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Molecular Identification and Characterization of Lipolytic Bacteria Isolated from Palm Oil Mill Effluent in Osun State, Nigeria

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ABSTRACT
This study focused on the molecular characterization of lipolytic bacteria isolated from *Agbogbo* stream impacted by Palm Oil Mill Effluent (POME) in Nigeria, utilizing 16S rRNA gene sequencing. Lipolytic bacteria play a crucial role in the degradation of lipids present in POME, offering potential for bioremediation. The study identifies and characterizes bacterial isolates with lipolytic activity, providing insights into their potential for bioremediation in POME-impacted environments. Raw POME samples and water samples were collected from the palm oil mill processing unit and designated sampling location respectively for bacteriological analysis. The pure bacterial isolates were further identified by microscopic and biochemical examination. Bacterial isolates were screened for lipolytic activity using tributyrin Agar by spot inoculation on modified agar medium and Molecular characterization was subsequently performed. A total of fifteen (15) bacterial species were isolated from palm oil processing effluent and screened for lipase production while ten (10) bacterial isolates were selected as the lipase-producing organisms and subjected to polymerase chain reaction amplification and 16S rRNA gene sequencing. The results showed that *Geobacillus* sp, *Bacillus altitudinis*, *Staphylococcus equoroum*, *Morganella morganii*, *Staphylococcus Saprophyticus*, *Acinetobacter johnsonii*, *Bacillus cabrialesii*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Bacillus cereus* were isolated across the stations of *Agbogbo* stream. The presence of diverse lipolytic bacteria in the POME-impacted stream suggests their potential role in the natural degradation of lipids, may possess significant bioremediation significances.

Keywords: Palm Oil Mill Effluent, Lipolytic Bacteria, Lipase activity, 16S rRNA sequencing and Bioremediation

INTRODUCTION

Palm Oil Mill Effluent (POME) poses a serious significant environmental challenge due to its high organic load and high acidic content due to its high content of free fatty acids, indicating that bacteria living in environment may have evolved to surviving under acidic conditions (Saputera *et al.*, 2021). Lipolytic bacteria are among microorganism found in POME. Lipolytic bacteria, offer a promising solution by hydrolyzing lipids capable of producing lipase enzymes, which can break down fats or oils by hydrolyzing ester bonds in triacyclgycerols water-soluble fatty acids and glycerol (Okore *et al.*, 2017). Therefore, lipase enzymes play a role in decomposing organic materials in the form of Oil (Ansar *et al.*, 2025). Because of its potential for biotechnology, Lipolytic enzymes are currently gaining attention. They are among one of the most important groups of biocatalysts used in biotechnology applications (Ajadi *et al.*, 2024). The study evaluated the impact of the Palm Oil Mill Effluents on selected physicochemical parameters and further, isolated and screened lipolytic activity of bacteria from Agbogbo stream, the primary receptor of these effluents. Molecular characterization, particularly 16S rRNA gene sequencing, provides a powerful tool for identifying and classifying bacterial species (Bady *et al.*, 2021). This study aimed to characterize lipolytic bacteria isolated from a POME-impacted stream in Nigeria using 16S rRNA gene sequencing. It was hypothesized that the harsh physicochemical conditions associated with POME contamination would favor lipolytic bacteria adapted to acidic, lipid-rich environments, and that molecular characterization would reveal a predominance of *Bacillus* and related genera with known lipase-producing and bioremediation potential.

MATERIALS AND METHODS

Sample Collection

Raw POME samples were collected directly from the palm oil mill effluent processing unit at Obafemi Awolowo University Ile-ife, Osun State, Nigeria. Two sets of subsurface water samples were collected from each sampling location. Water samples for bacteriological analysis were transported to the laboratory in ice boxes (Korotkov & Sandkvist, 2019). Lipase-producing bacteria were isolated from the palm oil processing effluent (Nwuche *et al.*, 2014). Serial dilution of the collected samples was carried out, and 1 mL of the diluents was plated on nutrient agar (NA) (Nwuche *et al.*, 2014). Distinctive colonies were sub-cultured onto fresh Nutrient Agar (NA) plates to obtain pure isolates. The pure bacterial isolates were further identified by microscopic and biochemical examination (Ibiyemi *et al.*, 2022). Bacterial isolates were screened for lipolytic activity by spot inoculation on modified agar medium. Lipolysis was indicated by the appearance of a clear zone of hydrolysis around the spot of inoculation. The diameters of the clearance zones around the colonies were measured after 24, 48, and 72 hours. The isolate with the highest lipolytic activity was stored on agar slant at 4^{o}C for further use (Ibiyemi *et al.*, 2022).

