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Research Article

Isolation, identification and Screening of Fungal Isolates for Humic Acid Production

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ABSTRACT
Sustainable biotechnological processes have encouraged the search for efficient microorganisms capable of converting agricultural and organic wastes into valuable bio-products such as humic acids. Soil samples were obtained from dump sites of an oil palm processing farm in Obokun, Osun State. The soil sample was subjected to serial dilution and plated on Potato Dextrose agar plating technique to isolate distinct fungal colonies. Fungal isolates were purified, and identified by morphological and cultural characteristics and screened for growth performance based on colony diameter and fungal biomass, while humic acid production was assessed using a submerged fermentation process. Molecular analysis confirmed two isolates identified as *Aspergillus flavus* (PX097504) and *Fusarium* sp. Comparative growth analysis revealed that *A. flavus* demonstrated superior performance in both colony diameter (30.50 ± 0.09 mm) and fungal biomass (2.49 ± 0.02 g) when compared to *Fusarium* sp. (28.64 ± 0.04 mm and 1.98 ± 0.02 g, respectively). During fermentation, *Fusarium* sp. initially showed slightly higher humic acid production at the early stages (0.90 mg/L on day 1 and 1.10 mg/L on day 2); however, *A. flavus* outperformed it in subsequent days, achieving a maximum humic acid concentration of 2.20 mg/L by day 5. The findings demonstrate the potential of indigenous fungal isolates for bioconversion of organic wastes into humic substances, offering an environmentally-friendly approach to waste management and soil enrichment.

Keywords: Fermentation; Fungi; Humic acid; Oil palm; Waste management

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INTRODUCTION
In recent years, the sustainable management of agricultural waste and the enhancement of soil quality have gained paramount importance in the field of agricultural research (Badis *et al.,* 2019). The oil palm industry, a major contributor to global vegetable oil production, generates significant quantities of wastes, including the empty fruit bunches (EFBs) of all oil Palm (*Elaeis guineensis*). These Oil pam empty fruit bunches are rich in lignocellulosic materials and represent a substantial waste stream that can pose environmental challenges if not managed properly (Awogbemi *et al.*, 2020). Concurrently, the decline in soil fertility due to intensive agricultural practices necessitates innovative approaches to rejuvenate soil health and productivity (Lal, 2015). Humic acid ($C_{187}H_{186}O_{89}N_9S_1$), a complex mixture of organic molecules derived from the decomposition of plant and microbial residues, plays a vital role in soil structure, nutrient cycling, and

overall soil fertility. Its ability to improve soil water-holding capacity, cation exchange capacity, and nutrient availability makes it a valuable component of soil organic matter. Consequently, the search for sustainable methods to enhance humic acid content in soils has garnered significant attention. Oil palm (*Elaeis guineensis)* is one of the most important crops globally, primarily cultivated for its high oil yield. The oil palm industry generates a significant amount of wastes, and one of the major byproducts is the empty fruit bunches. OPEFB refers to the fibrous residue left after the extraction of palm oil from the fruit bunches (Harun *et al.*, 2016). Oil OPEFB are considered abundant and readily available waste materials from the palm oil mill processing. They have gained increasing attention due to their potential as a valuable resource for various applications. They are primarily composed of lignocellulosic materials, including cellulose, hemicellulose, and lignin. These components make OPEFB a promising feedstock for various bioconversion processes, including fermentation. The OPEFB has several characteristics that make it suitable for fermentation processes (Hassan *et al*., 2020). Firstly, it is rich in carbohydrates, particularly cellulose and hemicellulose, which can serve as a carbon source for microorganisms. The high cellulose content provides a potential substrate for the production of various value-added products, such as biofuels, enzymes, and organic acids. Additionally, they have a relatively low lignin content compared to other lignocellulosic materials, making it more accessible for microbial degradation and conversion. Studies have explored the use of OPEFB as a substrate for submerged fermentation to produce various products (Atiwesh *et al.,* 2021).

