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Lipolytic Fungal Diversity in Palm Oil Mill Soils

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ABSTRACT

Lipolytic fungi play important roles in agro-waste management and industrial enzyme production. Palm oil mill effluents (POME) are rich in lipids, making them a suitable niche for lipolytic microorganisms. Soil samples were collected from selected palm oil mills in Eyekanse, Ogun State, Nigeria. Fungal isolates were obtained on mineral salts agar and identified using morphological and molecular techniques (ITS rDNA sequencing). Lipase activity was screened on tributyrin agar, crude lipase was extracted via submerged fermentation, and enzyme activity was quantified titrimetrically. Sixteen fungal strains were isolated across seven genera. Ten isolates exhibited significant lipolysis, with Aspergillus flavus (LFA3) recording the highest lipase activity (31.3 IU/mL). Enzyme activity was optimal at 40 °C and pH 7.0. Molecular analysis confirmed Aspergillus niger, Rhizopus arrhizus, Aspergillus flavus, Trichoderma virens, and Trichoderma asperellum as prolific lipolytic fungi. Palm oil mill soils represent a rich source of industrially relevant lipolytic fungi. The optimised fungal lipases obtained are promising for sustainable biotechnological applications, including biodiesel production and agro-waste valorisation.

1. INTRODUCTİON

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are one of the most important classes of industrial enzymes because of their ability to catalyse the hydrolysis and synthesis of ester bonds in aqueous and non-aqueous environments (Gargouri A, 2020; Sharma *et al*., 2019). They are widely applied in the food, detergent, leather, pharmaceutical, and biofuel industries (Adebusoye *et al*., 2021; Ali, 2020; Khan, 2023).

Microorganisms such as fungi are an excellent source of lipases due to their extracellular secretion, ease of cultivation, and ability to grow on cheap substrates (Adewale *et al*., 2020; Javed, 2021; Nema *et al*., 2019). Palm oil mills produce large amounts of lipid-rich effluents, creating a favourable niche for lipolytic fungi (Alves *et al,*, 2020; Ezeji *et al*., 2021; Kurniawati *et al*., 2023). Exploring these natural environments provides an opportunity to isolate efficient lipase producers for industrial applications (Jones, 2022; Li, 2022; Zhao *et al*., 2023). Also, there is limited information on the specific types of lipolytic fungi present in palm oil mills in Nigeria and their capacity for lipase production.

The potential of lipolytic fungi from specific palm oil mills in Eyekanse, Yewa South, Ogun State, remains largely

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unexplored. Industrial demand for new lipase sources continues to stimulate the screening, characterisation of new lipolytic fungi (Khan, 2023). Hence, it will be of great value to isolate from the local environment and identify new diverse species of lipolytic fungi of highest potential for optimum lipase production and turning waste to wealth. This study aimed to characterize and highlight the diversity of lipolytic fungi isolated from palm oil mill soils in Eyekanse, Ogun State, Nigeria, for potential industrial and envinronmental applications.

2. MATERIALS AND METHODS

2.1 Sample Collection

Soil samples were collected using a clean hand trowel from the effluent dumpsites of four selected palm oil mills in Eyekanse, Ogun State, Nigeria from a depth of 4 – 6 cm and transported in clean, sterile polythene bags to the laboratory

2.2 Isolation and Identification of Fungi

Fungi were isolated on mineral salts agar supplemented with lipid substrates. The mineral salts agar medium contained palm oil 10 mL (emulsified in Tween 80 at 10 mL oil:1 mL Tween); $(NH_4)_2SO_4$, 5 g; Na_2HPO_4, 6 g; KH_2PO_4, 2 g; $MgSO_4$, 3 g; $CaCl_2$, 3 g and Agar. The pH of the medium was adjusted to 5.0 using citrate-phosphate buffer (0.5 M). A conical flask with 100 mL of sterile physiological saline contained ten grams of samples suspended in it. Subsequently, a rotary shaker was used to vigorously mix continuously for 30 minutes at 120 rpm in order to loosen soil clumps before allowing them to settle. After the supernatant was decanted, a serial dilution ten times larger was created. On the mineral salts agar medium, 100 µL of each dilution (10^{-2} - 10^{-4}) was spread-inoculated. Plates were incubated at 28 ºC for 5 – 7 days and checked every day for growth. Fungal colonies with distinct morphologies were purified by sub-culturing on Potato Dextrose Agar plates (Smith, 2021; Spatafora *et al*., 2019; Tedersoo *et al*., 2018).

