ± 0.4^{bc}
B4	12.2 ± 1.1^{c}
B5	8.7 ± 0.0^{bc}
B6	0.0 ± 0.0^{a}
B7	4.4 ± 0.4^{b}
B8	0.0 ± 0.0^{a}
F1	11.9 ± 0.3^{c}
F2	0.0 ± 0.0^{a}
F3	13.8 ± 0.8^{c}
F4	5.1 ± 0.8^{b}
F5	0.0 ± 0.0^{a}
Control *E. coli* (BL21)	14.3 ± 0.5^{c}

Values are presented as mean and standard deviation. The values without the same superscript are significantly different ($P < 0.05$).

Table 3: biochemical identification of isolates

Isolate	Morphological Identification					O	C	Ci	In	MR/ VP	U	Sugar fermentation				Shape/ Gram Stain
	Si	Sh	Co	Op	Con							G	L	M	S	
B1	Sm	R	Gr	Tp	M	+	+	+	-	-/-	-	-	-	-	-	Rods/-
B2	La	Ir	Cr	Tl	Bt	+	+	+	-	-/-	-	-	-	-	-	Rods/-
B3	Sm	R	Cr	Tl	D	-	-	+	-	-/-	-	A	-	-	-	Rod/+
B4	La	Ir	W	Tl	Bt	-	+	+	-	-/+	-	AG	-	AG	AG	Rods/+
B5	La	Ir	Cr	Tp	Bt	-	+	-	+	+/-	-	AG	AG	A	AG	Rods/-

Key: Si = Size; Sh = Shape; Co = Colour; Op = Opacity; Con = Consistency; Sm = Small; La = Large; R = Round; Ir = Irregular; Gr = Greenish; Cr = Cream; W = White; Tp = Transparent; Tl = Translucent; Mu = Mucoid; Bt = butrycious; D = Dry; O = Oxidase; C = Catalase; Ci = Citrate; In = Indole; MR = Methyl red; VP = Voges Proskauer; U = Urease, G = Glucose; L = Lactose; M = Maltose; S = Sucrose; A = Acid; AG = Acid/Gas production; + = Positive; - = Negative

Keratinase activity: The keratinase activity obtained from the 7 isolates after 21 days incubation revealed peak activity for all isolates between day 9 and 11. The control setup was done sui g the control *E. coli* BL21. The keratinase activity in this study was carried out on day 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 and 21 using the seven (7) isolates (B1, B2, B3, B4, B5, F1 and F3) that showed good proteolytic activity with a control set up without inoculum. For B1 and B4, highest activities of 42.48 U/ml and 49.36 U/ml were obtained at day 5, B2, B3 and B5 had highest activities of 40.88 U/ml, 24.32 and 19.36 on day 7.

For isolate F1, the highest activity 48.52 U/ml was obtained at day 9 while F3 showed highest activity of 45.04 U/ml at day 11. Control organism had highest activity of 102.96 U/ml at day 3. Least activities by all isolates were obtained after 24 hours (day 1) incubation. Generally, highest keratinase activities were mostly obtained at days 5 and 7 for bacterial isolates while at days 9 and 11 for fungi (Figure 1). In accordance with this result was the study conducted by Chauhan and Devi (2020) who reported similar trend in the keratinase production. The results reveal Bacillus cereus N14 showed maximum keratinase production of 19.56 U/mL at day 3 of incubation after which there was a decline in the enzyme produced with minimum enzymatic activities of 3.26 U/mL observed at day 13 of incubation. The results obtained shows that incubation period have significant impact on keratinases production. The decline in enzyme output may have resulted from the constant depletion of nutrients from the production medium, which could have caused microbial cells to die quickly and uniformly (Benavente-Valdes *et al*., 2016). The reduced enzyme activity as a result of prolonged incubation may also be as a result of irreversible adsorption of enzyme to substrate or feedback inhibition/denaturation of enzymes, resulted from the lesser cellular metabolism with time during fermentation (Zhao *et al*., 2024). In this present study, the low activity obtained from fungal isolates in the first 5 days of incubation and may be due to the lag phase of the fungal growth where not much metabolic or enzymatic activity is observed. The slow declination in enzyme activity observed after day 13 could also be as a result of depleting nutrient hence death of fungal cell. Supporting this result was a report by Bohacz and Korniłłowicz-Kowalska (2019) who noted that keratinase enzyme activity by selected fungi isolates were at their peak after 14 days incubation.

