

SCREENING AND MOLECULAR IDENTIFICATION OF LIPOLYTIC BACTERIA ASSOCIATED WITH A PALM OIL MILL EFFLUENT FROM A PALM OIL PROCESSING PLANT IN AGBOGBO, ILE-IFE, SOUTHWESTERN NIGERIA

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ABSTRACT

We characterized palm oil mill effluent (POME)-isolated bacteria capable of producing lipase. Water samples were collected and serially diluted, and bacteria were isolated from the diluted samples using standard methods. The bacteria were investigated for lipase production using tributyrin agar assay and subsequent fermentation and lipase enzyme analyses. Fifteen bacterial isolates were obtained from POME collected at Agbogbo village, Ile-Ife, Nigeria. Ten of the fifteen bacteria had lipolytic activity, with *Bacillus subtilis* PO13 having the highest zone of hydrolysis (39.67 ± 2.08 mm), followed by *Bacillus subtilis* PO1 (27.3 ± 1.53 mm) and *Acinetobacter johnsonii* PO3 (26.66 ± 1.53 mm). *Staphylococcus saprophyticus* had the smallest zone of hydrolysis of 11.00 ± 1.0 mm. Further analysis for lipolytic activity using submerged fermentation medium showed that *Morganella morganii* PO6 had the highest lipolytic activity of 20.67 ± 0.90 U/mL, followed by *Bacillus subtilis* PO13 with 19.13 ± 0.35 U/mL and *Bacillus subtilis* PO1 with 18.43 ± 0.57 U/mL, while *Bacillus cereus* PO15 had the least lipolytic activity of 3.20 ± 2.61 U/mL. The 10 lipase-producing bacteria characterized by colonial and biochemical characteristics were confirmed by 16S rRNA gene sequencing are *Bacillus subtilis* PO1 (PZ740870), *Bacillus altitudinis* PO2 (PZ740854), *Staphylococcus equorum* PO4 (PZ741005), *Staphylococcus saprophyticus* PO10 (PZ741035), *Acinetobacter johnsonii* PO3 (PZ740785), *Bacillus subtilis* PO13 (PZ740880), *Bacillus cereus* PO15 (PZ740867), *Klebsiella pneumoniae* PO9 (PZ740886), *Escherichia coli* PO7 (PZ740885), and *Morganella morganii* PO6 (PZ740970). The study concluded that the POME-receiving Agbogbo stream harbours lipase-producing bacteria that could be harnessed for oil-rich wastewater treatment.

INTRODUCTION

Lipases catalyze the hydrolysis of the ester bonds in triacylglycerol, yielding glycerol and free fatty acids (Patel *et al.*, 2019). Under specific conditions, lipases also catalyze reversible transformations, including esterification, interesterification, transesterification, alcoholysis, and aminolysis (Mukherjee, 2009). Lipases occur in all biospheres and are produced by microorganisms, plants, and animals (Ali *et al.*, 2023). However, microorganisms are preferred as sources of enzymes due to their shorter generation times, amenability to genetic manipulation, and the production of enzymes with stable, broad catalytic activities (Patel *et al.*, 2019). Lipases have attracted increasing interest due to their versatility as catalytic agents and their applicability in diverse industrial sectors, including food, agriculture, detergents, textiles, pharmaceuticals, biodegradation, and waste treatment (Patel *et al.*, 2019).

The palm oil processing industry is an agricultural enterprise in Nigeria and is primarily involved in extracting palm oil from the fruits borne on the bunch of the oil palm tree (Alhaji *et al.*, 2024). Typically, local palm oil processing industries are situated near perennial water sources, where water is

