EXPERIMENTAL ARTICLES

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ISOLATION AND IDENTIFICATION OF TANNASE-PRODUCING *Aspergillus* spp. FROM POULTRY DROPPINGS

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Aim. Tannases have found application in many industries. *Aspergillus* species are moulds largely isolated from poultry droppings and are major producers of tannase. This study aimed to isolate and identify tannase-producing *Aspergillus* spp. from poultry droppings.

Methods. Samples of poultry droppings were obtained from a poultry farm in Kuje Area Council, Federal Capital Territory, Abuja, Nigeria. *Aspergillus* spp. was isolated according to standard microbiological procedures. The ability of the isolated *Aspergillus* spp. to utilise tannic acid was assessed through a tannase assay using a standard method. The isolated *Aspergillus* spp. were identified using morphological characteristics and molecular identification methods.

Results. Twenty-one pure fungal isolates were obtained from the poultry droppings, and 17 of them were able to utilise and grow on constituted tannic acid agar with a diameter ranging from 3.5–7.0 cm. Five (5) isolates with the highest tannase activity were identified as *Aspergillus fumigatus* and *Aspergillus flavus*.

Conclusion. The study concluded that tannase-producing *A. fumigatus* and *A. flavus* can be obtained from poultry droppings and may be exploited for tannase production.

Keywords: tannase, *Aspergillus* spp., morphological identification, molecular identification.

Tannase (tannin acyl hydrolase, EC 3.1.1.20), an extracellularly induced hydrolase, catalyses the degradation of hydrolysable tannins and esters of gallic acid to produce value-added compounds like galloyl ester, glucose, and gallic acid [1, 2]. Tannase is generally employed in the industrial bioconversion of tannic acid to gallic acid [3, 4]. Fungi majorly produce tannase. *Aspergillus* and *Penicillium* spp. constitute about 58% of the tannase-producing fungi. Between the two genera, *Aspergillus* spp. is the larger tannase producer [5].

Aspergillus spp. play a critical role in the degradation of organic substrates and have been reported to secrete a number of biologically active compounds, including antibiotics and mycotoxins [6, 7], as well as essential enzymes [8, 9].They have been found mainly in poultry droppings. Residual nutrient and organic matter content of poultry droppings makes it moist, thus enabling mould to sporulate [10]. The sporulated mould is known as *Aspergillus*, and the two most observed species of *Aspergillus* present in poultry dropping are

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Aspergillus fumigatus and *Aspergillus flavus* [11, 12]. Morphological characterization encompasses both macroscopic and microscopic characteristics [13, 14]. Molecular methods of identification have become more effective due to the problems associated with overlapping morphological features of organisms [15, 16]. This study, therefore, aims to isolate and identify tannase-producing *Aspergillus* spp. from poultry droppings.

Materials and Methods

Collection of Samples: 5 g of poultry droppings was collected from a local poultry farm in Kuje Area Council, FCT, Abuja, Nigeria.

Isolation of Tannase-producing Fungi

Isolation of *Aspergillus* spp. was carried out according to standard microbiological procedures described by Al-Temimay *et al*. [17]. 1g of the sample was used to carry out a 10-fold serial dilution. 1 mL of dilutions 10^3 and 10^5 were aseptically plated on solidified, sterilized potato dextrose agar (PDA). The inoculated PDA plates were incubated at room temperature for 3–7 days.

Screening for Tannase-Producing Aspergillus spp.

Tannic acid agar (TAA) was prepared as described by Pinto *et al*. [18]. Each pure fungal isolate obtained was inoculated on TAA and screened for its ability to grow in the presence of tannic acid. Fungal isolates that grew in the presence of tannic acid (i.e., on tannic acid agar) were selected for further studies

Identification of Isolated *Aspergillus* spp.

Morphological Identification of Aspergillus spp.

Morphological identification of five (5) pure cultures with the highest tannase activity (A1, A2, A4, A8, and A15) was carried out via macroscopic and microscopic characteristics assessment as described by Gaddeyya et al. [19].

Molecular Identification of Isolated *Aspergillus* spp.

