Original Research Article

Screening, Characterization and Identification of sophorolipid-producing yeast isolated from Palm oil effluent polluted soil

Abstract

Palm oil mill activities in Nigeria have gained socio-economic value in recent times. The waste management challenges for handling effluent from these activities have remained unresolved. Palm oil mill effluent impacted soil were obtained (Elibrada and Rumuche) in Rivers State and analyzed for Physicochemical, Geotechnical and Microbiological qualities. The yeast isolates were screened for hydrocarbon degradation and sophorolipid production; and were identified using biochemical and 16S ITS molecular approaches. The pH of the POME-impacted soil ranged between pH 6.18 to 6.34 the electrical conductivity value for Elibrada and Rumuche were 20.84 µS/cm and 80.19 µS/cm respectively while that of the unpolluted soil was 220.13 µS/cm. Permeability of the soil from Elibrada was 2.7 cm/s, Rumuche had 1.3 cm/s while the unpolluted soil had 5.6 cm/s. Total organic carbon for soil samples from Rumuche was 4.92%, Elibrada had 6.13% while the unpolluted soil had 8.74%. Oil and grease component for the Rumuche soil was 10500 mg/kg, Elibrada had 7200 mg/kg and the unpolluted soil had 28.0 mg/kg. Total fungal count for Rumuche soil samples was 3.8×10^4 CFU/g while Elibrada had 3.2×10^4 CFU/g. Eighty percent (80%) of the isolates were *Candida* spp. while 20% were *Saccharomyces* sp. There were four bands separated which were denoted as ITS 600bp and a Ladder L which was 500bp. The phylogenetic construct showed that the Y8 was 70.2% similar to *Candida parapsilosis*. The isolate Y3 was 95.8% similar to *Candida haemulonis*. Isolate Y9 had 100% similarity with *Pichia Kudriavzevii* while Y13 had a 97.4% identity with *Saccharomyces cerevisiae* the GENBANK accession numbers were *Candida haemulonis* MW182014, *Candida parapsilosis* MW182015, *Pichia Kudriavzevii* MW182016 and *Saccharomyces cerevisiae* MW182017. There is an increasing concern to develop a process for microbial isolation and characterization for effective biotechnological advancement using impacted environmental media as a bioresource.

Kay words: Palm oil, polluted soil, Screening, sophorolipid, yeast isolated.

Introduction and Background

Sophorolipids are surfactant-like biopolymers. Surfactants are amphiphilic compounds that lower the system's free energy by displacing higher-energy bulk molecules at an interface (Santos *et al*., 2016; Rufino *et al.,* 2014). It is composed of a hydrophobic moiety that has a low affinity for the bulk aqueous medium and a hydrophilic portion that is attracted to it. Industrially, surfactants have been used as flocculating, wetting, and foaming agents, adhesives and de-emulsifiers, lubricants, and penetrants (Nishanthi *et al*., 2010). The ability of surfactants to reduce surface

tension is a critical property. Surfactants tend to accumulate at interfaces (air-water and oil-water) and on surfaces due to their amphiphilic nature. As a result, surfactants reduce the repulsion forces between dissimilar phases at interfaces or surfaces, allowing the two phases to mix more easily (Kale and Deore, 2017). Biosurfactants are a structurally diverse class of molecules with surface-active properties that are synthesized by living organisms. Their ability to reduce surface and interfacial tension while exhibiting low toxicity, high specificity, and biodegradability has sparked increased interest in these microbial products as potential replacements for chemical surfactants (Vijayakumar and Saravanan, 2015; Okoliegbe and Agarry 2012). Biosurfactants are amphiphilic compounds produced by a wide variety of microorganisms that either adhere to the cellular membrane or are excreted into the culture medium extracellularly (Liu *et al*., 2015). They can reduce the surface and interfacial tensions in oil-water systems and are used in a variety of industrial applications as moistening agents, dispersants, emulsifiers, foaming agents, and detergents (Elazzazy *et al.*, 2015). These compounds outperform conventional synthetic surfactants in terms of low toxicity, improved environmental compatibility, increased intersurface activity, increased foaming capability, increased selectivity, and improved biodegradability (Kavitha *et al.*, 2014; Souza *et al.*, 2014).

Microbial surfactants, also known as biosurfactants, are a class of surface-active molecules derived from a diverse array of microorganisms. These surface-active compounds produced by bacteria have the ability to decrease the surface and interfacial tension between two immiscible fluid phases. It is only in the last few decades that surface-active microbial molecules, dubbed biosurfactants, have garnered widespread interest (Praveesh *et al*., 2011). Secondary metabolites, biosurfactants are produced during the stationary phase of microbial growth (Yan *et al*., 2012; Banat et al., 2010). Glycolipids, lipoproteins or lipopeptides, phospholipids, fatty acids or natural lipids, polymeric surfactants, and particulate surfactants are the broad classifications (Moya *et al.*, 2015; Hoskova *et al*., 2013). Biosurfactant is a term that refers to microbial compounds that exhibit significant surface activity (Maneerat, 2005). Biosurfactants are unique amphipathic molecules that have been investigated for a variety of industrial and bioremediation applications (Bodour *et al*., 2003), pharmaceutical and food processing, and oil recovery. Global surfactant production exceeded 2.5 million tons in 2002, reached approximately 1,735.5 million USD in 2011, and was expected to reach 2,210.5 million USD in 2018, implying an average annual growth rate of 3.5 percent from 2011 to 2018. (Sekhon *et al*., 2014).

72 The production of biosurfactant can be carried out in batch or continuous mode. Batch

73 fermentation involves the addition of media and inoculum simultaneously to the bioreactor, and

74 the product is collected at the conclusion of the fermentation (Zouari *et al*., 2014). Conditions in

75 the bioreactor change throughout the process (i.e., nutrients and products and waste were

76 reduced). According to Rodrigues *et al*. (2017), using soybean waste as a substrate enabled the

77 production of biosurfactants and biomass at concentrations of 11.70 g/L and 11.5 g/L,

78 respectively. Pansiripat *et al*. (2010) used a carbon source with a 40:1 oil-glucose ratio. They

79 reported a 58.5 percent reduction in surface tension due to the biosurfactant. Additionally, batch-

80 type bioreactors have advantages. For instance, they can be used when a material is only

81 available during specified times and is suitable for high solids content (25 percent). While the

82 continuous process is ongoing, substrate streaming and product collection can begin at any time

83 after the maximum product concentration or substrate limits have been reached. In this case,

84 substrates and inocula can be continuously added, extending the exponential phase (Rusmana,

85 2008). Due to the difficulties associated with controlling substrate availability, studies on

86 biosurfactant production using this type of fermentation have not been widely reported.