Microorganisms are ubiquitous in the sense that they can be found in almost any natural habitat (soil, water, air, leaves, and tree trunks), with soil serving as a reservoir for a variety of microorganisms. Humic-acid producing microorganisms have been isolated from soil, the marine environment waste volatile substances (Muthumari *et al.*, 2016), palm-oil mill effluent, (Mohammad *et al*., 2021). The present study addresses the dual challenges of agricultural waste management and soil health improvement by focusing on the isolation, identification, and screening of humic acid-producing fungi from the soil environment of oil palm empty fruit bunches waste (Akita *et al.,* 2021).

MATERIALS AND METHODS

Collection of Samples

Two hundred (200) g of soil One (1) sample was collected at a local palm oil processing mill located in Boredun, Obokun Local Government Area, Osun State, Nigeria. The sample was collected from a depth ranging between 7.0 cm and 15.0 cm. This depth range was chosen to capture the soil characteristics in the immediate vicinity of the OPEFB pile.

Isolation of Fungi

Five-fold serial dilution of each soil sample was conducted and pour-plated with Potato Dextrose Agar (PDA). The inoculated plates were incubated at room temperature for 48 hours. Distinct colonies were sub-cultured to obtain pure cultures and maintained by periodically sub-culturing (Al- Jaradi *et al.*, 2018).

Screening of the Isolates for Humic Acid Production

Screening of the isolates for potential to produce humic acid was carried out on agar plates. Prepared OPEFB was mixed with distilled water in a big glass jar. The mixture was heated and boiled in a water bath for 1 hour to facilitate the extraction of soluble components from the biomass. During the boiling process, the soluble components in the OPEFB were released into the water, forming a liquid extract. After boiling, the mixture was allowed to cool down. The liquid extract was separated from the solid residues by passing it through a filtration process by using a fine mesh. Exactly 1 % w/v of the extract was then supplemented into medium of PDA. The medium was mixed properly and autoclaved at 121°C for 15 minutes. After autoclaving, the medium was allowed to cool and solidified after which fungal isolates were spot- inoculated onto the solidified plates of the prepared medium. The plates were properly sealed with foil to avoid contamination and incubated for 48 hours. The colony diameter and the fungal biomass (weight) of the fungi that grew were subsequently evaluated and determined (Rahim *et al*., 2019). The biomass was obtained by harvesting the fungal mycelia carefully using a sterilized spatula after which they were transferred to pre-weighed filter paper. The combined weight of the filter paper and the mycelia was measured and recorded. The weight of the empty filter paper was then subtracted from the

total weight to determine the weight of the mycelia. This process was done for the fungi that grew subsequently from the plate screening in duplicates to ensure accuracy.

Humic Acid Production Assay

Fungi isolates were inoculated separately into 250 ml Erlenmeyer flask containing 0.5 g/L $(NH4)_2SO_4$, 0.1 g/L KH_2PO_4, 0.1 g/L $MgSO_4.7H_2O$, and 0.01 g/L $FeSO_4.7H_2O$ and OPEFB extract as carbon source. Exactly 1% w/v of OPEFB extract was added to basal medium containing the aforementioned salts. The mixture was incubated on a rotary shaker at 200 rpm for 5 days. After incubation, the mixture was centrifuged and the supernatants were collected. The humic acid content in the supernatants was quantified by measuring the absorbance at 400 nm and calculating the humic acid concentration using a standard curve (Motta and Santana, 2013).

Identification of Fungi from OPEFB Soil environment

Fungal isolates were identified by observing their colonial characteristics (colour, shape, size and hyphae) and microscopic features using a microscope with a digital camera using a lactophenol cotton blue stained slide mounted with a small portion of the mycelium. The resulting view from the micrographs were captured and compared with those features from morphological atlas (Soňa *et al*., 2020).

