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Isolation, Identification and Screening of Humic Acid Producing Fungi from Soil Environment of Oil palm (*Elaeis guineensis*) Associated with Empty Fruit Bunches

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Abstract

The demand for sustainable and sustainable biotechnological processes has developed as a result of a growing interest in converting agricultural waste into valuable products. In this study, fungal strains were isolated from soil environment of oil palm empty fruit bunches and screened for humic acid production. In Osun state, Nigeria, soil sample was taken from a local palm oil mill. Different colonies of fungi were obtained for further research using the serial dilution plating procedure. Plate screening was used to evaluate the fungal biomass as well as colony diameter of the fungal isolates, and a submerged fermentation test was used to measure the amount of humic acid concentration the isolates produced. Fungal isolates were identified by using molecular methods and morphological analysis. The fungi species Aspergillus niger, Rhizopus stolonifer, and Penicillium chrysogenum were isolated from the soil environment. When compared to Rhizopus stolonifer, Aspergillus niger grew more superiorly in terms of zone diameter (26.5 1.50 mm) and biomass (2.23 0.16 g), while Penicillium chrysogenum showed no growth. Throughout the fermentation process, Aspergillus niger also produced a greater quantity of humic acid, with concentrations varying from 0.70 mg/l to 2.20 mg/l. Aspergillus niger strain HR18's existence was confirmed by molecular analysis. This experiment proved that Aspergillus niger can efficiently produce humic acid from OPEFB, providing a useful method for waste utilization and sustainable practices.

Keywords: Humic acid, Fungi, Fermentation, Waste management

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 waste, including the empty fruit bunches (EFBs) of Oil Palm (*Elaeis guineensis*). These EFBs, rich in lignocellulosic materials, 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 waste, and one of the major byproducts is the empty fruit bunches. Oil palm empty fruit bunch refers to the fibrous residue left after the extraction of palm oil from the fruit bunches (Harun *et al.*, 2016). Oil palm empty fruit bunches (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. 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, (Jagtap and Chobade, 2015). 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 EFB waste (Akita *et al.*, 2021).

MATERIALS AND METHODS

Sample Collection

Two hundred (200) g of soil sample was collected at a local palm oil processing mill located in Boredun, Obokun local government, Osun state, Nigeria. The samples were 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

A five (5) fold serial dilution plating method was employed to isolate fungi. A total of 50 g of sampled soil was prepared in 100 mL of 85% NaCl solution. The solution was thoroughly shaken to ensure proper mixing. Test tubes containing 9 ml of sterile water were prepared and a total of 1 mL of the soil-NaCl solution was then transferred to the first vial using a pipette. Then 1 ml of the solution from the first vial was transferred to the second vial, and this process was repeated until the last vial in the series was filled. After which 1 ml of the solution from the last vial was pipetted in petri dish and Potato Dextrose Agar (PDA) was poured onto the plate and gently mixed, then the plate was incubated at room temperature for 48 hours. After the incubation period, each distinct colony type was characterized and sub-cultured onto a new PDA plate to obtain a pure culture. The pure cultures of the Isolates were then maintained by periodically sub-culturing them onto new PDA plates (Al- Jaradi *et al.*, 2018).

Screening of the Isolates for Humic Acid Production

Plate Screening of 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 media was mixed properly and autoclaved at 121 ° C for 15 minutes. After autoclaving, the media was allowed to cool and solidified after which fungal isolates were spot-inoculated onto the agar media plates. 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 were 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; Volpi *et al.*, 2018).

Identification of Fungi from OPEFB soil environment

Fungal isolates were identified by observing their colonial characteristics (color, 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 Potent Fungal isolates

The Fungal isolate with potential ability to produce humic acid was further identified using molecular biology techniques after screening for humic acid production.