87 **MATERIALS AND METHODS**

88 **Sample collection**

89 **Soil samples:**

90 There (3) soil samples were obtained from major palm oil mills in Rumuche and Elibrada
91 Emohua within Rivers State. The soil samples were collected using a simple hand-held auger
92 which will be obtained from the department of Geography and Environmental management.
93 Sterile seal bags will be purchased from the Everyday Supermarket, Choba , Rivers State. The
94 sample bags were marked on the basis of site of sample collection. The samples were transported
95 in an ice chest to the Laboratory, Alakahia for physicochemical analysis. Samples points were
96 georeferenced using Geographic positioning system (GPS) receives.

97 **Geotechnical and physicochemical evaluation of POME-impacted soil**

98 Imhoff-cone approach coupled with agitation of the cones for 20 to 30 minutes was employed for
99 particle size determination. The weight of the particles was documented and presented in %w/w.
100 The method of ASTM D422 was used to determine the soil texture of the soil samples using
101 different sieve meshes. Specific Gravity of the soil samples were determined the using
102 pycnometer method ASTM D854. Permeability of the soil using the samples was determined
103 through the ASTM-D2434 while Atterberg Limit was determined using ASTM-6913. The pH of

104 the soil samples was determined using the electrochemical method (IITA, 1979). Electrical
105 conductivity was determined using the method of APHA-2510B. The modified method of Dewis
106 and Freitas (1970) was employed for soil alkalinity. The modified method of Agbaji (2020) was
107 employed for phosphate determination of the soil samples using the APHA-4500PC approach
108 while turbidometric approach was employed for sulphate determination. The exchangeable
109 ammonia or ammoniacal nitrogen was determined using the APHA-4500. The modified methods
110 of Effiong et al. (2020); Orhohoro et al. (2017) and Agbaji 2021 was employed in the analysis of
111 parameters such as heavy metals, hydrogen sulphides and salinity.

Microbiological analysis of soil samples

113 The culture medium for the total heterotrophic bacterial count was determined by suspending
114 twenty-eight grams nutrient agar in de-ionized water. The suspension was heated over a Bunsen
115 burner to dissolve the media completely and placed in an autoclave $121^{o}C$ for 15 minutes at 15
116 psi. After cooling to room temperature, 0.065g/ml of tetracycline was seeded to the media prior
117 to dispensing into petri dishes and allowed to solidify. One milliliter of enriched samples was
118 drawn from the setup and diluted using pre-sterilized normal saline and cultured using spread
119 plate technique. Colony count within 30-300 CFU/ml were employed in the determination of the
120 bacterial load. Similarly, the total fungal count was determined by suspending 0.1 ml of the
121 enriched samples and diluted in a 10-fold serial dilution, it was then plated on Potato dextrose
122 agar fortified with 0.1% lactic acid to inhibit bacterial contaminants. The inoculated plates would
123 be incubated at $25^{o}C$ for 3-7 days (Abu & Ogiji, 1996)

Isolation of Yeast species from palm oil impacted soil

125 Aliquots of the enrichment setup were diluted using 9.9 ml sterile normal saline. Glucose yeast
126 peptone media were prepared and 1.0% lactic acid was introduced into the media to inhibit
127 fungal contaminants, autoclaved at $121^{o}C$ and 15 p.s.i for 15minutes. Vacuum filtered palm oil
128 was used in the palm oil utilization test and the vapour-phase culturing technique was adopted by
129 soaking the sterile Whatman filter paper no1 with crude oil and Palm oil and aseptically placed
130 on the cover of the petri dishes. The plates were incubated at $37^{o}C$ for 48 hours. Yeast flora were
131 determined by inhibiting bacterial contaminants using 1.0µg/ml chloramphenicol (Orhorhoro et
132 al., 2018: Ekwuabu et al., 2016).

Biosurfactant Production Screening

Haemolytic activity

135 Pure cultures of the yeast isolates were streaked and incubated on Blood agar plates at $25^{o}C$ for
136 45 minutes. The glucose yeast peptone containing 10% fresh sheep blood to 80% nutrient agar
137 plates were allowed to solidify. The streaked platewere incubated for 24-48 hrs at $37^{o}C$. The
138 result was interpreted α, β and γ heamolysis (Santhini and Parthasarathi, 2014).

Oil spread activity

140 The yeast isolates with potential to degrade crude oil, were sub-cultured to attain pure cultures.
141 The yeast isolates were cultured at $37^{o}C$ for 24 hrs and dislodged with sterile distilled water. The

concentration of yeast cells was determined using 0.1Macfarland before incubation. Two milliliters (2ml) of inoculum suspension were added to 100ml mineral salt medium in a 250ml Erlenmeyer flask, incubated in an orbital shaker (Stuart Orbital Shaker S150) at 150 r.p.m, 37˚C for 7 days. The reaction was carried out in the ratio of 10:1:0.05 for water; BLCO; Biosurfactant (Ali, 2013).

Drop collapse method

This test is a quality measure of sophorolipids, it was done using 5.0 µl of the 48 hrs culture, before and after centrifugation at 12,000 xg for 5 min to separate biomass from the sophorolipid and transferred to the oil-coated 96 well micro titre plate. The drop size formed from the sophorolips was observed after 1 min with the aid of a magnifying glass. The result was considered positive for biosurfactant production when the drop was flat and those cultures that gave rounded drops were scored as negative, indicative of the lack of biosurfactant production.

Blue Agar Plate Assay (Cetyl Trimethyl Ammonium Bromide CTAB Method)

The modified method of Ndibe *et al*. (2019) was adopted for this study, as mineral salt agar medium supplemented with Cetyl Trimethyl Ammonium Bromide (CTAB: 0.8 mg/mL) and methylene blue (MB: 0.02%) was prepared to detect anionic biosurfactant. The crude sophorolipid was dispensed into wells (4.0mm) in the medium. The plate were incubated at 37°C for 3 days.

Emulsification index (E24%)

The modified method of Ndibe *et al*. (2019) using 2.0ml of cell free sophorolipid in the ratio of 1:1, the mixture is mixed vigorously for half a minute and left to stand for (24h). The ratio of the emulsion to the column was determined.

Mathematically;

Emulsification Index (E_{24}) = $\dfrac{Height\ of\ emulsion\ formed}{Height\ of\ aqueous\ phase}$ x100%

Hydrocarbon degradation screening of yeast Isolates.
In vitro crude oil biodegradation potential of the organisms was done using the modified method of Shekhar *et al*. (2015). The test was conducted using 1.0ml overnight culture suspension seeded into 90% mineral salt media fortified with 1% Bonny Light Crude Oil (BLCO). The setup was kept under standard conditions for 7 days. The setup was repeated using 2,6-Dichlorophenolindophenol. The growth was monitored through culture densities, by taking the O.D readings daily at 600nm against Bushnell Haas medium as blank. Corresponding daily reading of pH were taken and recorded.