Molecular identification of Humic-Acid Producing Fungal isolates

The fungal isolate that demonstrated the highest potential for humic acid production during the preliminary screening was subjected to molecular identification techniques. This process involved the extraction of genomic DNA from the fungal mycelium, followed by amplification of the internal transcribed spacer (ITS) region using polymerase chain reaction (PCR). The ITS region, which is highly conserved within fungal species but contains sufficient variability for species-level identification, was chosen as the genetic marker for analysis (Adewale et al., 2023). The PCR products were then purified and sequenced, and the resulting nucleotide sequences were compared with reference sequences in publicly available databases such as GenBank through the Basic Local Alignment Search Tool (BLAST). The molecular identification provided a precise taxonomic placement of the isolate, complementing the earlier macroscopic and microscopic characterization and ensuring accurate species determination for subsequent experimental applications.

Fungi DNA Extraction Protocol

Approximately,100 mg of fungi mycellia was ground with Dellaporta extraction buffer (100 mM Trls pH 8, 51 ml EDTA pH 8, 500 mM NaCI, 10mMmcrcaptoethanol) and DNA extracted as described briefly. Each sample was grinded in 1000 µL of the buffer in a sterilized sample bag. Mix was collected in sterile Eppendorf tube and 40 µL of 20% SDS was then added, this was followed by brief vortexing and incubated at 65 ^oC for 10 minutes. At room temperature, 160 µL of 5 M potassium acetate was then added vortexed and centrifuged at 10000 g for 10 minutes. Supernatant were collected in another Eppendorf tube and 400 µL of cold iso propanol was added mixed gently and kept at -20 ^oC for 60 minutes. Centrifugation was at 13000g for 10 minutes to precipitate the DNA after which supernatant was gently decanted and ensured that the pellet was not disturbed. DNA was then washed with 500 µL of 70 % ethanol by centrifuging at 10000g for 10 minutes. Ethanol was decanted and DNA air-dried at room temperature until no trace of ethanol was seen in the tube. Pellet was then re-suspended in 50 µL of Tris EDTA buffer to preserve and suspend the DNA.

PCR Analysis

To use the ITS gene for characterization of fungi, ITS universal primer set which flank the ITS1, 5.8S and ITS2 region can be used;PCR sequencing preparation cocktail consisted of 10 µL of 5x GoTaqcolourless reaction, 3 µL of 25mM MgCl2, 1 µL of 10 mM of dNTPs mix, 1 µL of 10 pmol each ITS 1: 5' TCC GTA GGT GAA CCT GCG G 3'and - ITS 4: 5' TCC TCC GCT TAT TGA TAT GC 3'primers and 0.3units of Taq DNA polymerase (Promega, USA) made up to 42 µL with sterile distilled water 8µl DNA template. PCR was carried out in a GeneAmp 9700 PCR System Thermalcycler (Applied Biosystem Inc., USA) with a PCR condition include a cycle of initial denaturation at 94^0C for 5 min, followed by 35cycles of each cycle comprised of 30secs denaturation at 940C, 30secs annealing of primer at 55^oC, 1.5 min extension at 72^0C and a final extension for 7min at 72^oC (Minarni *et al*., 2021).

Integrity

The integrity of the amplified about 1.5Mb gene fragment was checked on a 1.5% Agarose gel ran to

confirm amplification. The buffer (1X TAE buffer) was prepared and subsequently used to prepare 1.5% agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60°C and stained with 3µL of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Two microliter (2 µL) of 10X blue gel loading dye (which gives colour and density to the samples to make it easy to load into the wells and monitor the progress of the gel) was added to 4µL of each PCR product and loaded into the wells after the 100bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120V for 45 minutes visualized by ultraviolet transillumination and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was ran alongside experimental samples in the gel.