Extraction of deoxyribonucleic acid from Fungal Isolate

The extraction of the fungal isolate's genetic material (deoxyribonucleic acid, or DNA) was carried out using Zymo Research Fungi/Bacteria DNA Extraction Kits. Polymerase chain reaction was carried out for the Internal Transcribed Spacer (ITS) sequence amplification. The 25 µL reaction mixture contained about 200ng template DNA, 0.3 µM forward, 0.5 µM reverse primer, Dream *Taq* PCR master mix (12.5 µL) and 2 µL of 50 µg/mL bovine serum albumin (BSA). The protocols, according to the manufacturer [20], were strictly followed.

Polymerase Chain Reaction of the Fungal Ribosomal Deoxyribonucleic Acid

Polymerase chain reaction was carried out for the Internal Transcribed Spacer (ITS) sequence amplification.

Qualitative and Quantitative Analysis of Deoxyribonucleic Acid

A qualitative and quantitative assessment of the extracted DNA was carried out using agarose gel electrophoresis, as described by Welsh and McClelland [21].

Sequencing and Analysis of Isolated Deoxyribonucleic Acid

The PCR amplicons were cleaned using Zymo Research PCR cleanup kits and sequenced on an Applied Biosystems International (ABI) automated sequencer model ABI 3130. The resulting Sanger sequences were compared with other related sequences using a BLAST search in GenBank (NCBI). The sequences obtained were deposited in GenBank, and the accession numbers were obtained.

Results and Discussion

Isolated and Screened Fungal Isolates

Fig. 1 shows 21 pure fungal isolates obtained from the poultry droppings samples.

Macroscopic features observed in the isolated fungi include yellow-green, brownish, and turquoise colours. The colonies are slightly wrinkled and dense, spreading from the centre of the plate.

From the 21 pure fungal cultures screened, 17 grew and showed a clear zone in the constituted tannic acid agar (Fig. 2 and Table 1).

Identified Fungal Isolates from Poultry Droppings

The seventeen isolates that utilized tannic acid as a carbon source were subjected to solid-state fermentation (SSF) for tannase production. Evident by tannase enzyme activity, all the fungal isolates produced tannase in different proportions under SSF. Five fungal isolates (Fig. 3) with high tannase activity were selected and subjected to macroscopic, microscopic, and molecular identification.

Macroscopically Identified Pure Fungal Cultures

Growth sporulated culture was observed to have started from the centre and progressed radially, covering the surface of the media and forming a colony. As the sporulation spread outwards, a characteristic white border was observed on the sporulating mycelia. The isolate identified macroscopically as *A. flavus* had a yellowish to greenish colony that spread radially from the point of inoculation while *A. fumigatus* had a greyish to blue greyish colony. As the colony progressively grew, its centre became a floccose and rough (Fig. 3).