2.3 Screening for Lipolysis

Twenty grammes (20g) of Tributyrin Agar base was weighed in a litre of distilled water and 10mL of Tributyrin supplement, mixed up uniformly, homogenized and autoclaved at 121ºC for 15 minutes. With regular agitation, the agar was cooled to 50°C before being promptly put into sterile petri dishes to set quickly. The plates were surface-dried at 45ºC for one hour, inoculated with the isolated organisms and incubated at 35ºC (+-2ºC) for 48 - 120 hours (Adewale *et al*., 2023; Rohban, 2021). The clear zone of inhibition was visually observed after the incubation period. The diameter of the clear zone was measured with ruler in millimeter (mm). Only the fungal isolates which showed wide zone of clearance for lipolysis (lipase-prolific) were selected for further studies.

2.4 Lipase Activity Assay

Mature spore suspensions were made by using 5 ml of sterile saline to gently wash the surface of agar plates. Next, 5 mL of the diluted spore suspension was transferred into a conical flask that held 45 mL of the mineral salts medium without agar. For 72 hours, the flasks were incubated at 28ºC and 100 rpm. After that, the culture broth was filtered through Whatman No. 1 filter paper and a double-layered muslin cloth (Meng *et al*., 2021; Nema *et al*., 2019). The filtered crude enzyme was combined with an emulsion (cocktail) made up of 10% v/v glycerol tributyrate emulsified in the solution of 5% v/v gum acacia dissolved in 20 mL 0.1 M Sodium phosphate buffer pH 7.0 and 2.2% w/v calcium chloride ($CaCl_2$). The resulting mixture was incubated at 30ºC and 100 rpm for one hour in an orbital shaker incubator. After one hour of incubation, the enzyme activity was terminated and fatty acid was extracted from the reaction mixture by adding 1.0 mL of Acetone-Ethanol (1:1) mixture solution. Three drops of phenolphthalein indicator

solution were added and the combination was titrated to an end-point of pink colour with a 0.05 M NaOH (Sodium hydroxide) solution (Li *et al*., 2019; Li *et al*., 2020). The blank contained 1.0 mL of boiled enzyme extract that was similarly treated as the enzyme extract above. The lipase activity was measured in micromoles of free fatty acids generated per milliliter of crude lipase enzyme from glycerol tributyrate as follows:

Lipase Activity (IU/mL) = (Vs- Vb) x N x 1000/ S

Vs = volume of 0.05M NaOH solution for Sample (enzyme-substrate cocktail)

Vb = volume of 0.05M NaOH solution for Blank (substrate cocktail control)

N = Normality of NaOH (0.05M)

S = volume of cocktail

The quantity of lipase needed to release one micromole (µmole) of fatty acids from triglycerides is known as one unit (u) of the enzyme (Li *et al*., 2019; Li *et al*., 2020).

2.5 Molecular Characterisation

DNA was extracted, and ITS rDNA was amplified and sequenced. Phylogenetic analysis was performed using MEGA X software (Kumar et al., 2018; White, 2022).