Purification of Amplified Product

After gel integrity, the amplified fragments were ethanol purified in order to remove the PCR reagents. Briefly, 7.6 µL of Na acetate 3M and 240 µL of 95% ethanol was added to each about 40µL PCR amplified product in a new sterile 1.5 µL tube Eppendorf, mix thoroughly by vortexing and kept at 20°C for at least 30 min. Centrifugation for 10 min at 13000 g and 4°C followed by removal of supernatant (invert tube on trash once) after which the pellet was washed by adding 150 µL of 70% ethanol and mix then centrifuge for 15 min at 7500 g and 4°C. Again, remove all supernatant (invert tube on trash) and invert tube on paper tissue and let it dry in the fume hood at room temperature for 1015 min. then resuspend with 20 µL of sterile distilled water and kept in 20°C prior to sequencing. The purified fragment was checked on a 1.5% Agarose gel ran on a voltage of 110V for about 1hr as previous, to confirm the presence of the purified product and quantified using a nanodrop of model 2000 from thermo scientific.

Sequencing

The amplified fragments were sequenced using a Genetic Analyzer 3130xl sequencer from Applied Biosystems using manufacturers' manual while the sequencing kit used was that of Big Dye terminator v3.1 cycle sequencing kit. Bio Edit software was used for all sequence editing and cluster alignment while and MEGA 6 was used for all genetic analysis.

Evolutionary relationships

The evolutionary relationships among the fungal isolates were inferred using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), a widely used distance-based clustering method that assumes equal evolutionary rates among lineages. This approach enables the construction of phylogenetic trees that provide insight into the genetic relatedness of isolates based on sequence similarity. In this study, the optimal phylogenetic tree produced had a total branch length of 2.774 and was drawn to scale, with branch lengths corresponding to evolutionary distances computed using the Maximum Composite Likelihood (MCL) method. The MCL approach is considered robust for estimating pairwise nucleotide substitutions per site, as it maximizes the composite likelihood across all sequence pairs. A total of 22 nucleotide sequences were analysed, including positions from the first, second, and third codon sites, as well as non-coding regions. Ambiguously aligned positions were excluded using the pairwise deletion option, resulting in a final dataset containing 1,084 informative sites. All computations, including distance estimation and tree construction, were conducted using MEGA version 12, which offers enhanced accuracy, advanced model selection capabilities, and improved visualization options suitable for publication-quality outputs.

RESULTS

Occurrence of Isolated Fungi from Soil Sample

There were isolation Two fungal isolates were successfully obtained and identified based on their macroscopic and cultural characteristics. These were *Aspergillus flavus* and *Fusarium* sp. *Aspergillus flavus* exhibited a raised colony morphology with a cottony to fluffy texture. The colony pigmentation appeared yellowish-green, which is a distinctive feature of the species. In contrast, *Fusarium* sp. displayed a spreading colony with a cottony to slightly fluffy texture, showing a characteristic pinkish to reddish pigmentation that is typical of the genus. Molecular characterization was performed on the isolate with sample ID MHK. Sequence analysis of the ITS region and BLAST comparison with the NCBI GenBank

database showed a 99.66% identity with *Aspergillus flavus* (Accession number: PX097504), with 99% query coverage, an E-value of 0, and a maximum score of 1085. This confirms the isolate as *Aspergillus flavus*.

Reaction of Fungal Isolates for Humic Acid Production

The results showed that *Aspergillu flavus* recorded the highest colony diameter (30.50 ± 0.09 mm) and fungal biomass (2.49 ± 0.02 g), while *Fusarium sp.* exhibited slightly lower colony diameter (28.64 ± 0.04 mm) and biomass (1.98 ± 0.02 g) as presented in Table 2.

Screening and Quantification of Humic Acid Production by Fungal Isolates

At the initial stage (day 1 and 2), *Fusarium* sp. exhibited higher humic acid concentration (0.90 mg/L and 1.10 mg/L, respectively) compared to *Aspergillus flavus* (0.70 mg/L and 1.00 mg/L). However, as fermentation progressed, the production trend reversed. From day 3 onwards, *Aspergillus flavus* outperformed *Fusarium* sp., recording a steady increase up to 2.20 mg/L by day 5, whereas *Fusarium* sp. peaked early and then declined to 0.80 mg/L at day 5 (Table 3).