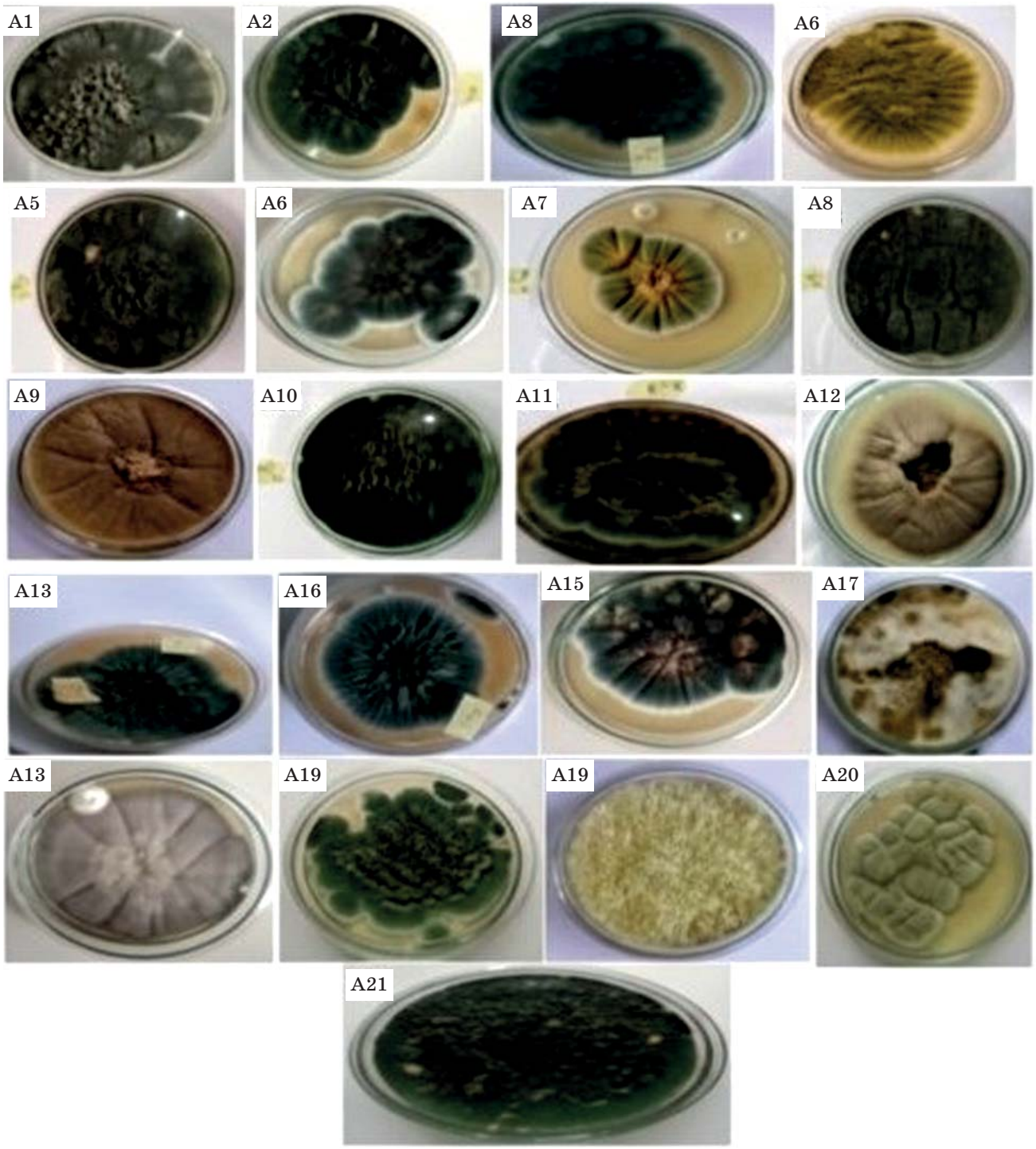


Fig. 1. **Pure fungal isolates**

Table 1. **Growth of *Aspergillus* spp. on tannic acid agar**

Fungal Isolate	Status	Diameter (cm)
A1	*	3.5
A2	*	4.8
A3	*	5.0
A4	*	5.0
A5	*	4.5
A6	*	4.5
A7	*	5.8
A8	*	5.5
A9	***	–
A10	*	6.8
A11	*	4.2
A12	*	5.5
A13	*	3.0
A14	*	4.8
A15	*	4.0
A16	*	3.2
A17	*	7.0
A18	NG	–
A19	NG	–
A20	NG	–
A21	NG	–

* — Growth; *** — massive growth; NG = No growth

Microscopically Identified Pure Fungal Cultures

Fig. 4 and 5 display the microscopic characteristics of the fungal isolate slides when viewed under the microscope. Biseriate colonies with phialides spreading from all sides of the metulae were borne on subglobose or globose vesicles of variable sizes (Fig. 4). Fig. 5 shows conidiophores ending with oval vesicles that bear a single series of sterigmata covering almost half of the vesicle. The sterigmata bore a series of oval, rough-walled conidia.

Molecularly Identified Pure Fungal Cultures

Fig. 6 and 7 show the agarose gel pictures of the extracted fungal isolates' deoxyribonucleic acid (DNA) and polyacrylamide chain reaction (PCR) amplicons, respectively. Table II shows the identified fungal isolates, along with their percentage identity. Various sequences from the NCBI GenBank database were used to construct a phylogenetic tree, which was then used to determine the phylogenetic position of the observed strains (Fig. 8).

The selected homologs from the alignment results revealed that the five morphologically distinct organisms were genetically recognised as two species (*Aspergillus fumigatus and Aspergillus flavus*).