3. RESULTS

3.1 Diversity of Fungal Isolates

Sixteen isolates were obtained and distributed across genera including *Aspergillus, Rhizopus, Penicillium,* and *Trichoderma* (Figure 1). Different *Aspergillus* spp. including *Aspergillus niger, Aspergillus flavus, Aspergillus restrictus, Aspergillus terreus* and *Aspergillus tamarii* were found in three soil samples collected from the three of the four palm oil mills. Different *Penicillium* spp. including *Penicillium roquefortii, Penicillium restrictum* and *Penicillium spinulosum* were also found in three of the soil samples collected from the three of the four palm oil mills. Two *Rhizopus* spp. namely *Rhizopus arrhizus and Rhizopus microsporus* were found in two of the soil samples collected from the two of the four palm oil mills. Similarly, two *Trichoderma* spp. including *Trichoderma virens* and *Trichoderma asperellum* were recovered from two of the soil samples obtained from the two of the four palm oil mills. Two *Mucor* spp. specifically *Mucor racemosus* and *Mucor plumbeus* were isolated in two of the soil samples collected from the two of the four palm oil mills. *Aureobasidium pullulans* was the only member of its genus found in only one of the soil samples collected from one of the four palm oil mills. In the same vein, *Curvularia geniculata* was also the only member of its genus found in only one of the soil samples collected from one of the four palm oil mills.

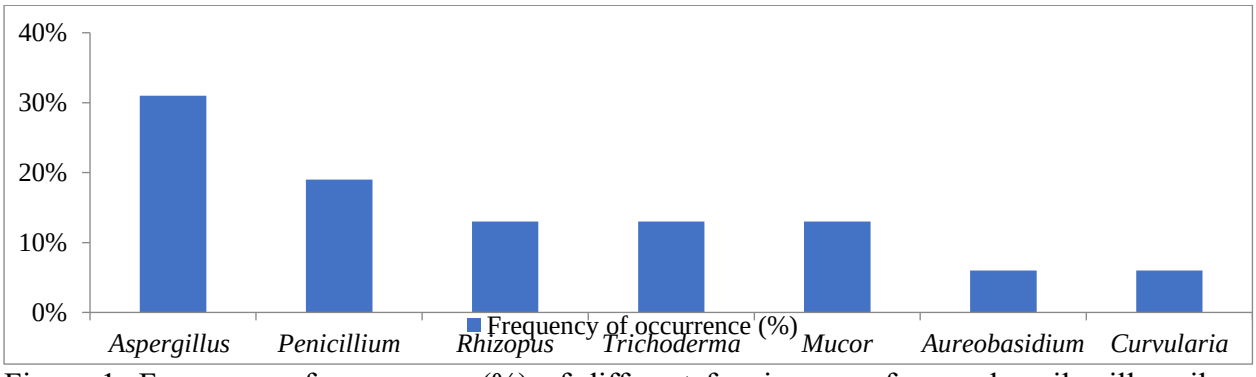


Figure 1: Frequency of occurrence (%) of different fungi genera from palm oil mills soil samples

Keys: Y-axis (vertical) – % frequency of occurrence of different fungi genera of isolates

X-axis (horizontal) – The seven named genera of fungi found in the soil samples

Screening for Lipolysis

Ten isolates showed significant lipolytic activity, as evidenced by clear zones on tributyrin agar (Figure 2).