Genomic DNA was extracted using the boiling method previously described (Akpoka *et al.*, 2020). The 16S rRNA gene sequencing was amplified using universal primers. PCR amplification of the bacterial 16S rRNA gene was carried out

in an Applied Biosystem 2720 Thermocycler. The amplified fragments were sequenced using a genetic analyzer ABI 3500 sequencer (Abdullah & hassonni, 2020). 16S rRNA gene sequences were analyzed using the Basic Local Alignment Search Tool (BLAST).

RESULTS AND DISCUSSION

A total of fifteen (15) bacterial species were isolated from palm oil processing effluent and screened for lipase production. Ten (10) bacterial isolates showed lipolytic activities when assessed both qualitatively (agar plate cultures) using tributryin agar and quantitatively using liquid culture. *Serratia marcescens* exhibited the highest zone of hydrolysis, with the largest diameter of 38.00 and 42.00 mm at 48 and 72 hours of incubation, respectively. *Morganella morganii* had the highest enzyme activity level of 109.9 µ/ml.

Table 1: Biochemical Characteristics of the Bacterial Isolates Obtained from Water Samples Collected from Agbogbo Stream Sites during the Period of Study

Isolate Codes	Cell Shape	Gram's Stain	Cat	TSI	SIM	CIT	MR	VP	GLU	MAL	MANN	SUC	LAC	O-F
1D7	LR	-	++	NcNcNc	- - -	+	-	+	Nc	Nc	Nc	Nc	N	F
2A8	LR	-	++	YNcNc	+- -	+	-	+	Y	Y	Nc	Y	Y	F
5C5	SR	-	+++	YNcNc	- - -	+	-	-	Y	Nc	Nc	Nc	Nc	None
2B4	SR	-	+	YNcNc	- - -	-	+	-	Y	Y	Nc	Nc	Nc	F
2B12	LR	-	+	YNcNc	- - -	-	+	+	Y	Y	Nc	Y	Nc	F
2C11	LR	-	+++	YG Nc +	- ++	+	+	-	YG	YG	YG	YG	YG	F
3D5	SR	-	+	NcNcNc	- - -	+	-	-	Nc	Nc	Nc	Nc	Nc	OX
7A11	SR	-	+++	NcNcNc	- - -	-	-	-	Nc	Nc	Nc	Nc	Nc	None
7B7	MLR	-	+	YGYNc	- + -	+	+	-	YG	YG	YG	YG	Nc	OX
4D6	Cocci	+	++	YYNc	- - -	+	+	-	Y	Y	Y	Y	Nc	OX
3A5	SR	-	+++	NcNcNc	- - -	+	-	-	Nc	Nc	Nc	Nc	Nc	OX
3A1	LR	-	+	NcNcNc	- + -	-	-	-	Nc	Nc	Nc	Nc	Nc	-
5B5	SR	-	+++	YGNcNc	- +-	+	+	+	Y	Y	Y	Y	Nc	F
6B3	SR	-	+++	YGNcNc	- - -	+	+	-	Y	Y	Y	Y	Y	F
9B10	Cocci	-	-	Y Nc Nc	- - -	-	-	-	Y	Y	Nc	y	Y	OX

The gel electrophoresis showed that the band size of all the selected isolates corresponded to the expected amplicon size of approximately 1400 bp as indicated in Figure 1.

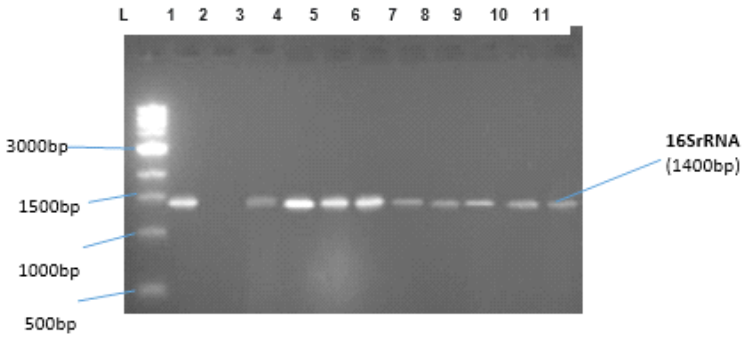

Figure 1: Agarose Gel Electrophoresis of PCR-Amplified 16S rRNA Gene Fragments

Discussion

The bacterial isolates recovered in this study showed marked phenotypic and taxonomic variation. Biochemical characterization revealed a predominance of rod-shaped bacteria, most of which were Gram-negative and catalase-positive, although Gram-positive and coccoid isolates were also recovered. The isolates differed considerably in carbohydrate utilization, TSI and SIM reactions, and oxidative–fermentative patterns, indicating variation in their metabolic capacities. Such heterogeneity is expected in environmental samples containing organic substrates of different composition and may account, at least in part, for the differences observed in their ability to produce extracellular lipase. However, the biochemical reactions should be viewed primarily as preliminary phenotypic characteristics because several bacterial taxa can share similar biochemical profiles.