Table 1: Cultural and Morphological Characteristics of Fungal Isolates

Fungal Isolates	Elevation	Appearance	Pigmentation	Conidial head	Phialide	Conidiophores	Rhizoids/ Foot cells	Inferential Organism	Confirmed Organism
Fi1 (MHK)	Raised	Cottony/fluffy, becoming granular with age	Yellowish-green on surface, pale to reddish-gold on reverse	Radiate, loosely columnar, globose head	Biseriate phialides covering the vesicle	Rough-walled, hyaline to light brown, arising from foot cells	Foot cells present, rhizoids absent	*Aspergillus flavus*	*Aspergillus flavus* (**Sample I.D**: MHK, **Accession No**: PX097504)
Fi2	Slightly raised, spreading	Cottony to woolly	Initially white, turning pinkish to reddish with purple tinge; reddish pigmentation on reverse	Not globose, micro- and macroconidia present	Phialides short, unbranched, borne on branched conidiophores	Slender, branched conidiophores producing abundant conidia	Foot cells present, no rhizoids	*Fusarium* sp.	Not molecularly confirmed

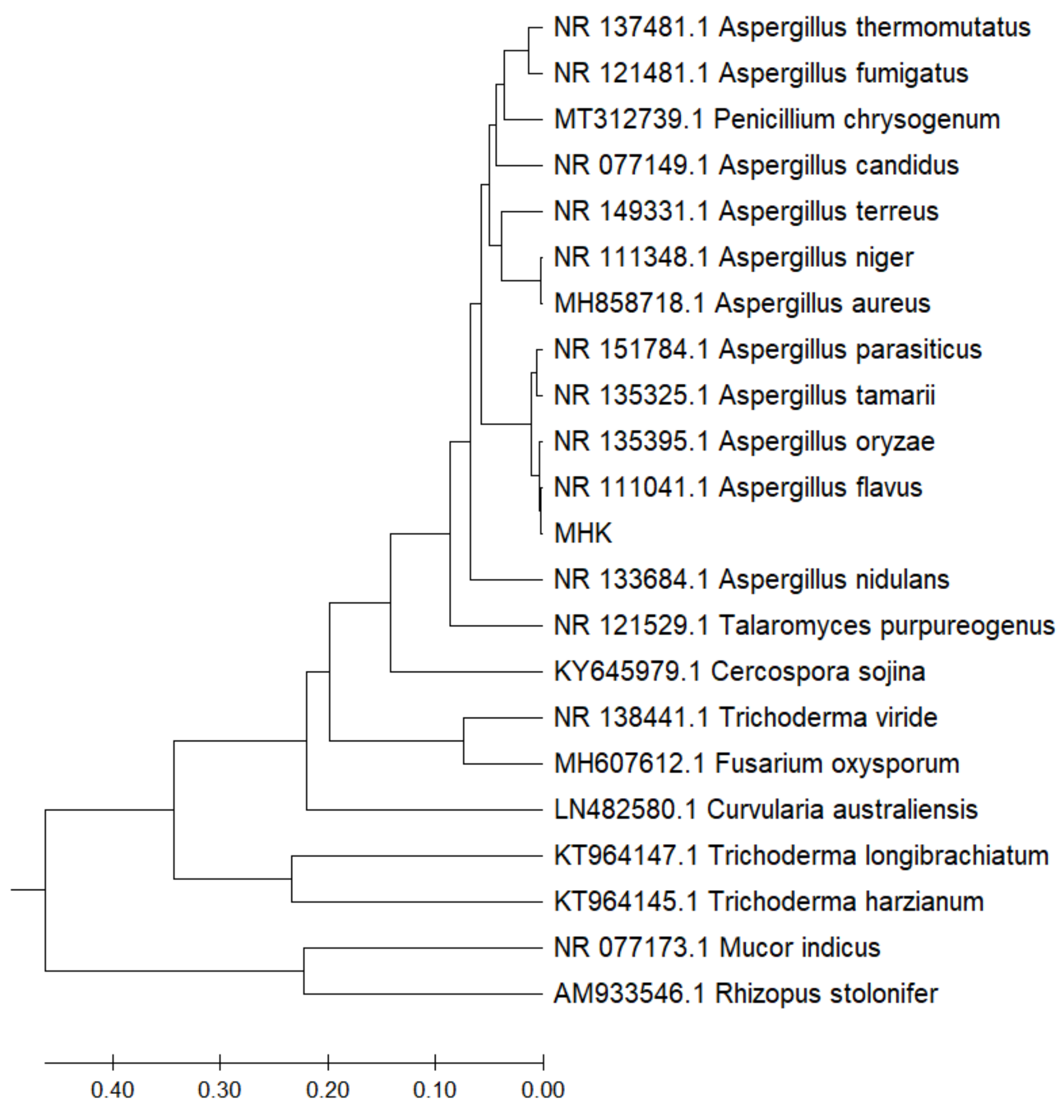

Figure 1: Phylogenetic Tree Constructed by Neighbor-Joining Method Showing the Relationship of the Isolate (*Aspergillus flavus*, Sample ID MHK, Accession Number PX097504) with Closely Related Species

Table 2: Diameter of Fungal Growth and Biomass

Fungal isolates	Colony diameter (mm)	Biomass (g)
Aspergillusniger	30.50 ± 0.09	2.49 ± 0.02
Fusariumsp	28.64 ± 0.04	1.98 ± 0.02

Values are means of duplicate readings ± SD

Table 3: Humic Acid Concentration Produced by Fungal Isolates During Fermentation

Fermentation Period (Days)	*Aspergillus flavus (mg/L)*	*Fusarium sp. (mg/L)*
0	0.00 ± 0.00	0.00 ± 0.00
1	0.70 ± 0.05	0.90 ± 0.06
2	1.00 ± 0.07	1.10 ± 0.08