Isolated and Screened Fungal Isolates

Morphological identification of the fungal isolates from poultry droppings revealed that most of the isolates were *Aspergillus* spp.

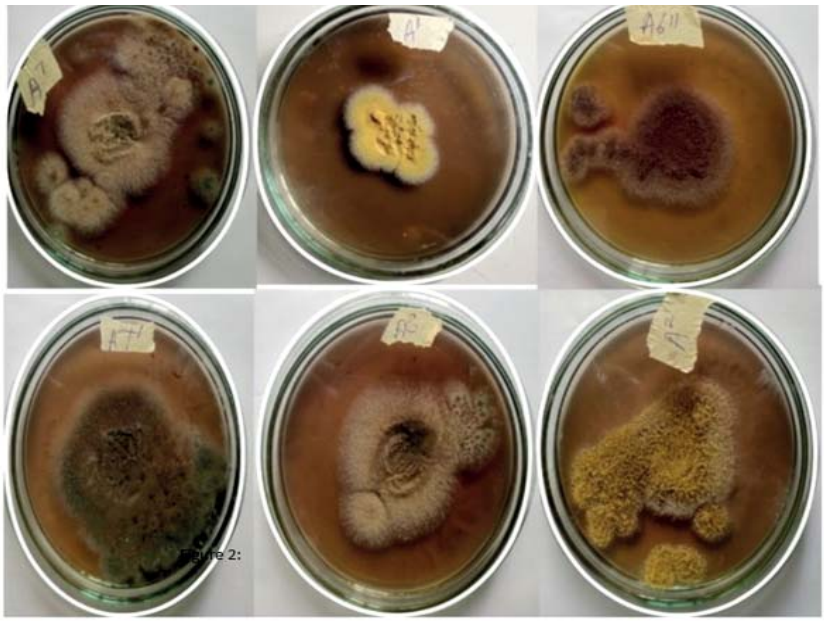


Fig. 2. **Pure fungal isolates with growth on tannic acid agar**

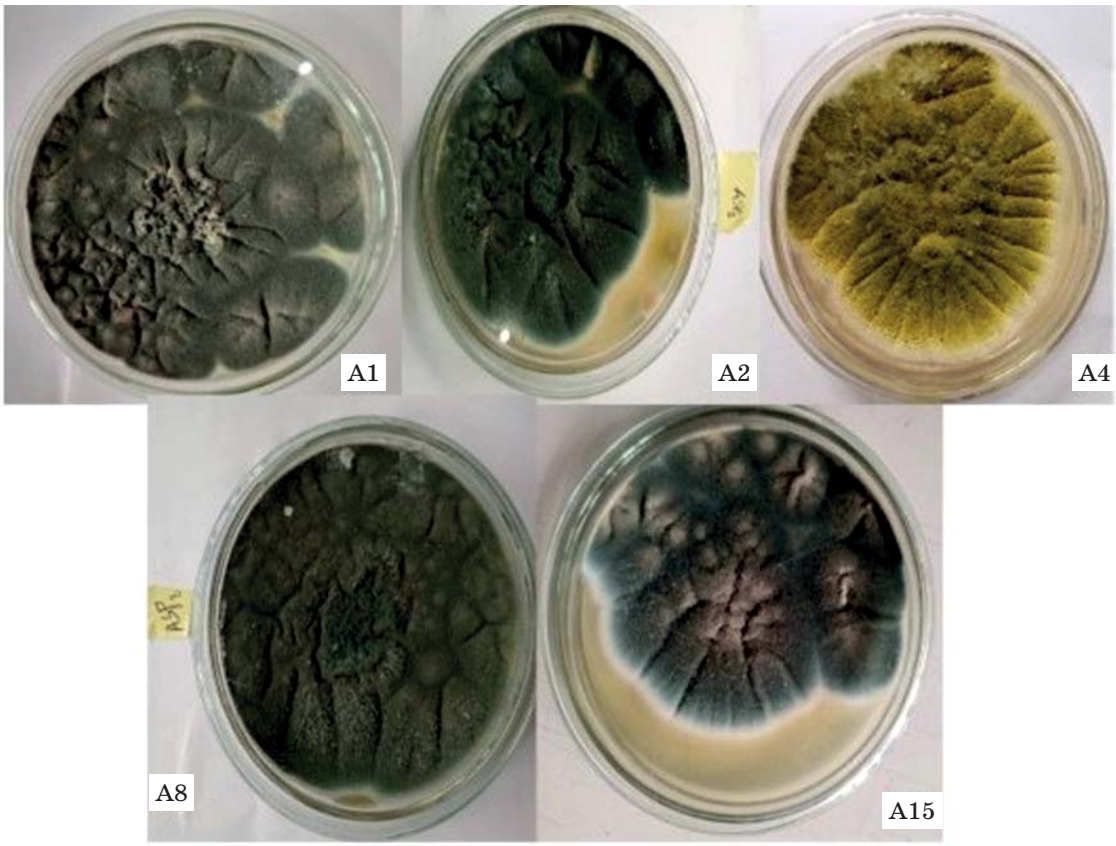


Fig. 3. **Selected *Aspergillus* isolates with very high tannase activity**

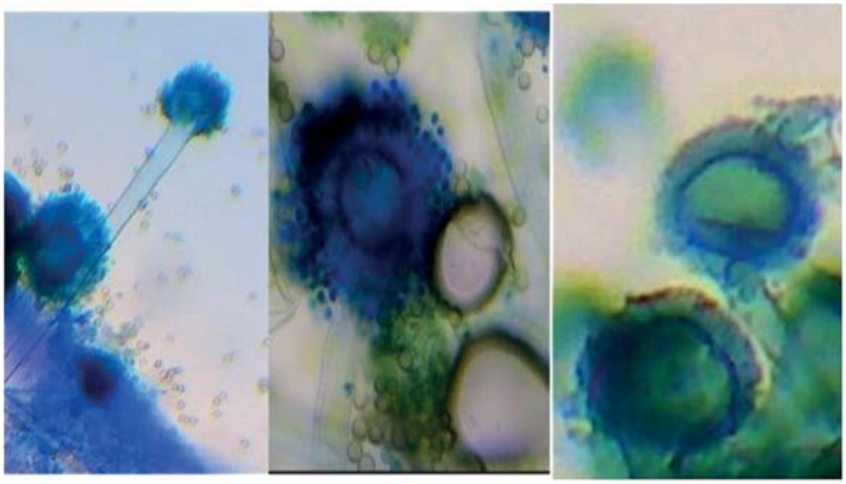


Fig. 4. **Microscopic characteristics of isolated** *Aspergillus flavus*

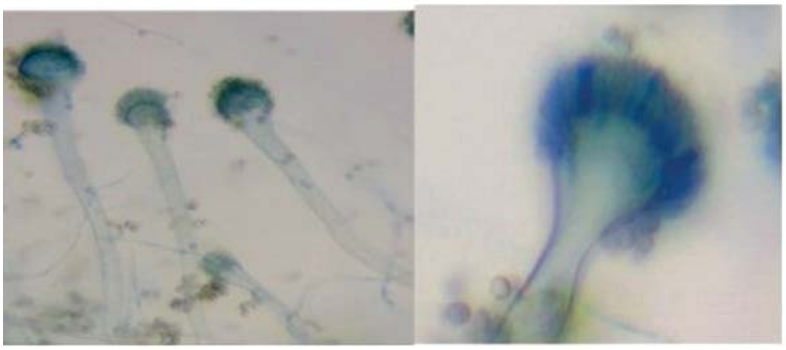
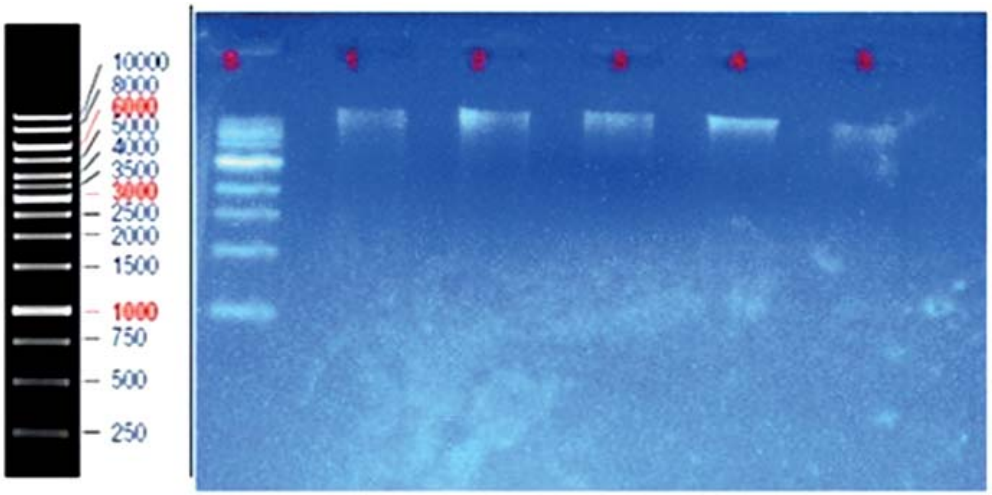
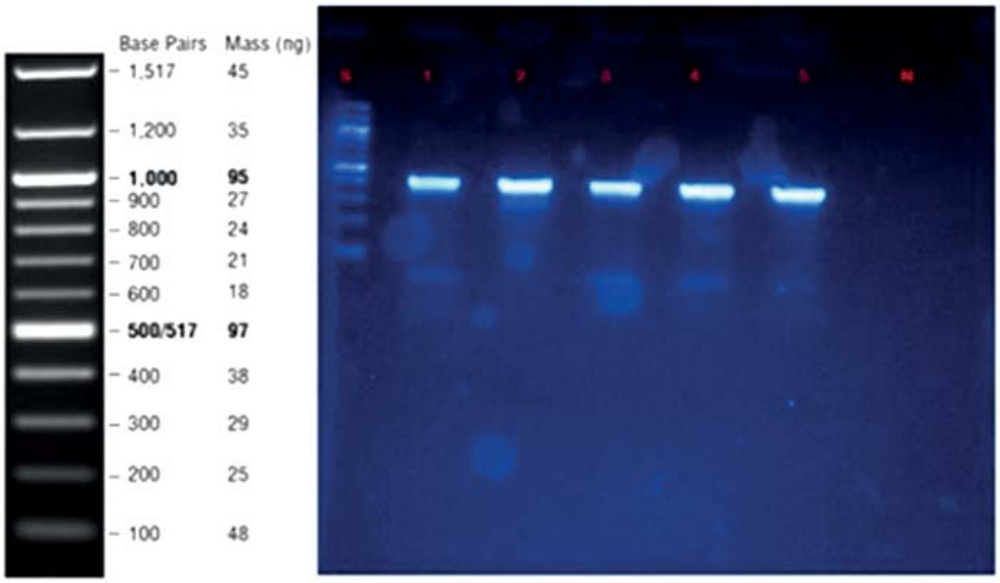


Fig. 5. **Microscopic view of** *Aspergillus fumigatus*



Key: S- Step ladder (1kb) Line 1-5 represents the different samples

Fig. 6. **Electrophoregram of DNA extracted from *Aspergillus* spp.**



Key: S- Step ladder (100 bp), Line 1-5 represents the different samples and N is negative Control

Fig. 7. **Electrophoregram of isolated fungal amplicons**

This finding is in agreement with the work of Byrd et al. [22], which reported that fungal cultures isolated from the ceca of commercial poultry were mainly *Aspergillus* spp. (about 78% of the isolates). Also, Al-Temimay et al. [17] reported in their study that *Aspergillus* spp. Constituted about 28.24% of the entire isolates. The four (4) isolates out of the 21 pure fungal isolates that did not grow on the constituted tannic acid agar in this study may be due to the antifungal action of tannic acid [23–24]. The observed growth of the 17 isolates on tannic acid agar is an indication of their ability to produce tannase, leading to the breakdown of depside and ester bonds in tannic acid and the subsequent release of gallic acid and glucose. This result aligns with the findings reported by Chhokar et al. [25] and Saad et al. [26]. They investigated and noted that the ability of some microorganisms to degrade tannic acid in screening medium indicates the presence of tannase produced by the organisms.