The 16S rRNA gene analysis provided further resolution of the isolates and showed affiliation with *Geobacillus*, *Bacillus*, *Staphylococcus*, *Morganella*, *Acinetobacter*, *Klebsiella* and *Escherichia*. Sequence similarities with the closest GenBank entries ranged from 97.33 to 99.38%. Isolate 3D5 showed the highest similarity (99.38%) to *Escherichia coli*, followed by 1D10 (98.69%) in relation to *Bacillus cereus*. Other isolates were closely related to *Geobacillus sp.* (98.31%), *Bacillus altitudinis* (98.29%), *Staphylococcus sp.* (98.50%), *Morganella morganii* (98.14%), *Acinetobacter johnsonii* (97.58%), *Bacillus cabrialesii* (97.76%) and *Klebsiella pneumoniae* (97.33%). These results confirm that the isolates represented several bacterial lineages rather than a single dominant taxonomic group which also agrees with the findings (Ibiyemi & julius, 2022).

The sequence similarities, however, require careful interpretation. Although 16S rRNA sequencing remains an important first-line method for bacterial identification, it does not resolve all species boundaries equally well. A recent analysis of more than 19,000 type strains showed substantial

overlap in 16S rRNA sequence identity between species, and earlier genome-based comparisons placed the commonly used species-level boundary around 98.65%, while recommending whole-genome measures such as average nucleotide identity (ANI) for greater resolution. Accordingly, isolates such as 3D5, with 99.38% similarity to *E. coli*, have stronger support for species-level affiliation than isolates showing similarities of 97–98%. In particular, the 97.33% similarity observed for isolate 7B7 should be interpreted as close affiliation with *Klebsiella*, rather than definitive confirmation of *K. pneumoniae*. The same caution applies to other isolates for which the similarity falls below the commonly applied species-level range. This distinction is important because the purpose of molecular characterization is not simply to assign a name to an isolate, but to establish its taxonomic position at a level supported by the available evidence.

The molecular results also provide useful context for the lipase-screening findings. Ten of the 15 isolates produced detectable lipolytic activity, indicating that lipase production was relatively frequent among the culturable bacteria recovered in the study. Nevertheless, lipolytic activity varied substantially between isolates, showing that enzyme production was not uniform even among closely related organisms. This strain-level variation is biologically plausible because lipase expression depends on the genetic composition of individual strains and is strongly influenced by the carbon source, inducer concentration, pH, temperature, oxygen availability and other culture conditions. Thus, the presence of a bacterial taxon previously associated with lipase production does not, by itself, predict the level of enzyme production by a particular isolate.

The most striking result was the different ranking of isolates in the qualitative and quantitative assays. *Serratia marcescens* produced the largest tributyrin hydrolysis zones, reaching 38.00 mm at 48 h and 42.00 mm at 72 h, whereas the *Morganella morganii*-affiliated isolate recorded the highest quantitative lipase activity of 109.9 U/mL. This difference should not be regarded as contradictory. A hydrolysis zone on tributyrin agar reflects extracellular substrate degradation, but its diameter is also influenced by colony growth, enzyme diffusion through the agar, substrate accessibility and the physical properties of the medium. Quantitative enzyme assays measure catalytic activity under defined reaction conditions and therefore provide a different measure of enzyme performance. The present results consequently support the use of plate screening as an initial selection step, followed by quantitative assays for more reliable comparison of promising isolates.

The strong lipolytic response of *S. marcescens* is consistent with previous evidence that this species is capable of secreting extracellular lipase. The organism has been extensively studied as a source of bacterial lipase, and its extracellular enzyme secretion is associated with a dedicated secretion system. The strong tributyrin hydrolysis observed in the present study therefore agrees with the established lipolytic capacity of the species. However, the 42.00-mm hydrolysis zone obtained here should not be compared directly with zone diameters from other studies unless the substrate concentration, medium composition, inoculum size and incubation conditions are comparable. Differences in these factors can substantially alter the apparent size of the hydrolysis zone and may explain variations between studies.

The quantitative activity of 109.9 U/mL recorded for the *M. morganii*-affiliated isolate is particularly noteworthy because *Morganella* has received considerably less attention as a lipase-producing genus than commonly investigated groups such as *Bacillus*, *Pseudomonas* and *Serratia*. The result suggests that environmental screening can reveal strains with useful enzymatic properties outside the taxa traditionally emphasized in enzyme research. Nevertheless, the activity should be regarded as a property of the isolate under the conditions used in this study rather than as a characteristic of *M. morganii* generally. Additional experiments involving fermentation optimization, enzyme purification and biochemical characterization are necessary to establish whether the observed activity is reproducible and whether the enzyme possesses properties desirable for industrial application.