collected for use. The palm oil mills generate large quantities of extremely oily effluents, which are mostly discharged into surface water without treatment (Anieke *et al.*, 2016). Effluent discharge significantly pollutes water bodies, with the degree of impact depending on the type, source, and toxicity of the pollutants (Obi and Adebayo, 2019). In Southern Nigeria, palm oil mill effluent is a major pollutant in aquatic ecosystems. It has been documented to affect the eco-physiology of aquatic organisms and contributes to bioaccumulation in local water bodies (Anieke *et al.*, 2016). Palm oil mill effluent (POME) is a brownish colloidal slurry containing oil, suspended solids, fatty acids, proteins, carbohydrates, and plant materials. It has high organic nutrient content, which leads to substantial oxygen depletion in water. Thus, it poses a grave environmental threat (Anieke *et al.*, 2016; Obi and Adebayo, 2019). Pollution from POME is linked to its high biological oxygen demand, chemical oxygen demand, turbidity, and low pH (Adegbola *et al.*, 2020; Okereke and Ginikanwa, 2020). It causes dissolved oxygen depletion, produces undesirable odours, and triggers biological activities that negatively impact water bodies (Adegbola *et al.*, 2020). Indiscriminate discharge causes the death of aquatic organisms,

while excessive nutrients promote rapid aquatic growth and eutrophication (Okereke and Ginikanwa, 2020). The effluent can also contaminate groundwater by infiltrating through soil (Olatunji and Falode, 2018).

The search for new microbial strains with better copious amounts of lipase production potential for diverse industrial and biotechnological applications is a continuous process. As a result of its high lipid content, POME is a good source of lipolytic bacteria with high potential for lipase production that could be used for the treatment of the wastewater before disposal to the environment, or bioremediation of oil-degraded environments (Adegbola *et al.*, 2020). Several bacterial species, such as *Bacillus aryabhattai* SE3-PB (Adetunji and Olaniran, 2018), *Pseudomonas formosensis* TB5 (Sahoo *et al.*, 2021), and *Acinetobacter* sp. AU07 (Gururaj *et al.*, 2016) have been reported to express copious amounts of lipase. Therefore, this study entails the identification of lipolytic bacteria from POME collected from the palm oil processing plants in Agbogbo Village, Ile-Ife, Nigeria.

MATERIALS AND METHODS

Collection of samples

Raw POME samples were aseptically collected from the Palm Oil Processing Plant in Agbogbo Village at Ile-Ife, Osun State, Nigeria, using sterile bottles. The samples were kept in an ice box and taken to the Laboratory for bacteriological analysis.

Bacterial isolation

Bacteria were isolated from the effluent sample by serial dilution and the pour plate method. The serial dilution of the effluent sample was carried out from 10^{-1} to 10^{-6} dilution. One millilitre of each dilution was pipetted into each sterile Petri dish to which sterile nutrient agar was poured, allowed to solidify, and the Petri dishes were incubated at 37 °C for 24 h. After incubation, distinct colonies were selected and successively subcultured onto fresh nutrient agar plates until pure cultures of each isolate were obtained. The isolates were inoculated on nutrient agar slants and maintained at 4 °C for further studies.

Preliminary characterization of bacterial isolates

The bacteria were characterized based on their morphological and biochemical properties with reference to Harrigan and McCance (1976) and Cowan (1985). The morphological characteristics determined were Gram stain and spore stain reactions, while the biochemical characteristics were catalase, coagulase, citrate utilisation, starch hydrolysis, oxidative/fermentative, and nitrate

reduction tests. Others were the methyl red, Voges-Proskauer, sulphide-indole-motility, hydrogen sulphide, and sugar fermentation tests (Olutiola *et al.*, 2018).

Primary screening of bacterial isolates for lipase production

The bacterial strains were primarily screened for lipolytic activity (Stathopoulou *et al.*, 2013). The modified agar medium contained (in g/L): 10.0 g peptone, 5.0 g NaCl, 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 15.0 g agar, 0.5 g Congo red, and 1.0 mL Tween 80. The agar plates were spot-inoculated with each of the bacteria, and the plates were incubated at 35 °C for 48 h. Clear zones around the bacterial growth were indicative of lipase production. Distinct colonies that showed activity were picked and point-inoculated on tributyrin agar plates, which were incubated at 35 °C for 48 hours. Clear zones of hydrolysis around each colony was noted and measured. The bacterial isolates showing lipolytic activity by clearance zones of hydrolysis on the tributyrin agar plate were selected for further screening for lipase production using the submerged fermentation medium.