Extraction of DNA

The DNA Extraction Buffer (DEB) was added to sterile mortal, 1 ml of proteinase K (0.05 mg/ml)-containing DEB was added, and the mixture was macerated with a sterile pestle. After that, the extract was put into a 1.5 ml Eppendorf tube. This Eppendorf tube was filled with 50 l of 20% Sodium Dodecyl Sulphate (SDS), which was then incubated for 30 minutes at 65°C in a water bath before being allowed to cool to room temperature. Potassium acetate in the concentration of 7.5 M was added and quickly mixed. After a ten-minute centrifugation at 13,000 rpm, the supernatant was poured into brand-new, autoclaved tubes. About 2/3 volumes of cold isopropanol / isopropyl alcohol was added to the supernatant. The tubes were inverted for 3–5 times gently and incubated at -20º C for 1 hour. It was then centrifuged at 13,000 rpm for 10 minutes and the supernatant was discarded. 500 µl of 70% ethanol was added and centrifuged for 5 minutes at 13,000 rpm. The supernatant was discarded carefully with the DNA pellet intact. Remaining traces of ethanol was removed and the DNA pellets was dried at 37ºC for 10–15 minutes. DNA pellets was re-suspended in 50 µl of Tris-EDTA (TE) buffer. Aliquot DNA was stored at -20º C for further lab analysis (Victor *et al.*, 2021).

PCR Amplification

The PCR amplifications were conducted in a final volume of 20 l, which included 10 l of master mixes (rnps, polymerase enzymes, magnesium chloride, and reaction buffer), 5 l of PCR grade water, 3 l of DNA, and 1 l of each of the ITS1 and ITS4 primers. ITS1 and ITS4 primer sequences were 5'-TCCGTAGGTGAACCTGCGG-3' and 5'-TCCTCCGCTTATTGATATGC-3', respectively. The following temperature profile was used for the PCR amplification: an initial step of 5 minutes at 94 °C, 40 cycles of 50 seconds at 94 °C, 30 seconds at 56 °C, and 30 seconds at 72 °C, and a final step of 5 minutes at 72 °C. The products were then purified and sequenced (Victor *et al.*, 2012).

Gel Electrophoresis and Purification of DNA fragments

The integrity of a 600 base pair DNA fragment was checked on a 1.5% agarose gel. The agarose gel was prepared using a buffer and molten agarose, then heated and stained with ethidium bromide. The PCR products were loaded into the gel and electrophoresed. The sizes of the PCR products were estimated by comparing them to a molecular weight ladder. The amplified fragments were purified in ethanol to remove PCR reagents. The purified fragment was checked on a 1.5% Agarose gel and quantified using a nanodrop of model 2000 from thermo scientific. The purified fragment was then stored at -20°C before sequencing.

ITS Region Sequencing for Identification of *Aspergillus* species

The amplified fragments were sequenced using an Applied Biosystems Genetic Analyzer 3130xl sequencer and the Big Dye Terminator v3.1 Cycle Sequencing kit according to the manufacturer's instructions. Sequences were discovered, deposited in Genbank, and accession numbers were assigned. DNA was kept at 20 °C until it was needed.

Phylogenetic Analysis

Based on the consensus sequence of the ITS1 and ITS4 gene regions, a phylogenetic tree was produced using MEGA X (M11) software to evaluate the similarity and analyze the evolutionary connection between the identified fungi and selected fungi strains obtained from the Gene Bank of The National Center for Biotechnology Information (NCBI). The Neighbor-Joining method was used to infer the evolutionary history. The evolutionary distances were calculated using the Maximum Composite Likelihood method and are in base substitutions per site. Tamura et al. (2002) removed all ambiguous positions for each sequence pair (pairwise deletion option).

RESULTS

Reaction of Fungal Isolates for Humic Acid Production

Aspergillus niger displayed a larger colony diameter (mm) of 26.50 ± 1.5 and a higher fungal biomass (g) of 2.23 ± 0.16 compared to *Rhizopus stolonifer* which showed a smaller colony diameter of 23.00 ± 1.00 (mm) and a lower fungal biomass of 2.06 ± 0.06 (g) while *Penicillium chrysogenum* exhibited no growth as shown in Table 1.

