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Isolation of Microorganisms Associated with Palm Oil Contaminated Soil

Popoola BM*, Famakinwa PB, Adeyemi OA

Ajayi Crowther University, Oyo, Oyo State, Nigeria

***Corresponding author:** Popoola BM, Ajayi Crowther University, Oyo, Oyo State, Nigeria. Tel: 08028428461, Email: bm.popoola@acu.edu.ng

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Abstract

Aim: Palm oil processing generally generates lots of wastewater (palm oil mill effluent), this is usually discharged into the environment in the untreated form and subsequently causes several environmental issues. There is therefore need to isolate microorganisms that can be used to clean up the palm oil contaminated environment especially the soil.

Methods and Results: Palm oil contaminated soil was obtained from Oba Adeyemi palm oil mill in Oyo, Oyo State, Nigeria, other soil samples which were purposely contaminated with palm oil, were obtained from Ajayi Crowther University Oyo, Oyo State. Isolation, characterization and identification of microorganisms were carried out using morphological and biochemical characterization. The isolates were preliminarily screened for lipolytic activities, this was confirmed by growth on the mineral salt medium after 7 days, signifying hydrolysis. One of the prominent isolates was further identified by sequences analysis of 16S rRNA genes. Forty-one bacterial isolates were identified, which included species of *Bacillus* (80 %), *Pseudomonas* (20 %) in the oil mill contaminated soil sample and *Bacillus* spp. (100 %) in the purposely contaminated soils. Twenty-nine fungal isolates including species of *Aspergillus, Oidiodendron, Geotrichum, Penicillum, Saccharomyces* were isolated with *Aspergillus fumigatus* having the highest frequency of occurrence (37.5 %) in artificially contaminated soil and *Saccharomyces* spp. having the highest frequency of occurrence (91 %) in palm oil contaminated soil from the palm oil mill. Sequencing of the 16S rRNA of one of the prominent isolates showed that it was identified as MN607220 *Saccharomyces cerevisae*. All the bacterial and fungal isolates had lipolytic activities except *Bacillus mycoides* and *Oidiodendron* sp. respectively. Nine of the ten *Saccharomyces* sp. had lipolytic activities.

Conclusion: These screened organisms could therefore be employed for the cleanup of palm oil contamination in the environment.

Significance and Impact of Study: Thereby ridding the environment of possible toxic effects especially in areas of need like Malaysia.

Key words: Palm Oil, *Saccharomyces Cerevisae*, *Bacillus* Spp. Contaminated, Identification, Lipolytic Activity

Introduction

Oil palm (*Elaeis guineensis*) is one of the species of palm oil normally called African oil palm or "macaw fat" [1]. This has been known to be the main source of palm oil, having its origin from West and South Africa, especially Angola and Gambia. This tropical plant is majorly grown for its industrial manufacture of vegetative oil [2]. They are single stemmed plant and grow 20m tall. The leaves are pinnate and get to 3-5m [3]. In spite of their useful economic value to national growth, the processing technique of palm oil has also contributed adversely to environmental pollution. This is as a result of very large quantity of liquids and solids waste generated during the extraction process of oil. Industrial release of effluents, especially effluents that does not undergo treatment before being discharged into the environment, could have profound impact on the biological and physicochemical properties of the soil and hence to the soil fertility [4].

Figure 1: The African Oil Palm *Eales guineensis*

The palm oil industry is a major agro based industry in some parts of the world, for instance, in Indonesia, being the largest producer of palm oil in the world, Malaysia and Nigeria, where you find palm oil trees in the wild and also in plantations. In ripe fruits processing, during oil extraction, large quantities of wastewater called palm oil mill effluent (POME) is produced [5]. In developing countries like Nigeria, extraction of palm oil is dominance among subsistence farmers who make use of the crude method of extraction. The generated POME is usually discharged unguided into the environment particularly to farmlands close by the oil mill [6]. The POME mixes with water, this unrecoverable oil will subsequently float to the surface, which will form a wide-spread film hence resulting in atmospheric oxygen not able to permeate the soil. This may result in the water body becoming depleted with dissolved oxygen [7]. Communities with close proximity with palm oil processing plant might likely suffer from odour emissions generated from the aged POME as a result of hydrogen sulphide and aromatic gases released into the environment [8].