Secondary screening of bacterial isolates for lipase production

Quantitative screening of bacterial isolates for lipase production was carried out according to the method of Venkatesagowda *et al.* (2012) using the lipolytic fermentation medium containing (in g/L): peptone (0.2), $(\text{NH}_4)_2\text{HPO}_4$ (0.1), NaCl (0.25), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.04), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.04) and Tween 80 (2 drops) (pH 8.0). One millilitre of standardized bacterial inoculum was inoculated into 100 mL of lipolytic fermentation medium, incubated in an incubator shaker at 37 °C and 150 rpm for 24 h. Then, the broth culture was centrifuged at 10,000 rpm for 15 minutes, and the supernatant was used as a crude enzyme.

Assay for lipase

Lipase activity was determined by the measurement of p-nitrophenol release from p-nitrophenyl laurate cleavage (Winkler and Stuckmann, 1979). The assay mixture encompassed 700 μL para-nitrophenyl laurate solution and 300 μL crude lipase extract. The para-nitrophenyl laurate solution was prepared by adding solution A (0.001 g of p-nitrophenyl laurate dissolved in 1 mL isopropanol) and Solution B (0.01 g gum Arabic, 0.02 g Sodium deoxycholate, and 50 μL of Triton X-100 dissolved in 9 mL Tris-HCl buffer (pH 8.0). Absorbance of the reaction was measured at 410 nm over 3 minutes at 30 °C using a spectrophotometer. Enzyme activity was deduced

from the change in absorbance. A non-enzymatic reaction was taken as the control by replacing the enzyme with distilled water. Under the standard assay conditions, one unit of lipase represents the amount of enzyme that liberated 1.0 μmol p-nitrophenol per millilitre per minute (ϵ : 15000 cm^2/mol).

Molecular identification

The extraction of genomic DNA of the bacteria was performed by the method in the ZYMO Research DNA Purification Kit. The DNA was amplified using universal 16S rRNA gene primers: 27F: 5'(AGAGTTTGTGATCMTGGCTCAG) 3' and 1525R: 5' (AAGGAGGTGWTCCARCCGCA) 3'. The PCR conditions for the detection of 16S rRNA bacterial species were as described by Omoboye *et al.* (2025). Sequences were analyzed using BioEdit and submitted to GenBank. Phylogenetic analysis of the nucleotide sequences of the lipase-producing bacterial isolates was performed using the neighbour-joining model in MEGA 11 (Omoboye *et al.*, 2025).

RESULTS

Isolation and presumptive identification of bacteria

Fifteen bacterial isolates were obtained from the POME collected from the Palm Oil Processing Plant in Agbogbo village, Ile-Ife, Nigeria. Both Gram-positive and Gram-negative bacterial species were presumptively identified based on their morphological and biochemical characteristics. The bacteria include *Bacillus subtilis* PO1, *Bacillus altitudinis* PO2, *Staphylococcus equorum* PO4, *Staphylococcus saprophyticus* PO10, *Acinetobacter johnsonii* PO3, *Bacillus subtilis* PO13, and *Bacillus cereus* PO15, *Klebsiella pneumoniae* PO9, *Escherichia coli* PO7, and *Morganella morganii* (Table 1).

Primary screening and selection of bacterial isolates for lipase production

Ten out of the fifteen bacterial isolates exhibited lipolytic activity in the primary screening of the bacterial isolates for lipolytic activity using the agar

plate assay with tributyrin agar (Table 2). *Bacillus subtilis* PO13 had the highest zone of hydrolysis (39.67 ± 2.08 mm), followed by *Bacillus subtilis* PO1 (27.3 ± 1.53 mm) and *Acinetobacter johnsonii* PO3 (26.66 ± 1.53 mm). *Staphylococcus saprophyticus* had the least zone of hydrolysis of 11.00 ± 1.0 mm. However, five of the isolates did not produce any noticeable lipolytic activity (Table 2). The ten lipolytic bacterial isolates showing lipolytic activity by clearance zones of hydrolysis on tributyrin agar plate were selected for further screening for lipase production using the submerged fermentation medium.

Secondary screening of lipase-producing bacteria

The ten bacteria that showed lipolytic activity in the tributyrin agar plate assay were further screened for lipolytic activity using submerged fermentation medium. *Morganella morganii* PO6 exhibited the highest lipolytic activity of 20.67 ± 0.90 U/mL, followed by *Bacillus subtilis* PO13 with 19.13 ± 0.35 U/mL and *Bacillus subtilis* PO1 with an activity of 18.43 ± 0.57 U/mL, while *Bacillus cereus* PO15 exhibited the least lipolytic activity of 3.20 ± 2.61 U/mL (Table 3).