Feather degradation rate: The weight of feather before and after 21 days incubation period was obtained as recorded (Table 5). The result obtained revealed that isolate B4 had the best percentage feather loss rate (69%) with B1, B2, F1 and F3 all also showing a good rate as compared to other isolates used in this study after 21 days incubation (Table 5). Control *E. coli* BL21 showed a massive percentage feather loss of 79%. A higher native feather decomposition rate was observed from the study of Bohacz, *et al*. (2020) who reported rates as high as 70% by *Aphanoascus keratinophilus* and *Chrysosporium tropicum*. Kumar *et al*. (2015) obtained a similarly high percentage of feather weight loss induced by *C. keratinophilum* and *C. tropicum* (78% and 82%, respectively) already after 30 days.

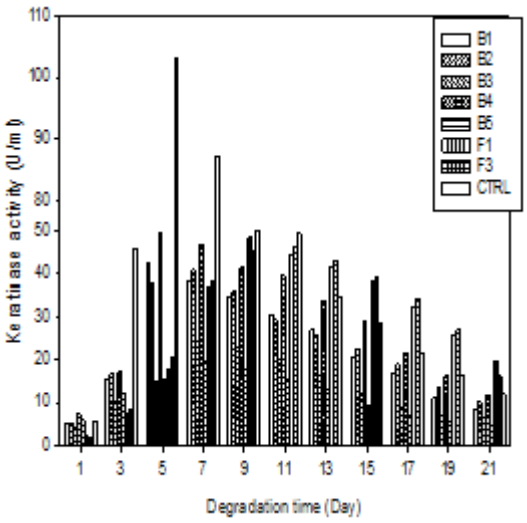


Fig 1: Keratinase activity of isolates after 21 days degradation

Table 4: Feather degradation rate

Isolate	Initial feather weight (g)	Final feather weight (g)	Percentage loss (%)
B1	1	0.51	49
B2	1	0.62	38
B3	1	0.94	6
B4	1	0.36	64
B5	1	0.91	9
F1	1	0.61	39
F3	1	0.43	57
E. coli BL21	1	0.21	79
CTRL (No inoculum)	1	0.98	2

Molecular identification of isolates: Five isolates (B1, B2, B4, F1 and F3) with best keratinase activity and feather degradation rate was identified using molecular technique. They were revealed to be *Pseudomonas aeruginosa*, *Alcaligenes faecalis*, *Bacillus cereus*, *Aspergillus niger* and *Aspergillus aculeatus.* Several researchers have reported some of the microorganisms to be good keratinase producers. Ramalingum *et al*. (2022), Dhiva *et al*. (2020), Moonnee *et al.* (2021) among others all reported *P. aeruginosa* has a keratinase producer. For *A. faecalis*, not much has been reported about its used for keratinase production although Yusuf *et al.* (2019) and Obinna (2023) reported its use in the production of keratinase. Significantly high numbers of researchers have reported production of keratinase from different species of Bacillus (Mazotto *et al*., 2022; Zhang *et al*., 2022; Chen *et al*., 2023). Aspergillus species have also have gained a lot of attentions in the area of keratinase production like Bacillus with researchers El-Ghonemy and Ali (2021), Preczeski *et al.* (2020) and many others

reporting their use, however, limited worked has been reported by *A. aculeatus*.

Conclusion: Among all the isolates tested, *P. aeruginosa*, *A. faecalis*, *B. cereus*, *A. niger* and *A. aculeatus* all showed high tendencies to degrade chicken feather keratin waste based on their keratinolytic activities as well as the feather weight loss results obtained from the study. These organisms were identified using molecular identification technique which involved DNA extraction and polymerase chain reaction. The genera of these identified microorganisms in relation to keratin degradation have previously reported, although not much have been reported on *A. aculeatus* as a good keratinase enzyme producer. It is therefore recommended that further research be carried out on this fungus to unveil more potential as regards keratinase producer as well as keratin substrate degradation.

Declaration of Conflict of Interest: The authors declare no conflict of interest.

Data Availability: Data are available upon request from the corresponding author.

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