An estimation was made that for 1 ton of produced crude palm oil, 5 - 7.5 tons of water eventually become POME [9]. An enormous quantity of the POME comes from the water used during processing [10]. POME, usually have high content of carbohydrates, proteins, fatty acids as well as other plant in its untreated form [11]. [12] stated that there is the alteration of environmental parameters such as dissolved oxygen, biological oxygen demand, chemical oxygen demand and carbon/nitrogen ratio as well as the general soil quality and moisture content when soil is exposed to POME. [13] showed that these parameters have effect on the microbial flora in the soil, which subsequently affects soil fertility.

Vegetable oils are primarily composed of fatty acids or triacylglycerols, these may be broken-down in their fresh state by marine bacteria [14]. The decomposition by the marine bacteria could be part of the reasons for the rancid odours, typical of vegetable oil spills [14]. Lipases, an enzyme responsible primarily for acylglycerides hydrolysis are responsible for this breakdown [15].

[16], demonstrated that bacteria could produce varied classes of lipolytic enzyme such as carboxylesterases, these hydrolyzes lipase and water-soluble esters known to hydrolyze substrates of long-chain triacylglycerol. Lipases of fungi have been considered as the best sources [17] due to their ability to produce lipase extracellularly [18]. Fungal lipases are more advantageous to bacterial lipases because the recent technology approves use of low-cost extraction methods and batch fermentation.

Lipases, because of their degradative abilities are of great importance in remediation especially in the degradation of lipid rich waste. However, due to the enzymes' thermal instability as well as the expensive cost of the single usage of such enzymes contributes to its drawbacks [19]. It is worth noting that initially both saturated and unsaturated fatty undergo biodegradation via β-oxidation process. Indeed, material biodegradation is also determined by the nature of the environment [20], where for instance, pH changes of soils, was noted to have detectable impact on the biodegradation of certain compounds [20].

The usefulness of microorganisms in protecting the environment cannot be overemphasized. The lipolytic activity of microorganism with diverse physiology could be used for the degradation of oil spills in the environment. It is therefore worth noting that oil palm and its processing set out as a substantial environmental challenge, which is of great economic relevance. Therefore, microorganisms are valuable in preserving the environment. It is of notable importance to isolate and identify high potential microbes for the biodegradation of pollutants such as palm oil mill effluent on land.

Hence, the aim of this study is to,

- Isolate microbes of high potential for the biodegradation of palm oil.

The objectives of this study therefore are,

- Isolation from palm oil contaminated soils.
- Characterization of the microbial isolates from the contaminated soil samples.
- Identification of the microorganisms.
- Preliminary screening of the isolates for lipolytic activity.

Materials and Methods

Sample Collection

Soil Samples: Soil samples were collected around the Department of Biological Sciences, Ajayi Crowther University. Samples were also collected from a palm oil mills factory located: Mobolaji Area, of 7°51" N,3°55" E Oyo, during the dry season. Samples were taken using a soil auger at various locations on site at a depth of about 10cm to ensure a broad spectrum of naturally -occurring microorganisms. Five hundred grams of the sample were weighed into 6 different clean bowls (holes were made beneath the bowl to allow for aeration).

Oil Samples: One liter of palm oil was purchased from the palm oil mill, 20ml of palm oil was thoroughly mixed into 2 bowls (20ml in each bowl), 10ml of palm oil was also mixed into 2 bowls and the 2 last bowls were left untouched serving as the control, the 6 bowls were left for 2months to check for the main effect of bioremediation [21].