16S rRNA confirmed the bacterial identities

Gel electrophoresis showed the amplified products as presented in Figure 1. The sequencing of the 16S rRNA gene partial sequences confirmed the identity of ten lipase-producing isolates with their corresponding accession numbers after deposition in GenBank. The identified bacteria via phylogenetic analysis using similar strains in the NCBI database, included *Bacillus subtilis* PO1 (PZ740870), *Bacillus altitudinis* PO2 (PZ740854), *Staphylococcus equorum* PO4 (PZ741005), *Staphylococcus saprophyticus* PO10 (PZ741035), *Acinetobacter johnsonii* PO3 (PZ740785), *Bacillus subtilis* PO13 (PZ740880), and *Bacillus cereus* PO15 (PZ740867) (Figure 2). The other bacteria are *Klebsiella pneumoniae* PO9 (PZ740886), *Escherichia coli* PO7 (PZ740885), and *Morganella morganii* PO6 (PZ740970) (Figure 3).

Table 1. Biochemical and morphological characteristics of the bacterial isolates obtained from the palm oil effluents collected from a Palm Oil Mill Processing Plant in Agbogbo village

Isolate Codes	Cell Shape	Gram's Stain	CAT	TSI	SIM	CIT	MR	VP	GLU	MAL	MANN	SUC	LAC	O-F	NIT	Possible Organism
PO1	LR	+	++	NcNcNc	--+	+	-	+	Y	Y	Y	Y	N	F	+	<i>Bacillus subtilis</i>
PO2	LR	+	++	YNcNc	+--	+	-	+	Y	Y	Nc	Y	Y	F	++	<i>Bacillus altitudinis</i>
PO3	SR	-	+++	YNcNc	---	+	-	-	Nc	Nc	Nc	Nc	Nc	None	-	<i>Acinetobacter johnsonii</i>
PO4	Cocci	+	+	YNcNc	---	-	+	-	Y	Y	Nc	Nc	Nc	F	++	<i>Staphylococcus equorum</i>
PO5	R	-	+	YNcNc	---	+	-	+	Y	Y	Nc	Y	Y	F	+	<i>Klebsiella</i> sp.
PO6	R	-	+++	YG Nc +	+++	-	+	-	YG	-	-	-	-	F	+	<i>Morganella morganii</i>
PO7	R	-	+	NcNcNc	+--	-	+	-	Nc	Nc	Nc	Nc	+	OX	+	<i>Escherichia coli</i>
PO8	SR	-	+++	NcNcNc	---	-	-	-	Nc	Nc	Nc	Nc	Nc	None	-	<i>Acinetobacter</i> sp.
PO9	SR	-	+	YGYNc	---	+	-	+	YG	YG	YG	YG	YG	OX	+	<i>Klebsiella pneumoniae</i>
PO10	Cocci	+	++	YYNc	---	+	+	-	Y	Y	Y	Y	Nc	OX	+	<i>Staphylococcus saprophyticus</i>
PO11	SR	-	+++	NcNcNc	---	+	-	-	Nc	Nc	Nc	Nc	Nc	-	+	<i>Pseudomonas</i> sp.
PO12	LR	-	+	NcNcNc	--+	-	-	-	Nc	Nc	Nc	Nc	Nc	-	-	<i>Alkaligenes</i> sp.
PO13	LR	+	+++	YGNcNc	++	+	+	+	Y	Y	Y	Y	Nc	F	+	<i>Bacillus subtilis</i>
PO14	SR	-	+++	YGNcNc	---	+	+	-	Y	Y	Y	Y	Y	F	+	<i>Chromobacterium</i> sp.
PO15	LR	+	+	Y Nc Nc	---	+	-	+	Y	Y	Nc	Y	y	OX	+	<i>Bacillus cereus</i>