Molecular Identification of Yeast
The isolates were identified molecularly using the method described by Onwumah et al (2019). The yeast DNA was extracted using an Inqaba South Africa-supplied ZR fungal DNA mini prep extraction kit. Pure and abundant yeast biomass was suspended in 200 l of isotonic buffer and

seeded into ZR Bashing Bead Lysis tubes using 500 l of lysis solution. Prior to assembly, the tubes were pre-fitted with a 2 ml column holder and beads and spun at maximum speed for 5 minutes. For 60 seconds, the ZR bashing bead lysis tube was spun at 10,000 xg. Approximately 400 l of the supernatant was transferred to a collection tube fitted with a Zymo-Spin IV spin Filter (orange top) and centrifuged at 7000 xg for 1 minute. 1200 l of fungal DNA binding buffer was added to the filtrate in the collection tubes, bringing the final volume to 1600 l. Around 800 l were then transferred to a Zymo-Spin IIC column contained in a collection tube and centrifuged for 1 minute at 10,000 xg. The collection tube's flow through was discarded. The remaining volume was transferred to the same Zymo-spin and spun; 200 l of the DNA Pre-Wash buffer was added to the Zymo-spin IIC in a new collection tube and centrifuged at 10,000 xg for 1 minute, followed by 500 l of fungal DNA Wash Buffer. Transferring the Zymo-spin IIC column to a clean 2.0 l centrifuge tube. To elute the DNA, 10.0 l of DNA elution buffer was added to the column matrix and centrifuged at 10,000 xg for 30 seconds. Following that, the ultra-pure DNA was stored at -20 oC for use in subsequent reactions.

DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer.

Internal transcribed space (ITS) amplification

The ITS region of the rRNA genes of the isolates were amplified using the ITS1(TCCGTAGGTGAACCTGCGG) and ITS4(TCCTCCGCTTATTGATATGC) primers on ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 µl for 35 cycles. The PCR mix contained twice Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, dNTPs, MgCl), the primers at a concentration of 0.4 M and the extracted DNA as template. The PCR conditions used are as follows: Initial denaturation, 95°C for 5 min; denaturation, 95° for 30 Sec; annealing, 53°C for 30 Sec; extension, 72°C for   30 sec and final extension, 72° for 5 min. The product was resolved on a 1.5 % agarose gel at 120 V for 15 min and visualized on a UV trans illuminator.

Sequencing

Sequencing was done using the Big Dye Terminator kit on a 3510 ABI sequencer (Inqaba Biotechnological, Pretoria South Africa).

Phylogenetic Analysis

The sequences were obtained after bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou and Nei, 1987). The bootstrap consensus tree was inferred from 500 replicates (Felsenstein, 1985) and taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the Jukes-Cantor method (1969).

Results and Discussion

Table 1 shows the physicochemical profile of the palm oil mill effluent impacted soil in Emohua Local Government, Rivers State, Nigeria. The pH of the unpolluted soil was 7.43, Rumuche

palm oil mill site had pH 6.18 while that of the Elibrada palm oil mill site was 6.34. The temperature of the soil samples was 31.2^{o}C, 31.9 ^{o}C and 32.01 ^{o}C for the unpolluted, Rumuche and Elibrada palm oil processing sites respectively. The Electrical conductivity for Elibrada soil sample was 20.84 μS/cm, the sample from Rumuche had electrical conductivity of 80.19 μS/cm while that of the unpolluted soil was 220.13 μS/cm. The salinity of the Elibrada soil sample was 2.022 mg/Kg while for the Rumuche and Unpolluted soil were 1.03 and 1.02 mg/Kg. The Alkalinity of the Elibrada soil sample was 50.74 mg/Kg, Rumuche soil had 30.51 mg/Kg while that of the unpolluted soil was 40.58 mg/Kg. Phosphate content of the soil samples were 3.57mg/kg, 3.81 mg/kg and 6.24 mg/kg for unpolluted soil, Rumuche and Elibrada palm oil mill effluent impacted sites respectively. Ammonia content of the Elibrada and Rumuche soil sample were 0.056 and 0.041 mg/Kg while the result for the unpolluted soil was 0.028 mg/Kg. Sulphates and hydrogen sulphide concentration of the soil sample from Rumuche location was 253.83 mg/Kg and 5.17 mg/Kg, for Elibrada location was 166.49 and 0.052 mg/Kg, while for the control unpolluted soil site it was 27.94 and 0.052 mg/Kg. The concentration of exchangeable cations Iron and Zinc was 5.10 and 3.01 mg/kg for unpolluted soil while for Elibrada it was 1.35 and 1.29 mg/Kg and Rumuche locations it was 1.83 and 1.59 mg/kg.

Table 2 shows the geotechnical properties of the samples obtained from the study area. The silt content in Rumuche soil sample was 2.57 % w/w, Elibrada was 2.38% while the unpolluted soil was 1.92%w/w. The sandy components were 96.15% w/w, 96.57% and 97.25% for Rumuche, Elibrada and unpolluted soil respectively. The moisture content of the Rumuche soil was 28.6 %, Elibrada soil was 15.83 %w/w while the unpolluted soil was 8.74%. Permeability of the soil from Elibrada was 2.7 cm/s, Rumuche had 1.3 cm/s while the unpolluted soil had 5.6 cm/s. Total organic carbon for soil samples from Rumuche was 4.92% w/w, Elibrada had 6.13% while the unpolluted soil had 8.74%. Oil and grease component for the Rumuche soil was 10500 mg/kg, Elibrada had 7200 mg/kg and the unpolluted soil had 28.0mg/kg. The plastic index was 8.0% and 13.0% for Rumuche and Elibrada respectively while the unpolluted soil had 11.0%

Microbial population of the soil samples

Table 3 shows the microbial population of the soil samples obtained during the study. The unpolluted soil had a total heterotrophic bacteria count of 1.32×10^5 CFU/g, while the samples obtained from Rumuche had a total heterotrophic bacterial count of 1.02×10^5 CFU/g. The impacted soil samples from Elibrada had 9.0×10^4 CFU/g. The Total fungal count for Rumuche soil samples was 3.8×10^4 CFU/g while Elibrada had 3.2×10^4 CFU/g.

Biochemical identification of yeast

Table 4 shows the biochemical identification of the yeast isolates mined from the palm oil mill effluent impacted soil. Eighty percent (80%) of the isolates were *Candida* spp. while 20% was *Saccharomyces* sp; Isolates Y1 (*Candida* sp.), Y3 (*Candida* sp.), Y8 (*Saccharomyces* sp.) and Y13 (*Candida* sp.).