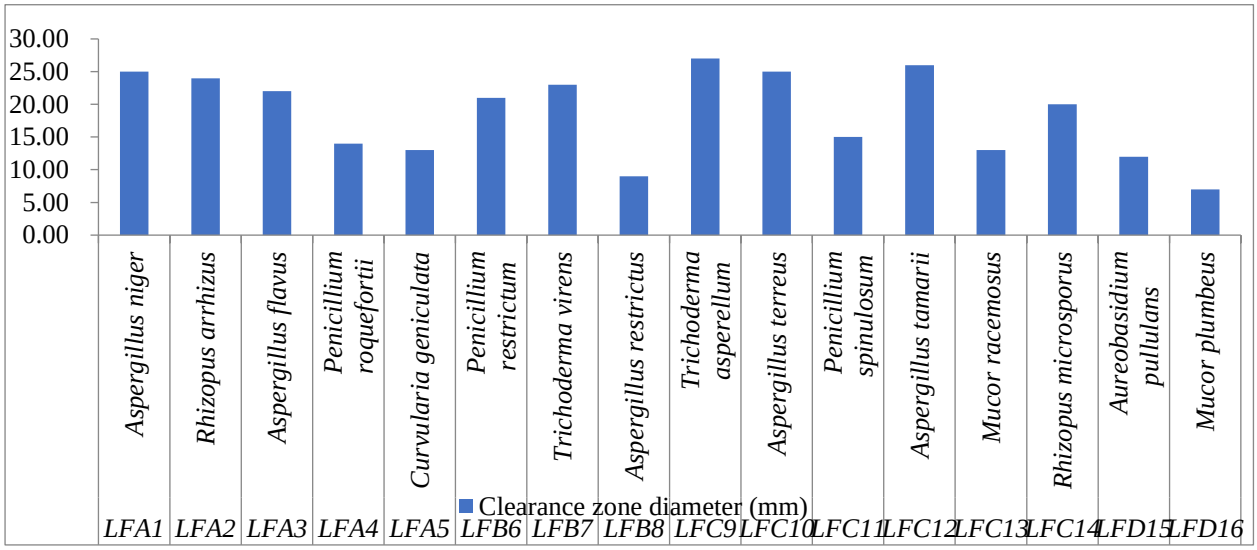


Figure 2: Lipolysis clearance zone of fungal isolates
Keys: LFA, B, C, D – Isolates code for Lipolytic Fungi from soil samples from palm oil mills A, B, C and D respectively
Y-axis (vertical) – Lipolysis clearance zone diameter values in mm of isolates
X-axis (horizontal) – Various isolates

3.2 Lipase Activity

Among the isolates, *Aspergillus flavus* (LFA3) showed the highest activity of 31.3 IU/mL (Figure 3).

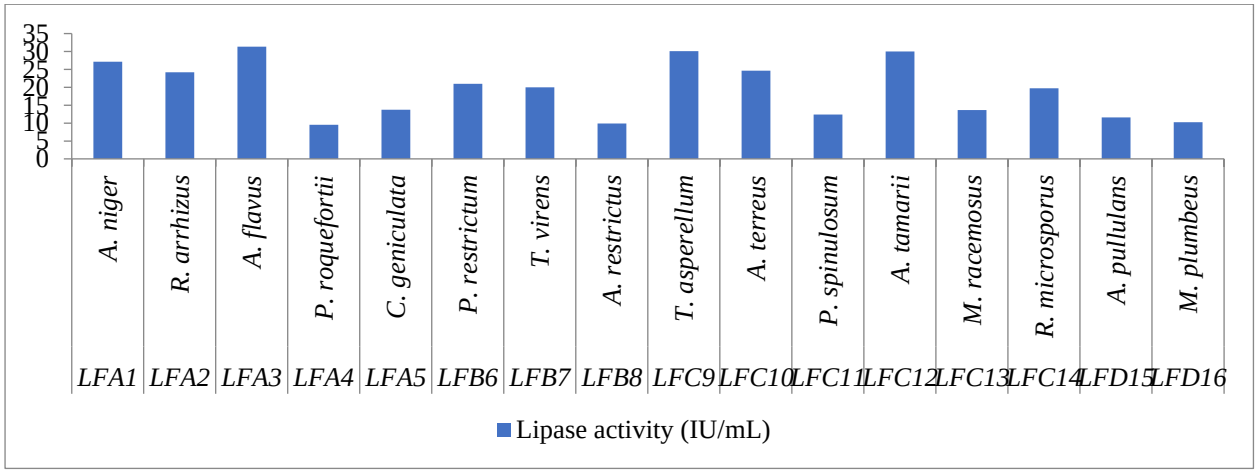


Figure 3: Lipase activity of fungal isolates
Keys: LFA, B, C, D – Isolates code Lipolytic Fungi from soil samples of palm oil mills A, B, C, D respectively
Y axis (vertical) – Lipase activity values (IU/mL) of isolates
X axis (horizontal) – Isolated lipolytic fungi

3.3 Effect of Temperature and pH

Lipase activity was optimal at 40 °C (Figure 4) and pH 7.0 (Figure 5), with reduced activity outside these ranges.