Isolation and Culture Methods

Isolation of Organisms: Ten grams of each soil sample collected from the different bowls and control bowls (uncontaminated soil) and that from an oil mill were weighed into 90 ml of sterile distilled water in a 250 ml conical flask each. These were shaken intermittently for a period of 30minutes to dislodge organisms adhering to the soil particles. One milliliter of the solution was aseptically taken from the stock solution using sterile pipette into a tube containing 9 ml of sterile distilled water to make a 10^{-1} diluent factor. This was also mixed thoroughly and the process was repeated until 10^{-8} diluent factor was reached [22]. One millilitre inoculum was aseptically transferred into a sterile Petri dish and pour-plated with the appropriate agar medium: Nutrient agar for bacterial isolation and potato dextrose agar for fungi isolation. The plates were incubated at room temperature ($27^{O}C \pm 2^{O}C$) for 24hours for bacterial isolates. Fungal isolates were also incubated at the same temperature for 3 days.

Maintenance of Pure Cultures: The pure cultures of the organisms were maintained on nutrient agar slants and potatoes dextrose agar slants for bacterial and fungal isolates respectively and preserved at $4^{o}C$.

Identification of Bacterial and Fungal Isolates

Bacterial isolates were identified using their colonial morphological characteristics such as shape, size, elevation, colour etc. on the agar plates. Smears were prepared and stained for Gram's reaction and spore morphology which were then examined under the microscope for cellular and spore morphological characteristics. They were further identified using various biochemical tests such as catalase test, oxidase test, starch hydrolysis, voges proskauer (VP) test, and sugar fermentation etc. The fungal isolates were identified according to their micro-morphology, as well as colour and morphology of the sporulating structures. Glass slides preparations were done using lactophenol blue [22]. Microscopic examination of the prepared slide was carried out by the use of low power objective, after which the 40X magnification objective lens was also used. Yeast was also identified using microscopic examination and selected biochemical tests such as sugar fermentation, urease test, nitrate assimilation and growth in different temperature.

Preliminary Screening for Lipolytic Activity

The isolates were screened preliminarily by the utilization of a modified method of [23]. A mineral salt medium (MSM) containing KH_2PO_4 - 7.584 (g/l), K_2HPO_4 - 0.80 (g/l), $MgSO_4.7H_20$ - 0.80 (g/l), $CaCl_2$ - 0.16 (g/l), $(NH_4)_2NO_3$ - 0.80 (g/l), $FeSO_4$ - 0.16 (g/l) and 2% palm oil as carbon source with pH maintained at 7.0 was used for the preliminary screening. Five hundred milliliter (500 ml) of the mineral salt was dispensed into conical flask and then sterilized inside the autoclave at 121 ^{o}C for 15 minutes. After sterilization the medium was allowed to cool before pouring into sterile petri-dishes, the plates were allowed to solidify before streaking the isolates on the mineral salt medium plates. The plates were then subsequently incubated at $37^{o}C$ for 7 days for visible growth [23].

Molecular characterization

DNA Extraction: The DNA extraction from the fungi mycelia was carried out by the method of [24].

PCR Amplification: The Fungal isolate was characterized using the amplification of their Internal Transcribed Spacers (ITS). The forward (ITS-1F) and reverse (ITS-4R) primers is as shown, (check Table 1). [25] described the use of the PCR reactions in amplifying the ITS region of the rRNA operon.

Primer Sequence	5' 3' →
ITS1 (forward)	CTTGGTCATTTAGAGGAAGTAA
ITS4 (reverse)	TCCTCCGCTTATTGATATGC

Table 1: The ITS primer pair used in this study

Results

In this study, 70 microorganisms were isolated and included species of *Bacillus, Pseudomonas, Aspergillus, Geotrichum, Penicillium* and *Saccharomyces.* The frequency of occurrence of bacterial and fungal isolates from artificially-contaminated and palm oil mill site soil samples, showed *Bacillus cereus*, *Bacillus subtilis, Bacillus circulans* and *Pseudomonas fluorescenes*, with *Bacillus circulans* having the highest frequency of occurrence (33 %) in the artificially-contaminated soil sample (Table 2). *Saccharomyces* spp. had the highest frequency of occurrence of fungal isolates with 91 % in palm oil mill soil sample while *Aspergillus fumigatus* had the highest frequency of occurrence (37.5 %) in the artificially-contaminated soil sample (Table 3).