Table 1: Physicochemical Composition of Soil samples

Parameter	Unpolluted soil	Rumuche Palm oil Mill Impacted soil	Elibrada Palm oil Mill Impacted soil	F-Stat Value
pH	7.43±0.01[c]	6.18±0.01[a]	6.34±0.01[b]	6284.78
Temperature (^{o}C)	31.2±0.04 [a]	31.9±0.00 [b]	32.01±0.01 [c]	864.86
Conductivity (µS/cm)	220.13±0.01 [c]	80.19±0.01 [b]	20.84±0.01 [a]	1.0×10^8
Salinity (mg/kg)	1.02±0.01 [a]	1.03±0.01 [a]	2.022±0.01 [b]	4356
Alkalinity (mg/kg)	40.58±0.01 [b]	30.51±0.01 [a]	50.74±0.01[c]	1.3×10^7
Phosphate (mg/kg)	3.57±0.01 [a]	3.81±0.01 [b]	6.24±0.01 [c]	29156.77
Ammonia (mg/kg)	0.028±0.01 [a]	0.041±0.01 [b]	0.056±0.01 [c]	201.09
Phenol (mg/kg)	0.066±0.002 [a]	0.464±0.004 [c]	0.38±0.007 [b]	3684.56
H_2S (mg/kg)	0.05183±0.01 [a]	5.17±0.01 [b]	8.02±0.01 [c]	387765.07
Sulphates (mg/kg)	27.94±0.01 [a]	253.83±0.06 [c]	166.49±0.01 [b]	1.7×10^8
Pb (mg/kg)	0.038±0.001 [a]	8.23±0.09 [b]	13.96±0.01 [c]	34031.34
V (mg/kg)	0.0001 [a]	0.61±0.01 [b]	2.0138±0.01 [c]	32203.0
Fe (mg/kg)	5.10 ±0.01 [c]	1.83±0.01 [b]	1.35±0.10 [a]	4700.75
Cr (mg/kg)	1.82±0.00 [a]	43.03±0.00 [b]	51.72±0.00 [c]	2.0×10^8
Zn(mg/kg)	3.01±0.01 [c]	1.59±0.014 [b]	1.29±0.01 [a]	2416.42

262 Data presented as Mean ± Standard Deviation; Similar superscripts in a column imply there was
263 no significant difference, those with different superscripts are significant at p-value <0.05

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272 **Table 2: Geotechnical composition of the soil samples**

Parameter	Unpolluted soil	Rumuche Palm oil Mill Impacted soil	Elibrada Palm oil Mill Impacted soil	F-Stat Value
Silt (%)	1.92±0.01 [a]	2.57±0.01 [c]	2.38±0.01 [b]	4468
Clay (%)	0.83±0.03 [a]	1.28±0.01 [c]	1.05±0.03 [b]	393.45
Sand (%)	97.25±0.01 [c]	96.15±0.00 [a]	96.57±0.05 [b]	678.62
Soil Type	Sandy	Sandy	Sandy	
Moisture Content (%)w/w	8.74±0.01 [a]	28.6±0.14 [c]	15.83±0.04 [b]	27931.97
Permeability (cm/s)	5.6±0.07 [c]	1.3±0.07 [a]	2.7±0.01 [b]	1829.33
Total Organic Carbon (%)	8.74±0.02 [c]	4.92±0.14 [a]	6.13±0.01 [b]	32781.5
Oil and Grease (mg/kg)	28.0±0.20 [a]	10500±0.28 [c]	7200±0.01 [b]	2128513135.6
Liquid Limit (%)	19.00±0.04 [a]	28.00±0.01 [b]	31.0±0.01 [c]	10378.4
Plastic Limit (%)	8.0±0.03 [a]	16.0 ±0.14 [b]	18.0 ±0.14 [c]	4188.5
Plastic Index	11±0.71 [b]	8.0 ±0.14 [a]	13.0±0.01 [c]	43.8
Plastic Description	Grey Sandy	Dark-Silty Sandy	Grey-Silty Sandy	
Permeability Description	Moderately rapid	Slow	Moderately slow	

Data presented as Mean ± Standard Deviation; Similar superscripts in a column imply there was no significant difference, those with different superscripts are significant at p-value <0.05

Table 3: Microbial population of palm oil mill impacted soil and pristine soil

Sample	THBC(CFU/g)	TFC(CFU/g)
Unpolluted soil	1.32×10^{5}	4.6×10^{4}
Rumuche Palm oil Mill Impacted soil	1.02×10^{5}	3.8×10^{4}
Elibrada Palm oil Mill Impacted soil	9.80×10^{4}	3.2×10^{4}

THBC= Total Heterotrophic Bacterial Count; TFC= Total Fungal Count; CFU/g= Colony Forming Unit per Gram

Table 4: Biochemical characteristics of yeast isolated from palm oil impacted soil

ISOLATE	GERM TUBE	GLUCOSE	LACTOSE	MALTOSE	GALACTOSE	SUCROSE	UREA	PROBABLE GENERAL
Y1	-	+	-	-	+	+	-	*Candida* sp.
Y2	-	+	-	-	+	+	-	*Candida* sp.
Y3	-	-	-	-	-	-	+	*Candida* sp.
Y7	-	-	-	-	-	-	+	*Candida* sp.
Y8	-	+	-	+	+	+	-	*Saccharomyces* sp.
Y13	-	+	-	-	+	+	-	*Candida* sp.
Y14		+	-	-	+	+	-	*Candida* sp.

287 Figure 1 shows the pattern of the growth, biomass accumulation of the yeast isolates during the
288 hydrocarbon degradation screening. The *Candida* sp. isolate Y3 was observed to perform
289 optimally under the hydrocarbon spiked medium. The organism Y3 was observed to have a brief
290 lag phase between the first day of the study. The exponential phase was recorded between the 1st
291 and 5th day. Isolate Y13 was observed to have a lag phase between the 1st day and the 3rd day.
292 Then, there was a steady peak into the Log phase between the third days. The control was
293 unaffected or did not accumulate biomass during the biodegradation of Bonny light crude oil.