Table 1: Diameter of Fungal Growth and Biomass (Plate Screening)

Fungal isolates	Zone of Colony diameter (mm)	Fungal biomass (g)
Aspergillu sniger	26.50 ± 1.50	2.23 ± 0.16
Rhizopus stolonifer	23.00 ± 1.00	2.06 ± 0.06
Penicillium chrysogenum	0.00	0.00

Values are means of duplicate readings ±SD of fungal growth diameter and biomass

Humic Acid Production by the Fungal Isolates

Aspergillus niger produced higher concentration of humic acid compared to *Rhizopus stolonifer* throughout the 5-day fermentation period with values ranging from 0.70 ± 0.05 mg/l to 2.20 ± 0.05 mg/l as opposed to range of *Rhizopus stolonifer* 0.28 ± 0.04 mg/l to 0.75 ± 0.06 mg/l as shown in Figure 1.

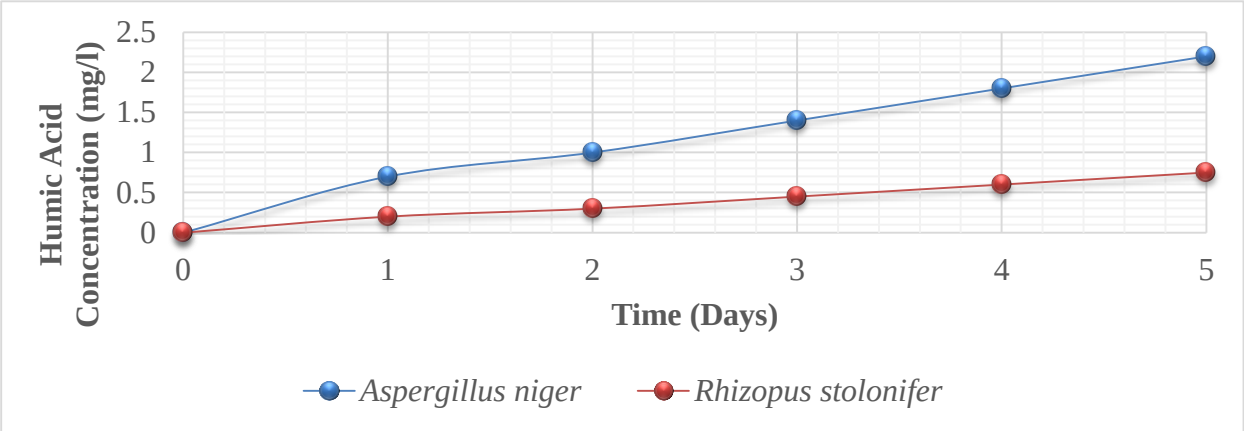


Figure 1: Humic acid Production by Fungal Species

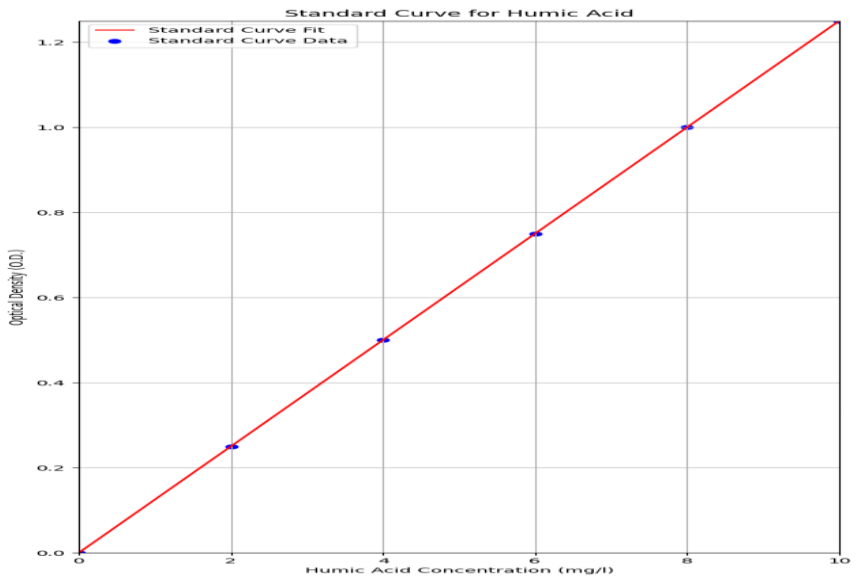


Figure 2: Standard Curve for Humic Acid Quantification Identification of Fungi

Three (3) fungi were obtained namely, *Aspergillus niger, Rhizopus stolonifer* and *Penicillium chrysogenum*. The *Aspergillus niger* exhibited a raised growth pattern with a cottony/fluffy texture. *Rhizopus stolonifer* shows a slightly raised and spreading growth pattern with a fluffy texture. It has a coloration that ranges from grayish white to dark gray. *Penicillium chrysogenum* shows a raised growth pattern with a velvety/powdery texture and displays a distinctive greenish-blue pigmentation as shown in Table 2.