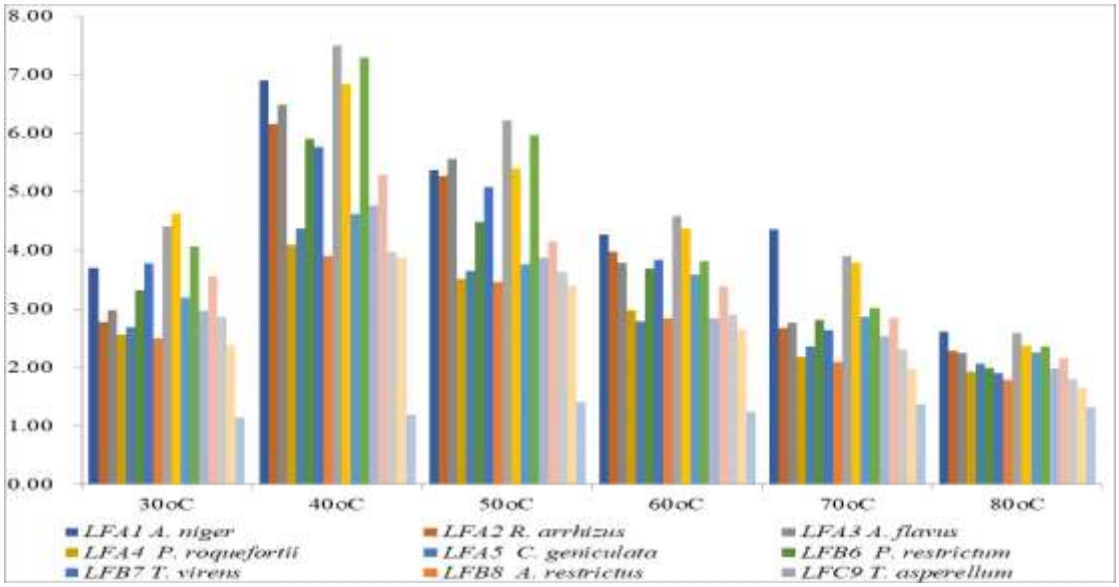


Figure 4: Effect of temperature on enzyme activity of fungal lipase
Keys: LFA, B, C, D – Isolates code for Lipolytic Fungi from soil samples from palm oil mills A, B, C and D respectively
Y-axis (vertical) – Enzyme activity mean values per mL of isolates
X-axis (horizontal) – Temperature range 30ºC – 80ºC