294 Table 5 shows the sophorolipid production potential of the yeast isolates obtained from the study
295 area. The isolate Y3 (*Candida* sp) was observed to have a gamma heamolysis, an excellent
296 hydrocarbon degradation potential with obvious decolorization of Dichlorophenyl indo-phenol
297 with methylene blue dye. The oil spread was 28.3 mm using bonny light crude oil. The
298 Emulsification index ($E.I_{24}$) was 52.9% while the reaction with CTAB was moderately positive
299 and the surface tension was 30.0 (unit). The Y8 (*Saccharomyces* sp.) also had a gamma
300 heamolysis with an oil spread potential of 34.43 and a higher Emulsification potential of 61.1%.
301 The cell free or crude sphoroplipid material obtained from the isolate had a positive hydrocarbon
302 degradation potential, Declourization of DCPIP, drop collapse and CTAB activity and a surface
303 tension of 30.0 (unit). The isolate Y13 (*Candida* sp.) had an alpha blood heamolysis with an oil
304 spread potential of 33.5 and an emulsification index of 66.7 with no CTAB activity and surficial
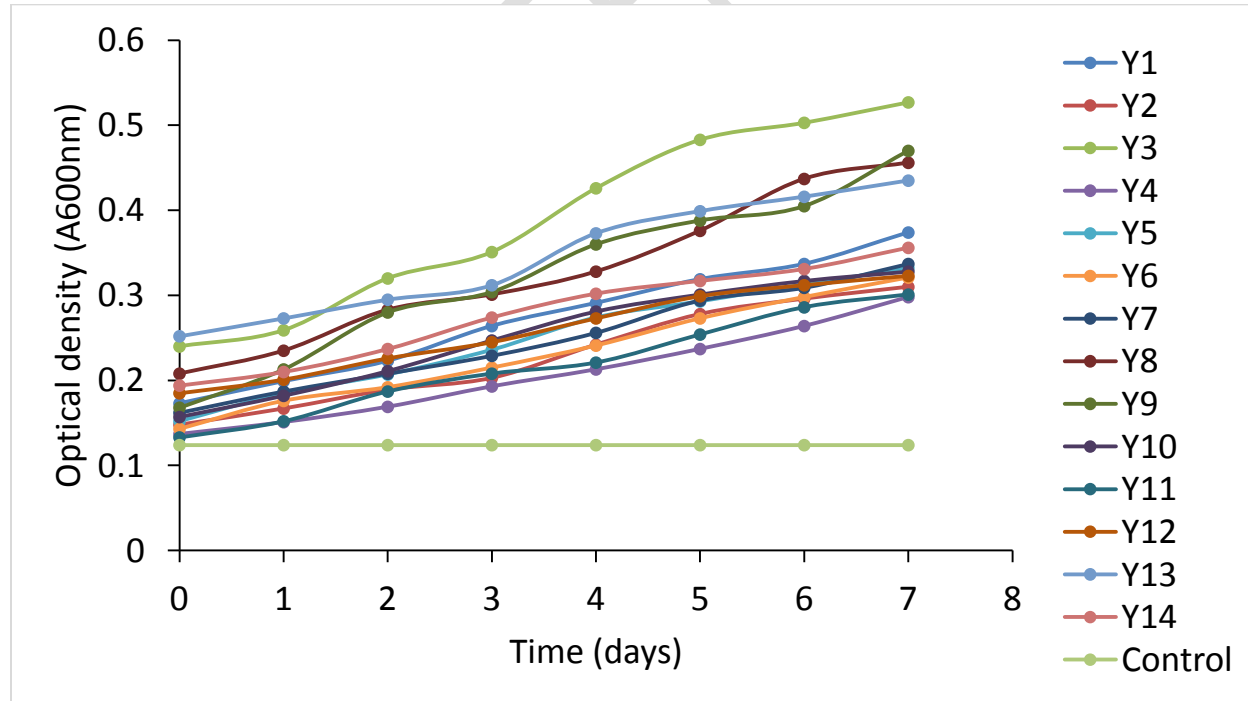305 tension of 50.0

307 **Figure 1: Growth pattern of yeast isolates for hydrocarbon degradation**
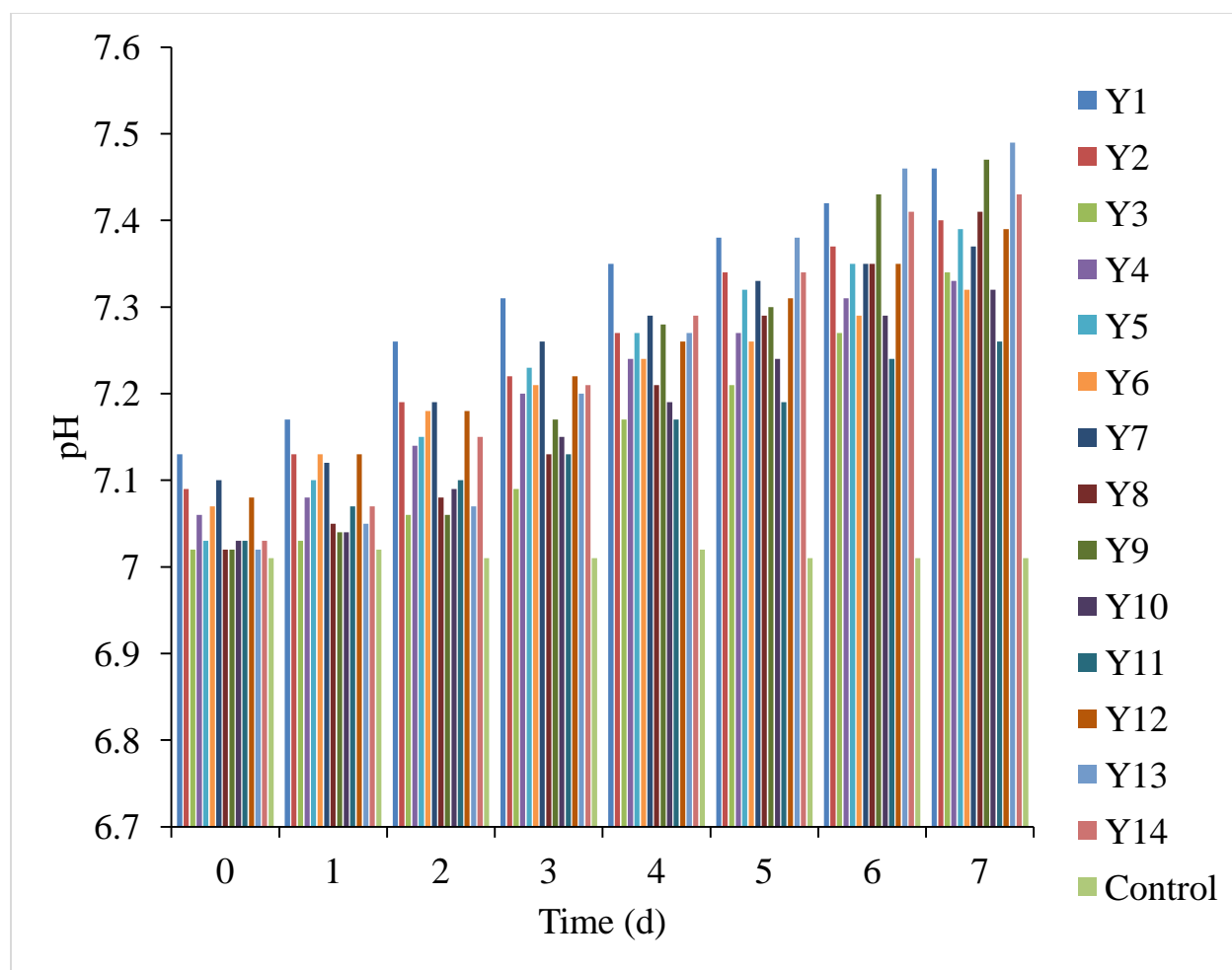
Figure 2: Effect of pH during hydrocarbon degradation screening