The total heterotrophic count of microorganisms isolated from palm oil mill site (Oba Adeyemi palm mill, Mabolaje) had bacterial count of 19.6591($\log_{10}$Cfu/ml) and a fungal count of 16.0944 ($\log_{10}$Cfu/ml) (Table 4).

	TYPE OF SOIL/FREQUENCY OF OCCURRENCE		
Isolate Name	Control soil sample	Artificially-contaminated	Contaminated soil from oil mill site
Pseudomonas fluorescens	-	-	2(20)
Bacillus cereus	-	1(6)	-
Bacillus circulans	3(23)	6(33)	2(20)
Bacillus licheniformis	2(15)	1(6)	2(20)
Bacillus megaterium	1(8)	2(11)	-
Bacillus mycoides	6(46)	1(6)	1(10)
Bacillus pumilus	-	-	1(10)
Bacillus subtilis	-	3(17)	2(20)
Bacillus sp.	1(8)	4(22)	-
Total	13(100)	18(100)	10(100)

Values in parentheses represent percentage of occurrence Key, - = no occurrence

Table 2: Frequency of occurrence of bacterial isolates from artificially-contaminated and oil mill site soil samples

	TYPE OF SOIL/FREQUENCY OF OCCURRENCE		
Isolate Name	Control soil sample	Artificially-Contaminated	Contaminated soil from oil mill site
Aspergillus niger	-	-	1(9)
Aspergillus fumigatus	2(20)	3(37.5)	-
Geotrichum candidum	1(10)	-	-
Aspergillus ochraceous	2(20)	1(12.5)	-
Aspergillus sp.	-	1(12.5)	-
Penicillium sp.	3(30)	-	-
Oidiodendron sp.	-	1(12.5)	-
Penicillium sp.	1(10)	1(12.5)	-
Saccharomyces spp.	1(10)	1(12.5)	10(91)
Total	10(100)	8(100)	11(100)

Values in parentheses represent percentage of occurrence Keys, - = no occurrence

Table 3: Frequency of occurrence of fungal isolates from artificially-contaminated and oil mill site soil samples

Microbial Group	Total Count (log_{10}Cfu/ml)
Bacteria	19.6591
Fungi	16.0944

Table 4: Total heterotrophic counts of isolates of soil sample from the oil mill

Microbial group/count (log_{10}Cfu/ml)				
Sample description/ Vol. of palm oil	Heterotrophic bacteria count Heterotrophic fungal count		Day of sample analysis Day of sample analysis	
	Day 0	Day 60	Day 0	Day 60
Sample B	22.2730	20.0719	10.3779	10.2527
Sample C	21.9338	15.3389	10.0496	6.7663
Sample D	18.6755	13.8629	9.6951	3.2958

Key, Sample B- Control, Sample C- Soil sample contaminated with 10ml of palm oil, Sample D- Soil sample contaminated with 20ml of palm oil.

Table 5: Total microbial count in soil samples contaminated with different measurement of palm oil

The qualitative preliminary screening for the lipolytic activity of bacterial, fungal and yeast isolates are shown on table 6. Table 7 shows the molecular characterization of the *Saccharomyces cerevisiae* isolate (SA9), the result yielded a close proximity to *Saccharomyces cerevisae* showing 99 % identity with accession number MN545451.1.

ISOLATE	LIPOLYTIC ACTIVITY
Bacillus licheniformis	+
Bacillus circulans	+
Pseudomonas fluorescens	+
Bacillus mycoides	-
Bacillus licheniformis	+
Pseudomonas fluorescens	+
Bacillus circulans	+
Bacillus subtilis	+
Bacillus subtilis	+
Bacillus pumilus	+
Aspergillus niger	+
SA1	+
SA2	+
SA3	+
SA4	-
SA5	+
SA6	+
SA7	+
SA8	+
SA9	+
SA10	+

Key: + Positive Presence of halos around the colonies, SA= *Saccharomyces* spp.