3	1.40 ± 0.08	1.20 ± 0.07
4	1.80 ± 0.10	1.00 ± 0.06
5	2.20 ± 0.05	0.80 ± 0.05

DISCUSSION

The study revealed significant variations in the growth pattern, morphology, and metabolic activity of the fungi examined. Two distinct fungal isolates were successfully obtained and identified based on their cultural and microscopic characteristics as *Aspergillus flavus* and *Fusarium* sp. These organisms demonstrated clear differences in their growth performance and capacity to synthesize humic acids during fermentation to reflect their unique physiological and biochemical properties.

The growth results showed that *Aspergillus flavus* exhibited the highest colony diameter and biomass accumulation, with values of 30.50 ± 0.09 mm and 2.49 ± 0.02 g, respectively, compared to *Fusarium* sp., which recorded 28.64 ± 0.04 mm and 1.98 ± 0.02 g. This suggests that *A. flavus* adapted better to the growth medium and utilized the available nutrients more efficiently. Its high growth rate may be linked to its ability to secrete extracellular enzymes such as cellulases and oxidases that accelerate the breakdown of complex organic substrates. According to Singh *et al*. (2022), species of *Aspergillus* are known for their rapid mycelial development and high enzymatic activity, which enable them to thrive even under limited nutrient conditions. The relatively lower biomass yield recorded by *Fusarium* sp. could be attributed to its slower metabolic rate and less efficient enzyme system. Kumar and Sharma (2021) similarly observed that *Fusarium* species generally exhibit slower biomass accumulation due to differences in carbon source utilization and lower resistance to fermentation by-products.