Table 2: Cultural Characteristics of fungal isolates

Fungal Isolates	Elevation	Appearance	Pigmentation	Conidial head	Phialide	Conidiophores	Rhizoids/Foot cells	Tentative organism	Confirmed Organism
Fi1	Raised	Cottony/fluffy	Initial white to black coloration	Black rounded sporangial head at the tips of the Sporangiophores		Unbranched Sporangiophores	Rhizoids and foot cell present	*Aspergillus niger*	*Aspergillus niger* strain HRL18
Fi2	Slightly raised and spreading	Fluffy	Grayish white to dark gray	Short, loosely and columnar	Biseratephialide, large and directly produced from vesicle surface	Conidiophores are Colorless and roughly textured , formed and terminated	Foot cell present	*Rhizopus stolonifer*	
Fi3	Raised	Velvety/powdery	greenish-blue	Glubose , rough walled brush like structure	Clusters of unbranched flask shapes phialide	Conidiophores produces a brush like structure terminated by clusters of flask shaped phialide	Foot cell present	*Penicillium chrysogenum*	

Key: Fi1 - Fungal isolate 1, Fi2- Fungal isolate 2, Fi3 - Fungal isolate 3.

Molecular Identification of Fungi

The molecular identification confirmed the presence of *Aspergillus niger* strain HRL18. The PCR amplification primers resulted in a fragment size of approximately 600 bp (Figure 3). The percentage (96 %) in which the associated taxa clustered together in the phylogenetic tree are shown below the branches (Figure 4).