The recovery of several *Bacillus*-affiliated isolates is also consistent with the established importance of this genus in microbial enzyme production. In the present study, isolates related to *B. altitudinis*, *B. cabrialesii* and *B. cereus* were detected. This finding is supported by recent work showing that *B. cereus* can produce extracellular lipase using agro-industrial residues, with enzyme yields varying considerably according to the substrate employed. One recent study reported activities of 41.2 ± 1.08 U/mL and 40.5 ± 0.97 U/mL when soybean extract and bagasse, respectively, were used as substrates. Another recent study described a *B. cereus* lipase that remained stable at pH 10 and 50 °C and demonstrated potential for biodiesel production and detergent applications. These reports support the biotechnological relevance of the *Bacillus*-affiliated isolates recovered in the present study, although their actual industrial potential cannot be inferred from taxonomic identity alone.

The occurrence of *Geobacillus* sp. is similarly relevant because members of this genus are known for producing enzymes capable of functioning under relatively demanding conditions. Its recovery from the study environment raises the possibility that the isolate may produce enzymes with useful thermal or alkaline stability. However, such a possibility requires experimental confirmation. The present study establishes the taxonomic affiliation of the isolate but does not demonstrate thermostability, alkaliphilic activity or other properties that would justify an industrial claim which also supports the findings made by (Nazina *et al.*, 2024).

The ecological context of the isolates is also relevant to their lipolytic potential. Where sampling environments contain substantial lipid or organic loads, microorganisms capable of hydrolysing complex organic substrates may have an advantage because extracellular hydrolytic enzymes allow them to access otherwise unavailable carbon sources. The recovery of multiple lipolytic bacteria from the study environment is therefore biologically plausible. However, it would be inappropriate to conclude from the present data that exposure to palm-oil-derived substrates directly selected for lipase-producing bacteria. Demonstrating such selection would require comparison with reference environments and measurement of environmental variables associated with lipid availability.

The results also demonstrate why taxonomic identification and functional screening should not be treated as interchangeable approaches. Several taxa identified in the present study have previously been associated with extracellular hydrolytic enzymes, yet only 10 of the 15 isolates showed detectable lipolysis under the screening conditions. Conversely, the strongest quantitative activity was associated with the *M. morganii*-affiliated isolate, a tax on that is less commonly highlighted in bacterial lipase studies. This finding reinforces the value of functional screening of individual environmental isolates rather than restricting bioprospecting to bacterial groups already recognized for a particular enzyme.

Taken together, the biochemical, molecular and enzymatic findings indicate that the study environment contained a diverse collection of bacteria with variable metabolic and lipolytic capacities. The 16S rRNA results provided useful phylogenetic support for the isolate identities, while the enzyme assays revealed differences that could not be predicted from taxonomy alone. The pronounced tributyrin hydrolysis by *S. marcescens* and the highest quantitative activity of the *M. morganii*-affiliated isolate identify these strains as the most promising candidates for further investigation. Their selection should, however, be based on quantitative activity and subsequent optimization rather than hydrolysis-zone diameter alone.

The findings provide a useful starting point for developing bacterial lipase production from environmental isolates, but several limitations should be recognized. The 16S rRNA gene provides valuable taxonomic information but may not resolve closely related species; therefore, isolates with borderline sequence similarities should be reported as closely related taxa unless additional genomic evidence is available. Furthermore, the enzyme activities reported here represent production under the experimental conditions used and do not establish enzyme stability, substrate specificity or suitability for a particular industrial process. Further work should therefore focus on optimizing culture conditions for the leading isolates, characterizing the physicochemical properties of their lipases, and, where species-level taxonomic resolution is important, applying genome-based analyses such as ANI or digital DNA–DNA hybridization.

CONCLUSION

This study demonstrated that the investigated environment harboured diverse bacteria with significant potential for lipase production. Of the 15 isolates recovered, 10 exhibited lipolytic activity, confirming the presence of promising extracellular lipase producers. *Serratia marcescens* produced the largest tributyrin hydrolysis zones, reaching 38.00 and 42.00 mm after 48 and 72 h, respectively, whereas the *Morganella morganii*-affiliated isolate recorded the highest quantitative lipase activity (109.9 U/mL). Molecular characterization further revealed considerable bacterial diversity, supporting the value of environmental isolates as a source of potentially useful biocatalysts. These findings are relevant to the present economy because microbial lipases are important industrial enzymes with applications in food processing, detergents, biodiesel production and other biotechnological processes, while exploiting locally available bacterial resources may support indigenous enzyme production and value addition. The study therefore provides a foundation for developing locally sourced bacterial lipases with potential industrial applications. Further studies should optimize fermentation conditions and purify and characterize the enzymes produced by the most promising isolates to establish their stability, substrate specificity and suitability for targeted industrial applications.

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