Morphological Identification of Fungal Isolates

Morphological identification of the isolated *Aspergillus* spp. The results obtained from this study revealed the presence of *A. flavus*,

A. niger, and A. fumigatus. Macroscopic characteristics, including globose, large vesicles, biseriate phialides, rough-walled conidia, and radiate, blackish conidial heads, are suspected features of *Aspergillus niger*, as described by EL-Shaer et al. [27]. Yellowish to greenish colonies, greyish to blue-greyish colony pigmentation, and microscopic features of biseriate colonies with phialides radiating from all sides of the metulae, observed among the fungal isolates in this study, are characteristics of A. flavus as reported by Abdelaziz et al. [28] and Arifah et al. [29]. While a macroscopic characteristic of greyish to blue-greyish colony pigmentation and microscopic features of smooth-walled conidiophores are characteristics observed in *A. fumigatus*, as reported by Diba et al. [30] and Abdelaziz et al. [28]. Thus, the morphological features presented by the five fungal isolates may inform their identification as *A. fumigatus, A. niger*, and *A. flavus*.

Molecular Characteristics of the Fungal Isolates

Molecular methods have been effectively applied in identifying microorganisms and also help overcome the bottlenecks associated with conventional methods [31, 32]. Molecular identification is therefore needed to validate morphological identification methods.

The DNA extraction of the samples yielded sharp, distinct genomic DNA that was amenable to polymerase chain reaction (PCR) and produced a strong and reliable amplification product, which conforms with the reports of Zarrin and Erfaninejad [33]. The amplified fragment sizes of 600-700 bp obtained in this study agree with previous reports [33, 34]. The ITS rDNA sequences obtained from the DNA extracted from the five fungal isolates in this study were compared to those in the NCBI database using the BLAST algorithm. The ITS rDNA region sequence has been considered a crucial tool for identifying fungal species [35].