Table 6: Qualitative lipolytic activity of the microbial isolates from palm oil mill

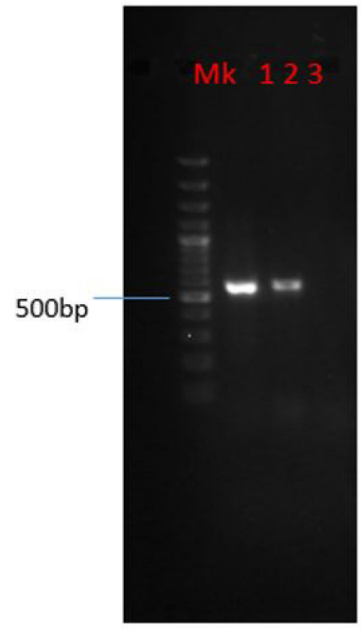

Figure 2: Agarose Gel Electrophoresis - indicating a positive amplification of fungi isolate using ITS region-specific Universal Primers

Band size of 550bp indicates a positive amplification in fungi samples.

KEY: Loading arrangement

MK- Molecular marker, 1-sample SA9, 2-positive control, 3-buffer control

S/N	Isolate code	Closely related fungal sequence	% identity	Accession no.	New accession no.	New strain name
1	SA9	*Saccharomyces cerevisiae*.	99.32	MN545451.1	MN607220	OYB-3

Table 7: Molecular characterization of the *Saccharomyces cerevisiae*

Plate 1: Qualitative lipolytic activity for *Saccharomyces spp*

Plate 2: Qualitative Lipolytic activity of *Aspergillus fumgatus, Aspergillus sp., Pencillium sp*

Discussion

In this study bacteria were the most predominant microorganisms with a frequency of occurrence of 80 % for *Bacillus* sp. *and* 20 % for *Pseudomonans fluorescens* in the soil sample from palm oil mill factory while *Saccharomyces* spp. (91 %) had the highest frequency of occurrence, with *Aspergillus niger* (9 %) having the lowest frequency of occurrence for fungal isolates from the palm oil mill, [26] found *Bacillus* sp. *and Apergillus niger* as the organisms with most frequent occurrence in a palm oil contaminated soil. Also [27] found *Saccharomyces cerevisae* to be one of the predominant terrestrial lipase-producing yeasts. This study also showed that the yeasts isolated had the capability to adapt to this changed environmental condition as they had high frequency of occurrence in the soil sample isolated from palm oil mill site, in the artificially-contaminated soil sample there was no yeast found on the day of isolation but after 60 days they were present.

In the artificially-contaminated soil samples, from which 100 % of *Bacillus* spp. was isolated, *Bacillus circulans* (33 %) had the highest frequency of occurrence for the bacterial isolates, while *Aspergillus fumigatus* (37.5 %) had the highest frequency of occurrence for the fungal isolates, this is in conformity with the work of [28] where *Bacillus* sp and *Aspergillus niger* had the highest frequency of occurrence for both the bacterial and fungal isolates.

As observed from this study, the microbial frequency of occurrence is so varied between the artificially contaminated soil and oil mill soil, this could be most likely due to the fact that natural degradation has been on in the sampling environment (oil mill soil) before this experiment as compared to the artificially contaminated whose time frame for biodegradation is shorter. The microbial isolates from the oil mill soil could therefore be more potent degraders as a result of their longer exposer to the contaminant.

The soil sample mixed with 10 ml of palm oil had a higher bacterial count after 60 days compared to the soil sample mixed with 20 ml of palm oil (Table 5) and this could be as a result of increase in the palm oil quantity, also these two soil samples had relatively low bacterial count than the control soil sample and this could be a function of the inability of some microbes to survive or adapt to the changed environmental conditions as a result of the introduction of palm oil.

Furthermore, the soil sample mixed with 10 ml of palm oil had a higher fungal count after 60 days compared to the soil sample mixed with 20 ml of palm oil, this could also be a function of the quantity of palm oil introduced, also these two soil samples had relatively low fungal count than the control soil sample, a function of some microorganisms inability to survive the environmental changes. The study shows that the soil where palm oil waste was frequently discharged had scanty fungal population and diversity this corroborates the work of [29].