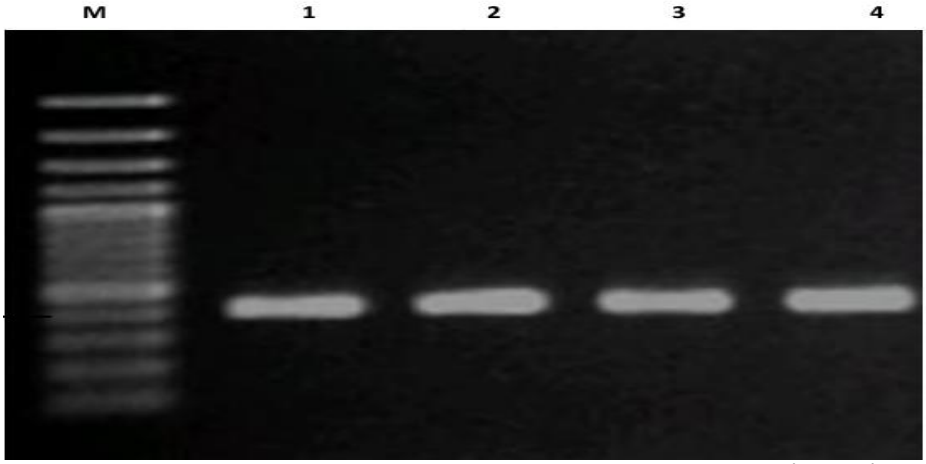
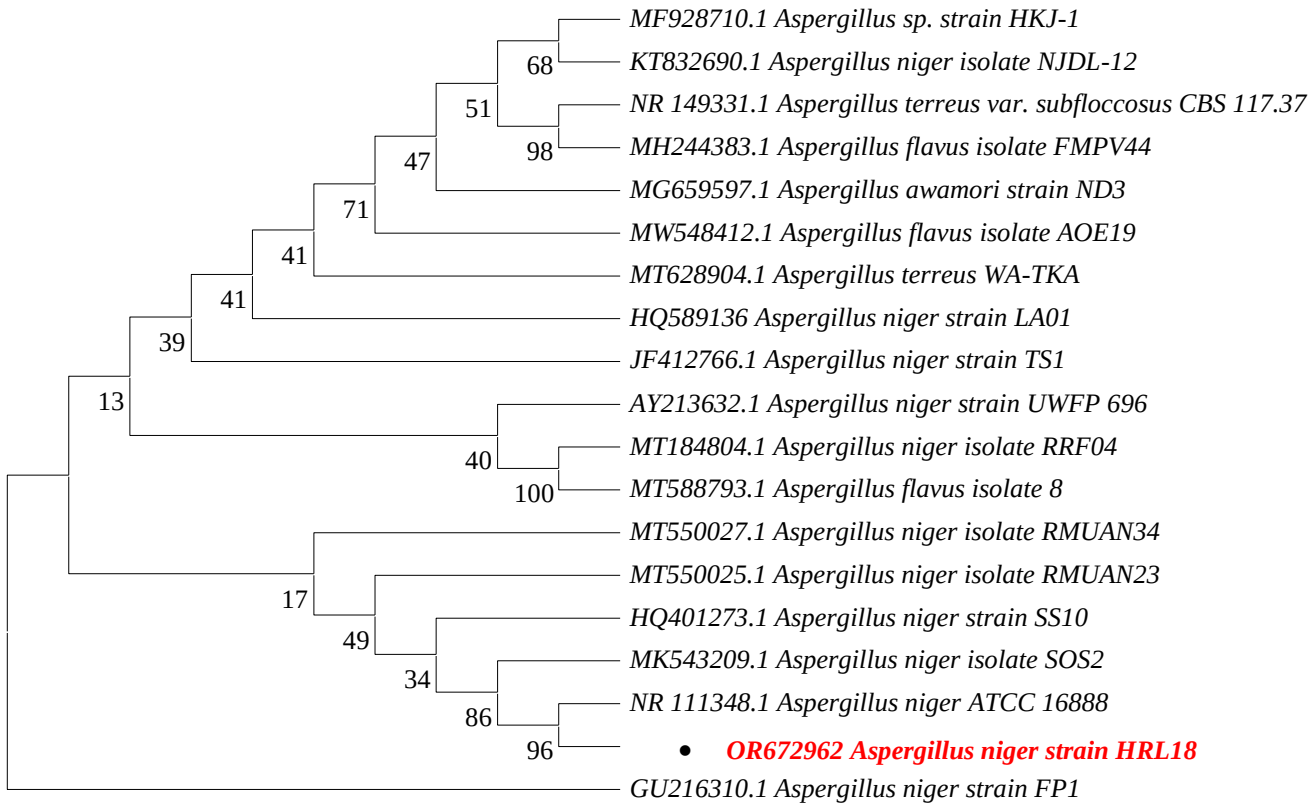


Figure 3: PCR amplification using ITS1 and ITS4 from *Aspergillu sniger*. Lane M: DNA Marker, Lane 1: *Aspergillus niger* (control), Lane 2-4 (*Aspergillus niger* isolates strain HRL18)



DISCUSSION

Aspergillus niger and *Rhizopus stolonifer* exhibited robust growth, characterized by well-defined mycelial growth and distinct morphological features (Table 1). In contrast, *Penicillium chrysogenum* did not show any observable growth on the OPEFB-supplemented medium in this study, suggesting a lack of compatibility with this substrate for humic acid production in this study (William *et al.*, 2020). The pronounced growth of *Aspergillus niger* and *Rhizopus stolonifer* on OPEFB-supplemented medium in this study indicates their ability to utilize the lignocellulosic material present in OPEFB as a carbon source for growth and metabolism. The capability of microbes to facilitate the decomposition of lignocellulosic biomass is measured with cellulolytic, lignolytic, and lignocellulolytic indices.