During the screening for humic acid production, both isolates showed the ability to synthesize measurable quantities of humic acids, though with different trends over time. At the early stage of fermentation (days 1 and 2), *Fusarium* sp. produced slightly higher concentrations of humic acid (0.90 mg/L and 1.10 mg/L, respectively) than *A. flavus* (0.70 mg/L and 1.00 mg/L). This early spike in production might be linked to rapid substrate oxidation and decomposition at the initial stage when nutrients were readily available. However, as fermentation progressed, *A. flavus* showed a consistent and steady increase in humic acid yield, reaching 2.20 mg/L by day 5, while *Fusarium* sp. peaked early and then declined to 0.80 mg/L by day 5. The gradual and sustained increase in *A. flavus* agrees with the findings of Zhang *et al*. (2020), who reported that *A. flavus* maintains prolonged metabolic activity, leading to the continuous synthesis of humic and fulvic acids during organic matter fermentation.

The decline in humic acid yield observed in *Fusarium* sp. after the third day may be due to nutrient depletion or accumulation of inhibitory metabolites in the medium. Oluwaseun *et al*. (2022) noted that organic acids produced during microbial fermentation can inhibit further humicacid synthesis when their concentrations exceed a tolerable limit. The sustained productivity of *A. flavus* could therefore be attributed to its tolerance to acidic conditions and its ability to regulate enzyme activity over longer fermentation periods. This adaptive trait makes *A. flavus* a more stable and efficient organism for humic acid production.

The morphological identification further confirmed the isolates based on distinctive colony features. *Aspergillus flavus* exhibited a raised colony morphology with a cottony to fluffy texture and yellowish-green pigmentation, characteristics consistent with those described by Samson *et al*. (2022). On the other hand, *Fusarium* sp. displayed a spreading, woolly colony with pinkish to reddish pigmentation, in line with the diagnostic descriptions of Leslie and Summerell (2020). The presence of biseriatephialides and rough-walled conidiophores in *Aspergillus flavus* also supported its identification. The accuracy of these observations underscores the reliability of morphological techniques in fungal identification, especially when complemented with cultural features.

The differences observed in both growth and humic acid production patterns reflect the distinct enzymatic capabilities and metabolic flexibility of the two isolates. *Aspergillus flavus* appears to be better suited for large-scale humic acid production due to its ability to maintain sustained enzyme activity and consistent growth under fermentation conditions. This agrees with the findings of Nwokocha and Eze (2023), who highlighted the efficiency of *A. niger* in the bioconversion of organic matter into humic substances, especially under controlled pH and temperature conditions. In contrast, *Fusarium* sp. may have a faster but shorter phase of metabolic activity, which makes it less efficient for prolonged fermentation processes.

The findings from this study demonstrate that both *Aspergillus flavus* and *Fusarium* sp. are capable of producing humic acids, but *A. flavus* exhibited superior performance in both growth and yield. Its consistent increase in humic acid concentration throughout the fermentation period suggests a robust enzymatic system capable of continuous transformation of organic substrates into humic materials. These results point to the potential use of *A. flavus* in the biotechnological production of humic acids from agricultural or organic wastes, providing an eco-friendly alternative to chemical synthesis. Future work should consider molecular identification, optimization of fermentation parameters such as pH, temperature, and carbon-nitrogen ratio, and scaling up the process for industrial application.

CONCLUSION

The study successfully isolated, identified, and screened fungal isolates for their ability to produce humic acid. Two fungal species, *Aspergillus flavus* and *Fusarium* sp., were obtained and characterized based on their cultural and microscopic features. Both isolates demonstrated the ability to synthesize humic acids, but *Aspergillus flavus* showed superior growth performance and a higher humic acid yield compared to *Fusarium* sp. The steady increase in humic acid concentration observed with *A. flavus* throughout fermentation indicates its strong enzymatic activity and adaptability to fermentation conditions. These findings suggest that *Aspergillus flavus* is a promising organism for the biotechnological production of humic acids from organic materials. Its ability to efficiently transform organic matter into valuable humic substances highlights its potential in sustainable agriculture, soil fertility improvement, and organic waste management. Further studies focusing on molecular identification, optimization of fermentation parameters, and large-scale production could enhance yield and commercial application of microbial humic acid production.

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