Key: SR = Short rod; MLR = Medium long rod; LR = Long rod; Y = Acid production only; YG = Acid and gas production; Nc = No change; M = Motility; F = Fermentative; Ox = Oxidative; H₂S = Hydrogen sulphide production; TSI: Triple Sugar Iron; SIM: Sulfide Indole Motility; CIT: Citrate; MR: Methyl red; VP: Voges Proskauer; GLU: Glucose; MAL: Maltose; MANN: Mannitol; SUC: Sucrose; LAC: Lactose; NIT: nitrate reduction; +++ = Vigorous Positive; + = Positive; - = Negative

Table 2 Primary screening and selection of bacterial isolates for lipase production

S/N	Code	Bacteria	Zone of clearance (mm)
1	PO1	<i>Bacillus subtilis</i>	27.3 ± 1.53
2	PO2	<i>Bacillus altitudinis</i>	19.33 ± 1.53
3	PO3	<i>Acinetobacter johnsonii</i>	26.66 ± 1.53
4	PO4	<i>Staphylococcus equorum</i>	13.33 ± 1.53
5	PO5	<i>Klebsiella</i> sp.	0
6	PO6	<i>Morganella morganii</i>	25.00 ± 1.00
7	PO7	<i>Escherichia coli</i>	16.67 ± 1.53
8	PO8	<i>Acinetobacter</i> sp.	0
9	PO9	<i>Klebsiella pneumoniae</i>	25.67 ± 1.15
10	PO10	<i>Staphylococcus saprophyticus</i>	11.00 ± 1.00
11	PO11	<i>Pseudomonas</i> sp.	0
12	PO12	<i>Alcaligenes</i> sp.	0
13	PO13	<i>Bacillus subtilis</i>	39.67 ± 2.08
14	PO14	<i>Chromobacterium</i> sp.	0
15	PO15	<i>Bacillus cereus</i>	15.00 ± 1.00

Table 3. Secondary screening of bacterial isolates for lipase production

S/N	Code	Bacteria	Lipase activity (U/mL)
1	PO1	<i>Bacillus subtilis</i>	18.43 ± 0.57
2	PO2	<i>Bacillus altitudinis</i>	13.50 ± 0.79
3	PO3	<i>Acinetobacter johnsonii</i>	7.90 ± 0.62
4	PO4	<i>Staphylococcus equorum</i>	12.30 ± 0.72
5	PO6	<i>Morganella morganii</i>	20.67 ± 0.90
6	PO7	<i>Escherichia coli</i>	16.03 ± 0.71
7	PO9	<i>Klebsiella pneumoniae</i>	13.90 ± 0.71
8	PO10	<i>Staphylococcus saprophyticus</i>	11.00 ± 1.00
9	PO13	<i>Bacillus subtilis</i>	19.13 ± 0.35
10	PO15	<i>Bacillus cereus</i>	3.20 ± 2.61