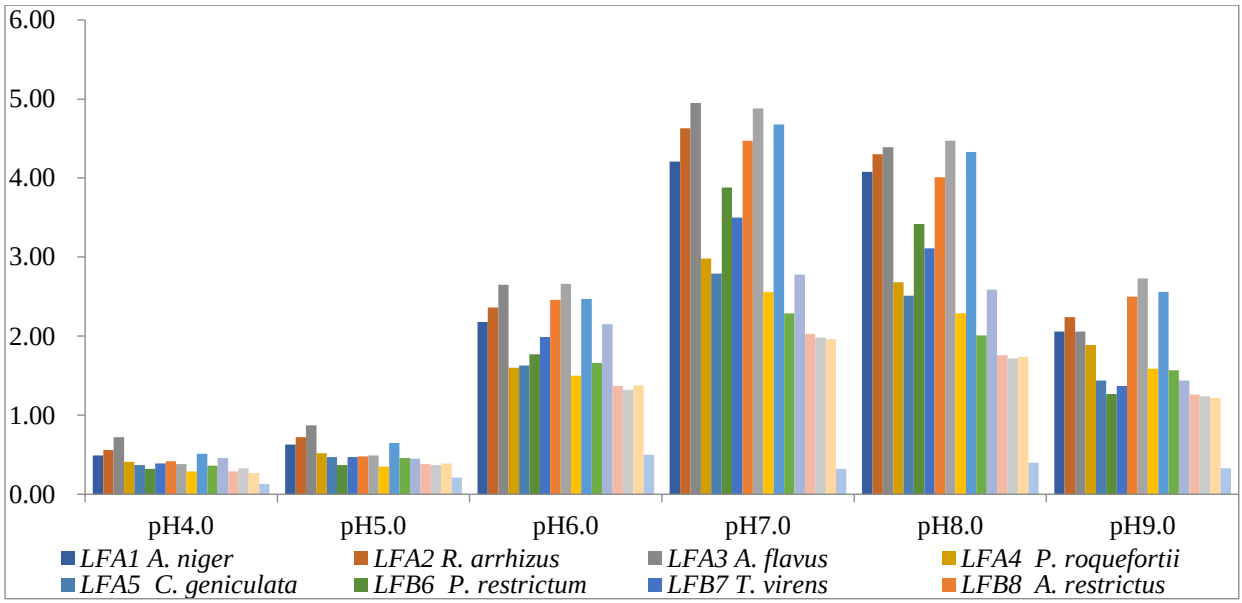


Figure 5: Effect of pH on enzyme activity of fungal lipase
Keys: LFA, B, C, D – Isolates code for Lipolytic Fungi from soil samples from palm oil mills A, B, C and D respectively
Y-axis (vertical) – Enzyme activity mean values per mL of isolates
X-axis (horizontal) – pH range 4.0 - 9.0

3.4 Molecular and Phylogenetic Analysis
Molecular sequencing confirmed five diverse key lipolytic fungi: *Aspergillus niger, Rhizopus arrhizus, Aspergillus flavus, Trichoderma virens,* and *Trichoderma asperellum* (Table 1). Phylogenetic trees showed close relationships with other known lipolytic strains (Figure 6).

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Table 1: The molecularly characterised lipolytic fungal isolates

Isolates code	Name of organisms	Accession number	% Sequence Identity
LFA1	*Aspergillus niger*	MN634444.1	89.00
LFA2	*Rhizopus arrhizus*	MN525245.1	99.00
LFA3	*Aspergillus flavus*	OR418540.1	98.00
LFB7	*Trichoderma virens*	MG650615.1	80.00
LFC9	*Trichoderma asperellum*	MN173868.1	90.00