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**Table 5: Sophorolipid production potential screening of yeast isolates obtained from palm oil impacted soil**

ISOLATE	Blood Haemolysis	HC- Deg potential	DCPIP Degradation	Drop Collapse	Oil Spread (mm)	Emulsification Index (%)	CTAB	Surface Tension
Y1	γ	+	+	+++	18.2	40.6	−	38
Y2	γ	+	+	+++	15.6	22.2	−	48.7
Y3	γ	+++	++	++	**28.3**	**52.9**	++	**30**
Y4	γ	+	+	+	30.4	39.3	+	33.5
Y5	γ	+	+	−	1.5	10.8	−	41
Y6	γ	+	+	+++	22.6	32.3	+	55.2
Y7	γ	+	+	+++	30.7	35.5	+	57
Y8	γ	++	++	+++	**34.3**	**61.1**	++	**30**
Y9	γ	++	++	++	**30.4**	**42.7**	+	**40**
Y10	γ	+	+	−	3.8	15.3	−	41.2
Y11	γ	+	+	+	20.1	46.9	+	52.6
Y12	γ	+	+	+	28.7	53.3	++	37.1
Y13	∝	++	++	++	**33.5**	**66.7**	-	**50**
Y14	γ	+	+	+	16.7	21.4	−	64.3

314

Molecular Characteristics of the yeast isolates.

The result presented in Figure 3 shows the gel electrophoresis of genomic extracts. There are four bands separated and denoted as ITS 600bp and a Ladder L which was 500bp. The bands were tagged or labeled Y3, Y8, Y9 and Y13.The molecular weight is a measure of the migration on the gel.

Figure 4 shows the phylogenetic construct of the isolates. The phylogenetic construct shows that the Y8 was 70.2% similar to the *Candida parapsilosis*. The isolate Y3 was 95.8% similar to the *Candida haemulonis*. Isolate Y9 had 100% similarity *Pichia Kudriavzevii* while Y13 had a 97.4% identity with *Saccharomyces cerevisiae*.

324 Table 6 shows the NCBI accession numbers for the four yeast isolates. The Y3 (*Candida*
325 *haemulonis*, Y8 (*Candida parapsilosis*), Y9 (*Pichia kudriavzevii*) and Y13 (*Saccharomyces*
326 *cerevisiae*) had accession number between MW182014 to MW182017

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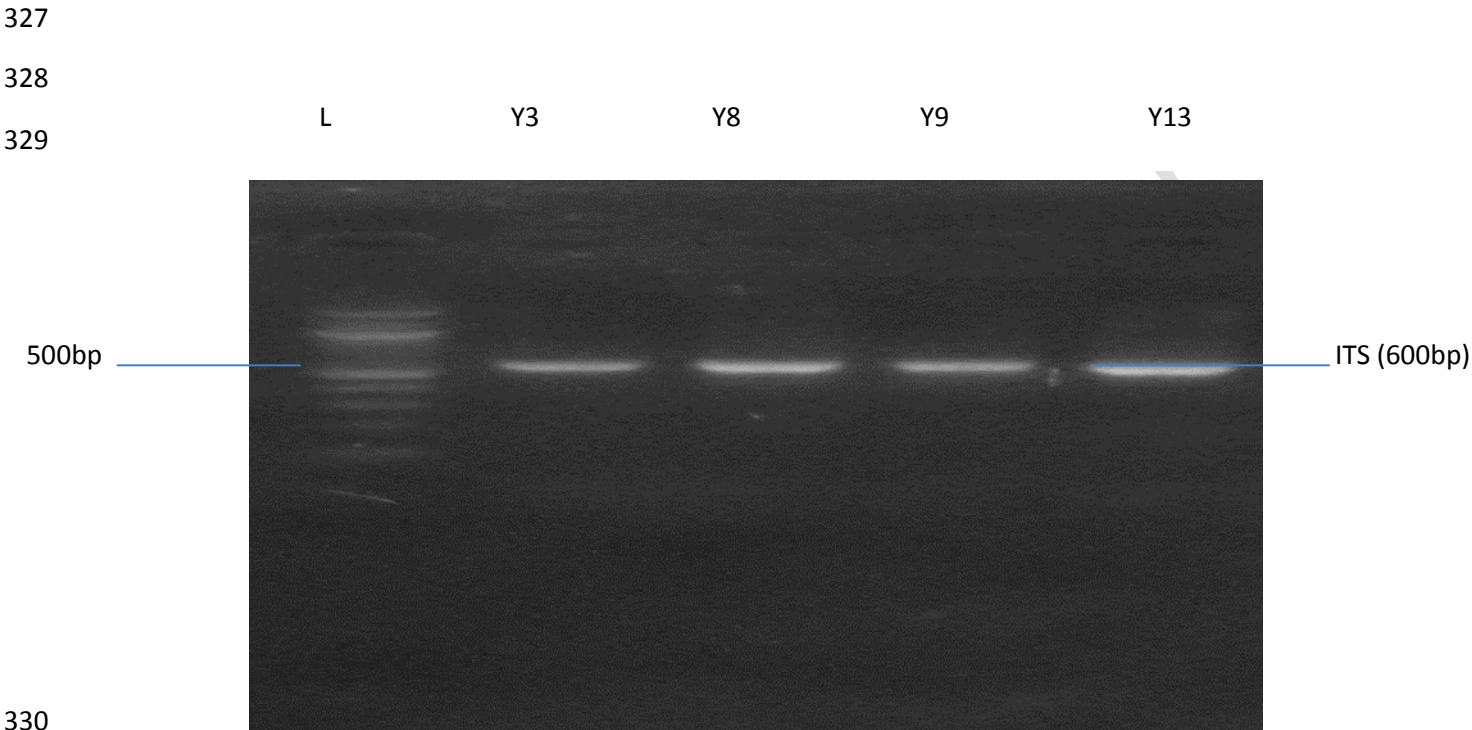
330