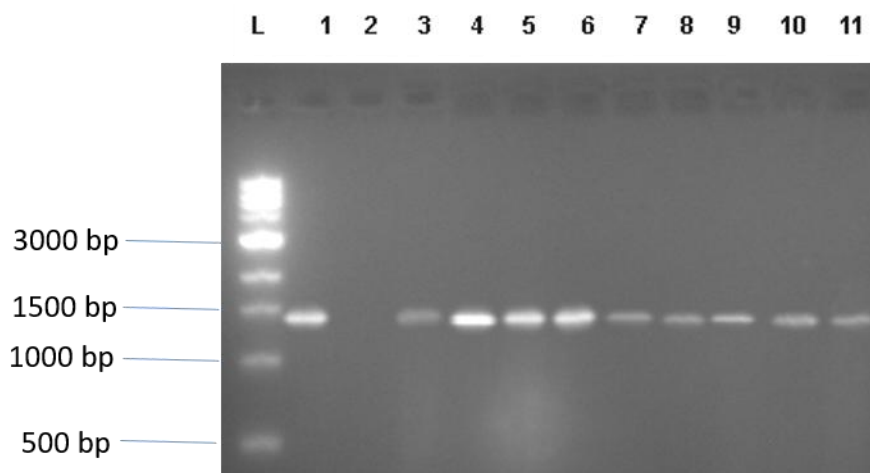


Figure 1. Agarose gel electrophoresis showing amplified products of 16S rRNA genes detected by PCR in bacterial isolates in water samples collected from Agbogbo Stream during the Period of study. Lane: 1 kb DNA ladder markers; Lane 1: *Bacillus subtilis* PO1; Lane 2: Blank (Sterile water); Lane 3: *Bacillus altitudinis* PO2; Lane 4: *Staphylococcus equorum* PO4; Lane 5: *Morganella morganii* PO6; Lane 6: *Staphylococcus Saprophyticus* PO10; Lane 7: *Acinetobacter johnsonii* PO3; Lane 8: *Bacillus subtilis* PO13; Lane 9: *Klebsiella pneumoniae* PO9; Lane 10: *Escherichia coli* PO7; Lane 11: *Bacillus cereus* PO15.

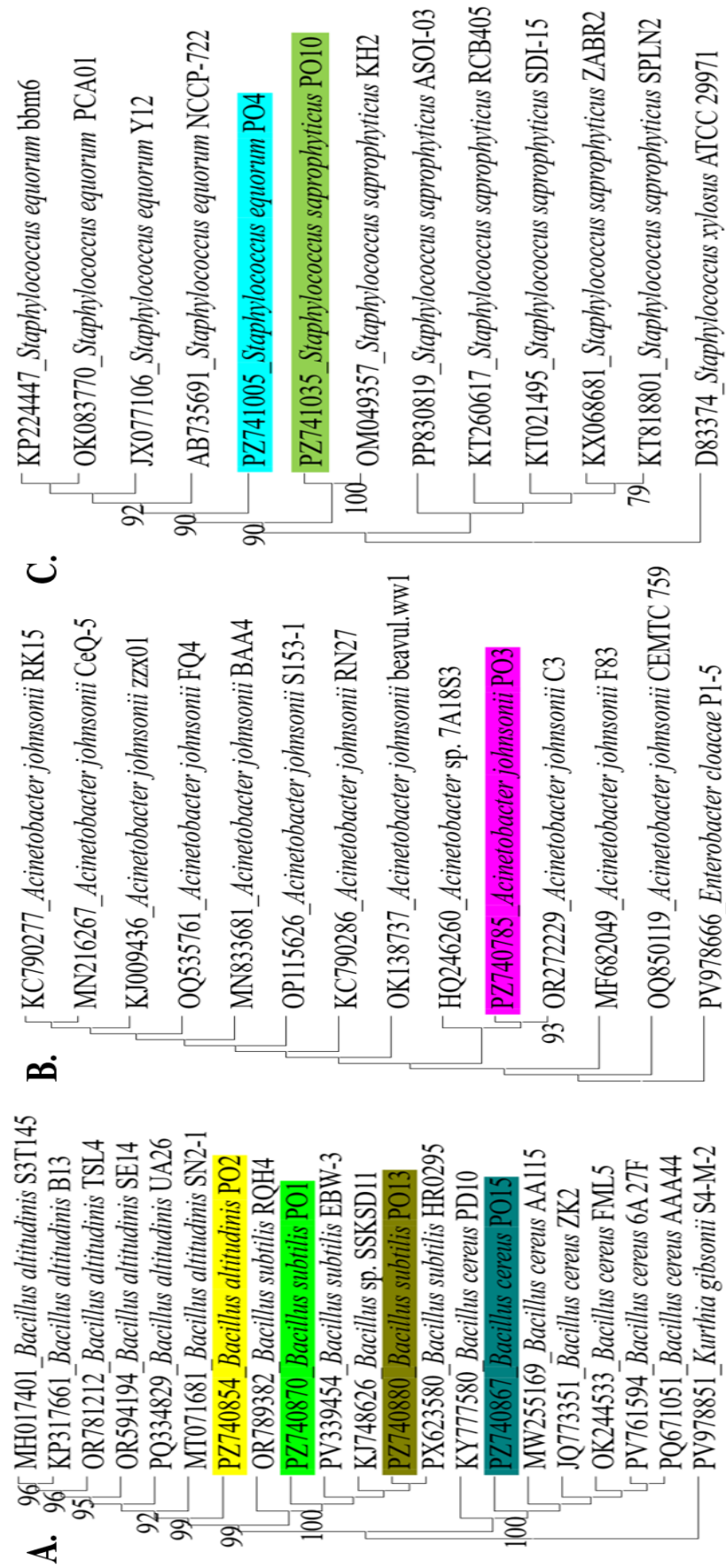


Figure 1. Bacterial 16S rRNA gene sequence-based phylogenetic tree of the study bacteria. Lipase-producing bacteria isolated and characterized in this study are highlighted in distinct colours. A. *Bacillus* strains. B. *Acinetobacter johnsonii*. C. *Staphylococcus* strains.