Generally, in this study species of *Bacillus* and *Pseudomonas, Oidiodendron, Geotrichum, Saccharomyces*, *Aspergillus* and *Penicillium* were isolated from palm oil contaminated soil samples, several researchers have isolated or reported various microbial species from palm oil contaminated soils similar to those isolated from this study, such as [29], [30], [26], [28], [31].

The qualitative preliminary screening for the lipolytic activity of the isolates was undertaken by carrying out assay using agar plate, different strategies for screening have actually been suggested for lipase activity determination, however, assay method by the use of agar plates are highly suggested in view of the fact that it is an easier method and relatively less expensive [32]. The use of agar plates for the assay method might also be advantageous because of the difficulty in the determination of lipase activities since it has to do with the action of a water-soluble enzyme on an insoluble substrate. However, quantitative enzymatic activity measurement (pH-stat titration) [33] have been generally used for quantifying lipolytic activity, but for this preliminary study, the qualitative method of determination was employed.

The bacterial isolates, *Bacillus circulans*, *Bacillus subtilis*, *Bacillus licheniformis, Bacillus megaterium* and *Pseudomonas fluorescens* had lipolytic activities and *Bacillus mycoides* had no lipolytic activity, this corroborates the findings of [28], where *Bacillus subtilis, Pseudomonas fluorescens* had lipolytic activities. The fungal isolates *Aspergillus fumigatus, Aspergillus ochraceous, Geotrichum candidum, Penicillium* sp., had lipolytic activities as well as *Saccharomyces* spp. whereas *Oidiodendron* sp. had a negative lipolytic activity signifying no lipolytic activity.

The 16S rDNA gene of isolate SA9 (being the most prominent) was amplified by PCR primer sequence ITS1 (50-CTTGGTCATTTAGAGGAAGTAA-30) and ITS4 (50-TCCTCCGCTTATTGATATGC-30). The purified amplicons and partial sequences of the genes were sequenced by the use of the forward and backward primer and were subsequently blasted on the NCBI database for further identification of isolate (SA9), the result yielded a close proximity to *Saccharomyces cerevisae* showing 99 % identity with accession number MN545451.1 and also the newly generated accession number from the NCBI (National Centre for Biotechnology Information) for the isolate is MN607220, check Table 6.

This research study showed that species of *Bacillus, Pseudomonas, Aspergillus, Penicillium, Geotrichum* and *Oidiodendron* were isolated from palm oil contaminated soil from Oba Adeyemi Palm Oil Mill, Oyo, Oyo State, Nigeria and artificially-contaminated soil samples from the Faculty of Natural Sciences, Ajayi Crowther University, Oyo, Oyo State, Nigeria. The result of the study revealed the possibility of isolating lipase producers that are capable of degrading the palm oil in the palm oil contaminated soils. The microbial isolates in this study proved capable of lipase production. Lipolytic activities have indeed been observed for pure cultures of all the microbial genera used in this study.\

Conclusion and Future Prospect

This preliminary study aimed at assessing potential microbial isolates which could be helpful in degrading lipid wastes from oil mills and oil contaminated soils. In the near future, this would definitely help to organise a cleaner environment with waste utilisation by these microbial species.

This implies that with time, given favourable conditions, these microorganisms could naturally aid the degradation process in vegetable oil polluted soil. Effective bioremediation could be achieved within 2 to 8 weeks of bio-treatment after which an additional measure like additional inoculum application would be required for prolonged biodegradation process.

These microbial lipases studied are very paramount group of valuable enzymes of great biotechnology relevance, hence more attention should be given to explore or investigate these microbial lipases towards fatty waste degradation, furthermore, these microorganisms producing lipase can therefore be usefully and gainfully employed in the treatment of fatty waste contaminated sites.

Conflict of Interest

The authors declared no conflict of interest in this manuscript.

Authors Contribution

First Author: Concept and design of the study, Methodology, Writing of the Manuscript, Part of Laboratory Analysis.

Second Author: Contributory towards Laboratory Analysis, Writing Part of Manuscript.

Third Author: Contributory towards Methodology, Writing Part of Manuscript.

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