This correspond with the work of Ariana and Candra (2017). The observed robust growth further supports their potential suitability for humic acid production under submerged fermentation conditions (Volpi *et al.*, 2019; Adewale *et al.*, 2023).

Over the five-day fermentation period, *Aspergillus niger* consistently demonstrated a higher humic acid concentration compared to *Rhizopus stolonifer*. The results indicate that *Aspergillus niger* has a higher humic acid-producing potential compared to *Rhizopus stolonifer* under the given fermentation conditions in this study (Figure 1). The observed differences in humic acid production between the two fungal isolates could be attributed to variations in their metabolic capabilities and enzymatic activities as highlighted in the work of Alberto *et al.* (2021). The results was also comparable with some results obtained by other researchers (Kluczek- Turpeinen *et al.*, 2005; Naranjo *et al.*, 2007). However, the steady increase in humic acid concentration over the five-day period for both isolates suggests ongoing microbial activity and organic matter decomposition, which are essential for humic acid synthesis (Siada *et al.*, 2021).

The fungi isolation and characterization from the OPEFB sample demonstrate the successful identification of distinct fungal isolates (Table 1). The identification of diverse fungal genera in the OPEFB sample is noteworthy as it highlights the rich fungal diversity associated with this waste material. *Aspergillus*, *Rhizopus*, and *Penicillium* are common fungi known for their involvement in various biological processes, including organic matter decomposition, nutrient cycling, and the production of bioactive compounds (Saeed *et al.*, 2021). Tao *et al.* (2021) noted that the presence of such fungal diversity in OPEFB sample can play a crucial role in the breakdown of lignocellulosic material, a constituent which is abundant in oil palm waste. Previous studies also reported a higher number of known fungal phyla, such as Basidiomycota and Ascomycota (Sulaiman , 2011). Tian *et al.* (2014) showed that the most abundant fungal phylum found in soil environment of OPEFB in Xishuangbanna, a tropical forest in China, was Zygomycota, Ascomycota, and Basidiomycota.

Aspergillus niger strain HRL18 was found through molecular analysis and has a 96% homology rate with *Aspergillus niger* ATCC 16888 (figure 2). This outcome is consistent with the research of Henry et al. (2000). In their research, they discovered an ITS1-5.8S-ITS2 amplicon with sizes ranging from 565 to 613 bp (Sarathambal *et al.*, 2021).

CONCLUSION

This study concentrated on isolating, identifying, and screening fungal isolates from the soil surrounding empty fruit bunches from oil palm trees. Through the use of both macroscopic and microscopic analysis, three different fungal isolates were successfully identified falling under the *Aspergillus niger*, *Rhizopus stolonifer*, and *Penicillium chrysogenum* genera. *Aspergillus niger* isolate strain HRL18 displayed great potential in utilizing OPEFB extract as substrate suggesting it feasibility for submerged fermentation for production of humic acid. With fungi being recognized for their involvement in the process of organic decomposition, nutrient cycling, and the generation of bioactive compounds, the presence of numerous fungal genera in the waste material highlights the significant amount of biodiversity of fungi associated with oil palm waste.

Contribution to Knowledge

This study sheds light on the diversity of fungi found in oil palm waste and offers insightful information about the ability of particular fungi to produce humic acid. The results advance knowledge of how important a role fungal strains like *Aspergillus niger* can play in transforming waste materials into products with added value. This study lays the groundwork for future study and application of the capabilities of fungi in the management of waste and bioresource development.

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