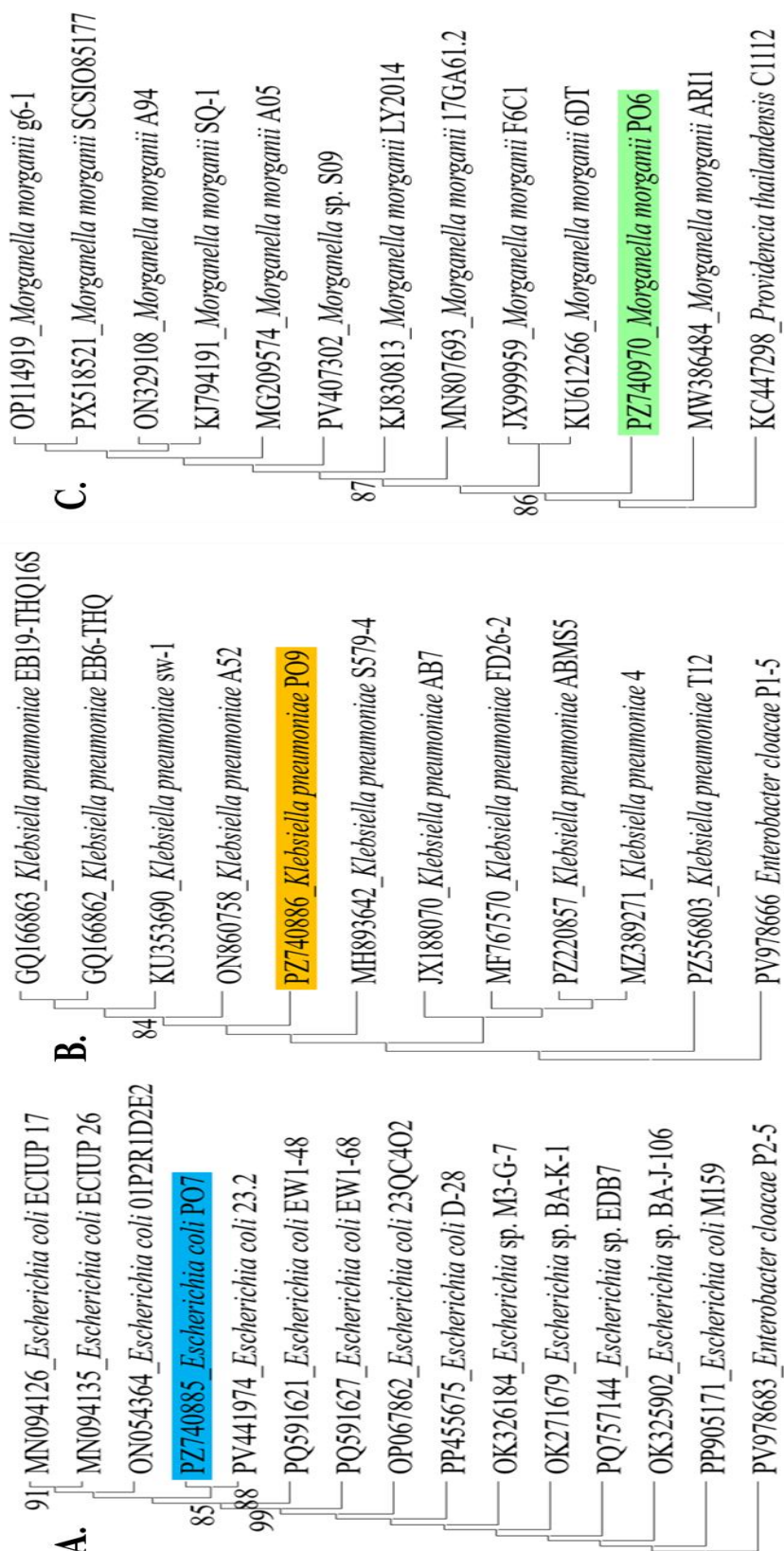


Figure 2. Bacterial 16S rRNA gene sequence-based phylogenetic tree of the study bacteria. Lipase-producing bacteria isolated and characterized in this study are highlighted in distinct colours. A. *Escherichia coli*. B. *Klebsiella pneumoniae*. C. *Morganella morganii*.