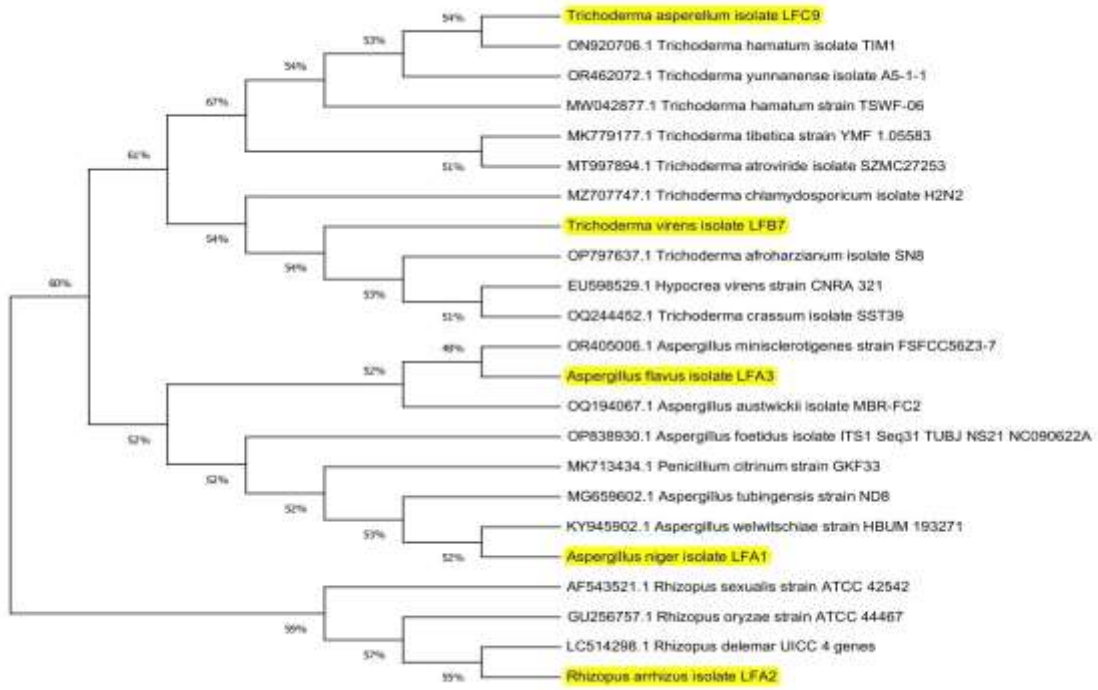


Figure 6: Phylogenetic tree of the molecularly characterised lipolytic fungal isolates

4. DISCUSSION

The dominance of *Aspergillus* and *Trichoderma* species among isolates agrees with previous studies on lipolytic fungi in oil-rich environments (Kumar, 2021; Wu, 2022). Several studies in the last few years have focused on the diversity of lipolytic fungi in various environments and found a comparable diversity of lipolytic fungi in soil samples, highlighting the importance of these fungi in lipid degradation processes (Sharma, 2019). The highest enzyme activity observed in *A. flavus* suggests its strong potential for industrial enzyme production (Gupta, 2023; Lee, 2024). Assessing lipolytic activity and the potential of microorganisms to produce lipases is a common practice in microbiology and biotechnology research (Javed, 2021). The observed variations highlight the diversity of lipase production capabilities among fungal isolates. Lipase activity under neutral pH and moderate

temperature aligns with industrial conditions for detergents and biodiesel production (Bedinger et al., 2019; Li, 2019).

The molecular confirmation of isolates enhances reliability and supports their future application in biotechnological processes (White, 2022). Species-level identification is crucial in understanding the functional diversity of lipolytic fungi. Recent studies (Gupta, 2023; Kumar, *et al*., 2018; Lee *et al*., 2021) have employed molecular techniques to identify lipolytic fungal species in various environments, enhancing our understanding of their metabolic capabilities and potential biotechnological applications.

Geographic variation in lipolytic fungal communities has been observed in other studies (Wu, 2022). Understanding this variation can aid in optimising the selection of fungal strains for specific biotechnological applications. These isolates can serve as potential sources of industrially valuable lipase enzymes.

5. CONCLUSION

A diverse array of lipolytic fungi were isolated from the soil samples of the palm oil mills, indicating a rich fungal biodiversity in these environments. The genus *Aspergillus* was the most frequently isolated, suggesting it may be particularly well-adapted to the conditions in palm oil mill soils and especially prolific in lipase production. *Trichoderma asperellum* (isolate LFC9) and *Aspergillus flavus* (isolate LFA3) exhibited the highest lipolytic activities, making them prime candidates for industrial applications. The characterisation and consequent identification of commercially viable lipolytic fungi abundantly replete in local environment from inexpensive renewable agro-resources will not only accelerate cottage industrial growth and create employment opportunities but can also become an additional source of revenue generation for government at all levels.

Molecular characterisation confirmed the identity of prolific lipolytic fungi, with high sequence identity percentages indicating more precise identification. This study demonstrates the rich diversity of lipolytic fungi in palm oil mills in Eyekanse and the potential for utilising these organisms in various biotechnological applications, particularly in lipase production and the treatment of palm oil mill effluents. Palm oil mill soils harbour diverse lipolytic fungi with strong potential for enzyme biotechnology.

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