331 **Figure 3: Gel electrophoresis pattern and band of the genomic DNA extracts obtained after**
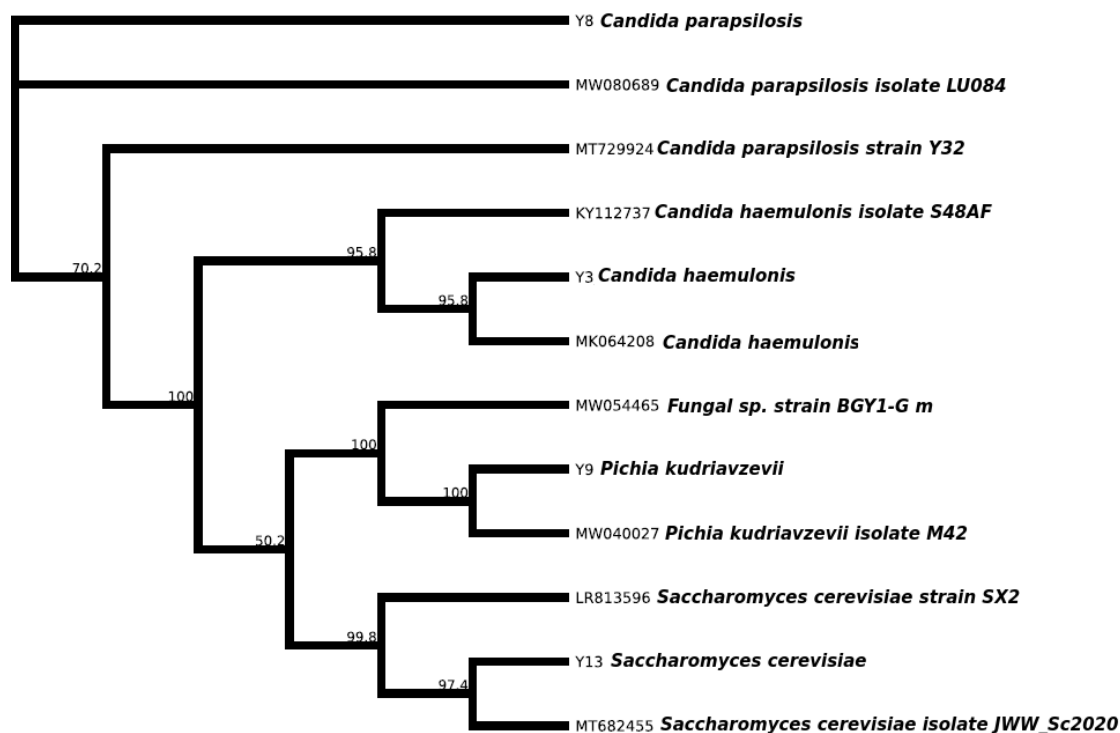332 **gene amplification**

Y8 *Candida parapsilosis*

MW080689 *Candida parapsilosis isolate LU084*

MT729924 *Candida parapsilosis strain Y32*

KY112737 *Candida haemulonis isolate S48AF*

Y3 *Candida haemulonis*

MK064208 *Candida haemulonis*

MW054465 *Fungal sp. strain BGY1-G m*

Y9 *Pichia kudriavzevii*

MW040027 *Pichia kudriavzevii isolate M42*

LR813596 *Saccharomyces cerevisiae strain SX2*

Y13 *Saccharomyces cerevisiae*

MT682455 *Saccharomyces cerevisiae isolate JWW_Sc2020*

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Figure 4: Phylogenetic tree showing the evolutionary distance between the fungal isolates

Table 6:   Bioinformatics details of yeast isolates

Sequence	Isolate Code	Percentage Similarity (%)	Identity	Gen Bank Accession Number
1	Y3	95.8	*Candida haemulonis*	MW182014
2	Y8	70.2	*Candida parapsilosis*	MW182015
3	Y9	100	*Pichia kudriavzevii*	MW182016
4	Y13	97.4	*Saccharomyces cerevisiae*	MW182017

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5.0   Discussion:

Palm oil mills in the southern part of Nigeria has remained a mainstay for a number of economic benefits. The activities from the milling have been reported in both cottage and commercial scale in many states of Nigeria. The need for proper treatment of POME have been advocated by researchers (Nwachukwu *et al*., 2018; Okute and Isu, 2007). There have been concerns that indiscriminate discharge of POME may impact on the physicochemical attributes such as the total hydrocarbon content, and level of dissolved oxygen as reported by Ohimain *et al*. (2012). The impact of these industrial activities on the soil have been reported to have a number of negative effects ranging from aesthetic loss to tainting and leaching of nutrients. The account of Iyakndue *et al*. (2017) suggests a marked change in the physicochemical and nutrient availability for the soil pre-exposed to palm oil mill effluent. Chikwendu and Ogbonna (2018) reported a high level of soil radicals. Their study also documented a change in their microbial flora.

The pH of the impacted soil ranged from 7.43 for the unpolluted soil to 6.18 for Rumuche and while pH 6.3 was recorded for Elibrada soil. The pH of any soil has been credited and correlated with the microbial activities, nutrient uptake, diversity and bioavailability. This range observed was in tandem with the report of Sakiah and Andri (2018) whose investigation reported pH 7.48. There was a steady decline in the pH values of the polluted soil making them slightly acidic. This observation was similar to the report of Iwuagwu and Ugwuanyi (2014) whose study reported a similar decline in the pH of the POME-impacted soil. In a related study, the report of Nnaji *et al*. (2016) also reported that there was a steady decline in the pH of an impacted soil, but also observed that the steadily returned to alkaline condition from the acidic condition caused by the palm oil mill effluent. POME-impacted soil has been widely reported to be acidic and could create a number of acidic soil challenges (Awotoye *et al*., 2011).

The electrical conductivity of the soil impacted by POME ranged from 20.84 µS/cm to 31.9 µS/cm, it was slightly more than the unpolluted soil. This was in agreement with the report of Nwachukwu *et al*. (2018) whose account suggested that the effluent could increase the level of ions in the soil and also affect the level of Electrical conductivity of the soil. In a related study, Chikwendu and Ogbonna (2018) reported an Electrical conductivity of 3.2 µS/cm. This agrees with the concentration of Iron which was 1.83 mg/Kg, Lead (Pb) was 8.23 and 13.96 mg/Kg for POME-impacted soil from Elibrada which also suggest a considerably significant presence of cations. These values obtained in the study are in tandem with the report of Osman *et al.* (2020) who reported a significant number of ions such as Lead, Chromium, Arsenic in palm oil mill effluent. Futhermore, Okereke and Ginikanwa (2020) also reported that palm oil mill Effluents and processing is associated with a number of ions.

Geotechnical evaluation revealed a number of soil specific profile about the study area. The Silt (%) was 2.57 for Rumuche and 2.38%w/w for Elibrada while sand (%) was 96.15 and 96.57% respectively. The soil type under evaluation were categorized as sandy soil with a moisture content of 28.6 % and 15.83% for Rumuche and Elibrada. The Oil and Grease values for the POME-polluted soil was 10500 mg/kg for Rumuche and 7200 mg/Kg for Elibrada

samples. The plastic limit was 16.0 % and 18% respectively for the POME-impacted soil samples. These findings support Awotoye *et al* (2011) findings that POME-mediated pollution can alter the geotechnical properties of soil. Although their study observed a similar index, they concluded that this type of pollution caused by a poor management profile and treatment technologies could leach into other non-target aquatic ecosystems, potentially resulting in blow-out situations. These findings corroborated Iyakndue et al. (2017), who also noted a difference in the level of fluxes in the geotechnical properties of POME-impacted soil.