DISCUSSION

Fifteen bacterial isolates were obtained from POME obtained at a Palm Oil Processing Plant in Agbogbo Village, Ile-Ife, Nigeria. The biochemical characterization of the isolates revealed that they comprise Gram-positive and Gram-negative bacteria. The lipase-producing bacteria are predominantly *Bacillus* species (40%), encompassing *Bacillus altitudinis*, *Bacillus subtilis*, and *Bacillus cereus*. The predominance of *Bacillus* species among the lipase producers is consistent with prior reports (Adegbola *et al.*, 2020; Ezeonu *et al.*, 2020). This is similar to the reports of Lee *et al.* (2015), Ibiyemi and Julius (2022) and Popoola *et al.* (2022), respectively, in which 90%, 57.2% and 80% of the total bacterial isolates were lipase-producing *Bacillus* strains. Interestingly, *Bacillus subtilis* PO13 had the highest zone of clearance of 39.67 mm as recorded from the primary screening for lipase production using tributyrin agar assay. This observation is similar to the study of Ibiyemi and Julius (2022) where *Bacillus licheniformis* had a zone of clearance value of 34 mm. The other lipase-producing bacteria are *Acinetobacter johnsonii*, *Staphylococcus equorum*, *Morganella morganii*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Staphylococcus saprophyticus*. The isolation of lipase-producing bacteria from the POME-impacted stream is particularly significant. The oil present in POME serves as a carbon substrate that favours the proliferation of lipolytic microorganisms.

Successive secondary lipase production screening assays, using submerged fermentation, further confirmed the bacterial ability to produce lipase, with enzyme activity ranging from 3.20 ± 2.61 U/mL for *Bacillus cereus* PO15 to 20.67 ± 0.90 U/mL for *Morganella morganii* PO6. The lipase-producing bacteria possess the potential for bioremediation of lipid-rich pollutants in POME-contaminated environments, including waterbodies receiving the effluents. These bacteria and their enzymes possess notable industrial and biotechnological applications (Ibiyemi and Julius, 2022), including the degradation of fats and treatment of industrial wastewater.

The identities of the lipolytic bacteria were confirmed established using the 16S rRNA gene sequencing. The 16S rRNA gene sequence is very reliable for bacterial identification (Omoboye *et al.*, 2025). 16S rRNA gene sequencing-based molecular identification provided a more reliable means of identifying the bacteria than conventional biochemical methods. Phylogenetic analysis revealed the relatedness of the isolated lipase producers with well-characterized strains in the GenBank and other

public databases. Molecular techniques enhance the accuracy of taxonomic classification of bacteria (Ezeonu *et al.*, 2020; Adepoju and Adegbola, 2019).

CONCLUSION

The presence of lipase-producing bacteria in the POME-impacted waterbody suggests a potential for bioremediation of the stream's lipid-based pollutants. This study showed that lipase-producing bacteria have the potential for application in bioremediation of the water body. The results from this study can serve as fundamental informed management strategies to mitigate the indiscriminate discharge of POME. Further study on the extraction and characterization of lipase produced by bacteria identified in this study is highly recommended.

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CONFLICT OF INTEREST

None

AUTHORS' CONTRIBUTIONS

Ajadi, F. A.: Conceptualization, Methodology, Validation and Writing – Review & Editing; Omoboye, O. O.: Data curation, Software, Visualization, Methodology, Writing – Original Draft and Writing – Review & Editing; Oyedeji, O.: Validation and Writing – Review & Editing; and Oluduro, O. A.: Conceptualization, Supervision and Writing – Review & Editing.

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