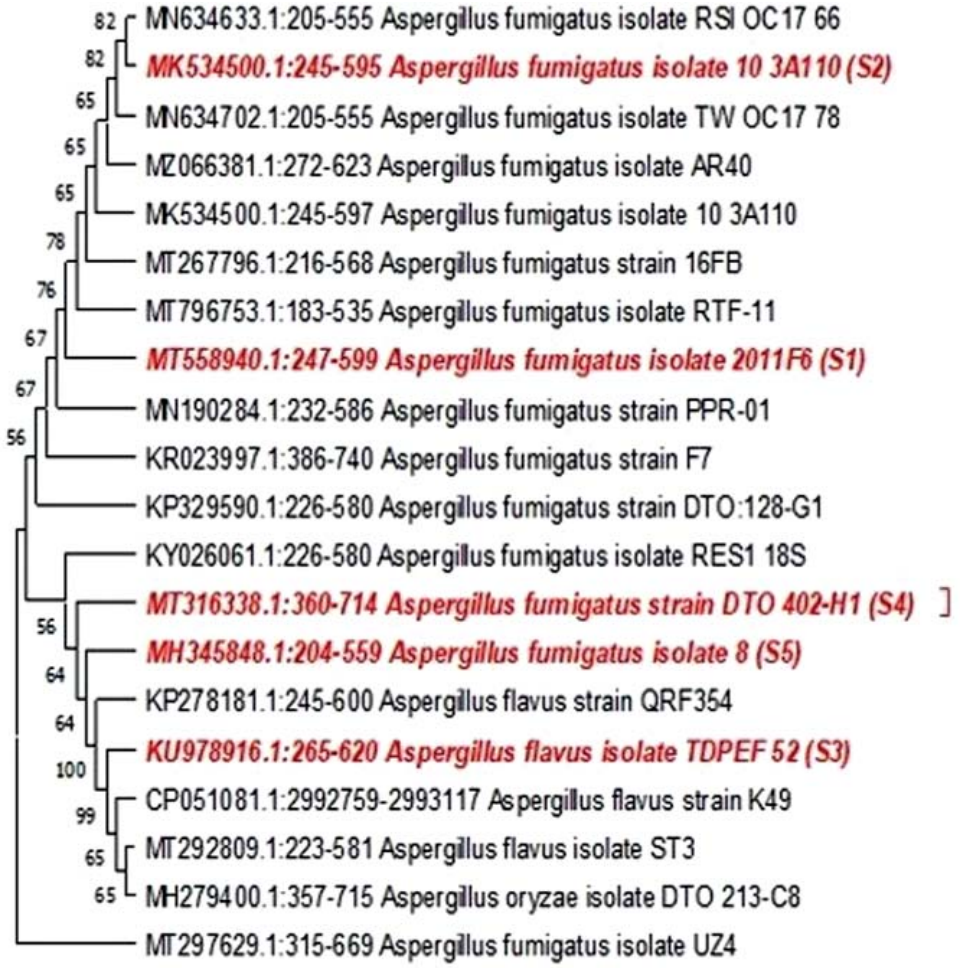


Fig. 8. **Phylogenetic tree using neighbour-joining phylogenetic expression**

Table 2. **Alignment profile of the sequences of fungal isolates**

Isolate	Species identified	Percentage Identity, %	Query cover, %	Total Score	Max Score	E value	Assertion Number
A1	*Aspergillus fumigatus*	99.72	99	647	647	0.0	PQ557285
A2	*Aspergillus fumigatus*	99.72	99	645	645	2e-180	PQ557286
A4	*Aspergillus flavus*	100.00	98	658	658	0.0	PQ557287
A8	*Aspergillus fumigatus*	99.15	99	641	641	3e-179	PQ557288
A15	*Aspergillus fumigatus*	99.44	99	649	649	0.0	PQ557289

ITS rRNA genes also serve in phylogenetic analysis due to their universal distribution, functional consistency, adequate conservation, and appropriate length, which helps provide sufficient evolutionary relationships [36]. Considering the ITS sequences, which showed that four of the fungal isolates exhibited 99.15–99.72% similarity to *A. fumigatus* and one exhibited 100% similarity to *A. flavus*. The fungal isolates were thus molecularly identified as *A. fumigatus* and *A. flavus*. The molecular identification of the *Aspergillus* spp. from this study as *A. flavus* and *A. fumigatus* is consistent with the report by Fagbohun et al. [37], who isolated and identified *A. flavus* and *A. fumigatus* from poultry samples. In the phylogenetic analysis, all the isolates clustered in the same clade as the reference strains.

Conclusion

The isolation and identification of filamentous fungi from poultry droppings revealed the presence and abundance of some economically essential fungi. The molecular identification of tannase-producing *Aspergillus fumigatus* and *Aspergillus flavus* in poultry droppings is a significant indicator that their tannase production can be further studied and optimized for maximum enzyme production.

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ВИДІЛЕННЯ ТА ІДЕНТИФІКАЦІЯ *Aspergillus* spp., ЩО ПРОДУКУЄ ТАНАЗУ, З ПТАШИНОГО ПОСЛІДУ

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Мета. Таннази знайшли застосування в багатьох галузях промисловості. Види *Aspergillus* — це цвіль, яка значною мірою виділяється з пташиного посліду та є основним продуцентом танази. Це дослідження мало на меті виділення та ідентифікацію видів *Aspergillus*, що продукують таназу, з пташиного посліду.

Методи. Зразки пташиного посліду були отримані з птахоферми в районній раді Кудже, Федеральна столична територія, Абуджа, Нігерія. Вид *Aspergillus* був виділений відповідно до стандартних мікробіологічних процедур. Здатність ізольованих видів *Aspergillus* використовувати дубильну кислоту була досліджена за допомогою аналізу танази з використанням стандартного методу. Ізольовані види *Aspergillus* були ідентифіковані за допомогою морфологічних характеристик та методів молекулярної ідентифікації.

Результати. Двадцять один (21) чистий грибковий ізолят було отримано з пташиного посліду, і 17 з них змогли використовувати та рости на конституйованому таніново-кислотному агарі діаметром 3,5–7,0 см. П'ять ізолятів з найвищою активністю танази були ідентифіковані як *Aspergillus fumigatus* та *Aspergillus flavus*.

Висновок. Дослідження показало, що продукуючі таназу *A. fumigatus* та *A. flavus* можна отримати з посліду птиці та використовувати для виробництва танази.

Ключові слова: танназа, *Aspergillus* spp., морфологічна ідентифікація, молекулярна ідентифікація.