Microbial diversity of POME-impacted soil has remained a constant worry to several scientific researchers. Total heterotrophic count of the soil samples from Rumuche and Elibrada was 1.02 $\times10^5$ and 9.80×10^4 CFU/g respectively while that of the Unpolluted soil had 1.3×10^5 CFU/g. This suggests that the microbial population of an unpolluted soil was at least 10 times higher than the POME-impacted soil. This implies that there could be a significant decline in the microbial community profile of the soil. However, Total fungal count of the soil was observed to remain fairly the same with little or no significant changes as the microbial population varied from 3.2×10^5 CFU/g to 4.6×10^4 CFU/g. Sakiah and Andri (2018) reported a Total heterotrophic count in POME-impacted soil of 25×10^5 CFU/mL while the unpolluted soil had a concentration of 1.86×10^6 CFU/mL. The work of Awotoye *et al*. (2011) reported a total heterotrophic count of POME-impacted soil to range from 1.8×10^6 CFU/g to 6.50×10^9 CFU/g, with a Total fungal count of 9.5×10^2 CFU/g to 2.5×10^5 CFU/g; their finding was in close proximity with the report obtained from the present study. The study may have varied only on the basis of the temperature of the effluent employed in the spiking of the soil. The temperature of the effluent and the geotechnics of the soil have been attributed to the changes in the microbial bioload as observed by Okwute and Isu (2007), their study observed that the changes in the Total Aerobic count varied on the basis of the temperature of the POME. The counts obtained from their study showed a steep decline in their Total Heterotrophic count although the account of Woods (1997) attributed the presence of organic matter for the stability of the fungal community while the heat associated with the effluent as a major reason for the dynamics in the heterotrophic bacterial count. Sekiah *et al*. (2017) reported that the activity of POME against certain soil or water microbiota could either be positive or negative as the nutritional components associated with the effluent.

Eighty percent (80%) of the isolates were *Candida* spp. while 20% was *Saccharomyces* sp. Isolates Y1 (*Candida* sp.), Y3 (*Candida* sp.), Y8 (*Saccharomyces* sp.) and Y13 (*Candida* sp.). The report of Okwute *et al*. (2014) reported *Pseudomonas*, *Bacillus*, *Proteus*, *Micrococcus*, Aspergillus, *Penicillium*, *Paecilomyces* and *Candida* as the microflora they observed from POME- polluted soil. These agrees with the findings of the present study that *Candida*, *Saccharomyces* sp, and other yeast isolates may be associated with this nature of industrial effluent. This agrees with the report of Bala *et al*. (2018) whose investigation revealed the presence of *Aspergillus fumigatus* 107PF, *Aspergillus nomius* 108PF, *Aspergillus niger* 109PF and *Meyerozyma guilliermondii* 110PF. Nebo and Abu (2015) reported that the yeast have the ability to withstand and grow on POME and also possess degradative and biosurfactant production potential. The report of Nwuche and Ogbonna (2011) isolated lipase producing fungi from POME-dumpsite in Nsukka, Nigeria and characterized isolates such as *Aspergillus* sp.,

420 *Penicillium* and *Mucor* genera. Sophorolipid production potentials of the yeast isolates showed
421 that the yeast isolates had a significant potential to induce reduction in the surficial tension of
422 effluent. The *Candida* isolate (Y3) was observed to have a positive reaction to the drop collapse
423 test; oil spread of 18.2 mm, Emulsification index of 52.9% and a moderate surface tension
424 activity. This agrees with the position of Nebo and Abu (2015) whose study identified a surface
425 tensioactive profile by the yeast isolates obtained POME-effluent. Their report documented an
426 appreciable yield in the sophorolipid produced by the yeast associated with POME-impacted
427 soil. Konishi *et al*. (2016) was able to assess the yeast isolates as possible tools for white
428 biotechnology and evaluated their potential and phylogeny. The report of Orji *et al*. (2006)
429 reported the isolates obtained from POME-impacted environment with the possibility of
430 producing diverse tensioactive substances. This supports the work of Silveira *et al*. (2019) who
431 also reported that these sophorolipids produced by yeast isolates such as *Starmerella bombicola*
432 can both function as emulsifiers and antimicrobial agents. The work of Archana *et al*. (2013)
433 identified and screened the potential of yeast from impacted environment with a yield of
434 4.23g/L; their report identified the sophorolipid production potentials of *Candida* species which
435 agreed very strongly with the findings of the present study. These surfactants have been
436 identified as a possible adaptive feature of microbes. The report of Claus and Bogaert (2017)
437 was also able to screen yeast producing sophorolipid and categorized them as environmentally
438 friendly but decried the faulty characterization protocol as major problem in niche-related
439 studies. Their study also developed a number of protocols in the assessment of sophorolipid
440 production potentials of yeast isolates.

441 Hydrocarbon degradation of the yeast isolates were identified during this study. The activity to
442 reduce the methylene blue indicator in DCPIP was observed during the study. There was a
443 remarkable reduction in the colour of the indicator during the study with a corresponding
444 increase in the biomass and pH of the medium. The molecular identification of the yeast isolates
445 showed that the isolates possess the ability required for mineralization of the petroleum
446 hydrocarbons. The phylogenetic construct shows that the Y8 was 70.2% similar to the *Candida*
447 *parapsilosis*. The isolate Y3 was 95.8% similar to the *Candida haemulonis*. Isolate Y9 had 100%
448 similarity *Pichia Kudriavzevii* while Y13 had a 97.4% identity with *Saccharomyces cerevisiae*.
449 The Y3 (*Candida haemulonis*, Y8 (*Candida parapsilosis*), Y9 (*Pichia kudriavzevii*) and Y13
450 (*Saccharomyces cerevisiae*) had accession number between MW182014 to MW182017.
451 These findings strongly agree with the report of Al-Dhabaan (2021) who reportedly isolated and
452 characterized yeast isolates with hydrocarbon degradation capacities from Kafji oil field in Saudi
453 Arabia, namely *Candida tropicalis*, *Rhodotorula mucilaginosa* and *Rhodosporidium toruloides*.
454 The account of Miranda *et al*. (2007) identified the degradation of petroleum hydrocarbon by
455 prolific yeast isolates *Rhodotorula auranticaca* and *Candida ernobii* However, Okerentugba *et*
456 *al*. (2016) reported the activity of hydrocarbon degradation potentials from palm wine yeast such
457 as *Candida adriatica* and *Candida taoyuanica*. In a related study Shumin *et al*. (2011) reported
458 that *Pichia omen* has the potential to degrade hydrocarbon fractions. It is common knowledge
459 that indigenous microorganisms grown in the soil for a long, they could adapt and function in
460 diverse ecosystems. This also validates that there could be a number of exogenous microbes
461 which could also play a vital role in eco-restoration. There have considerations that the
462 exogenous organisms may not be perfect eco-adapters in every terrestrial habitat situation

(Shumin *et al*. 2011, Mukherjee and Das, 2007 and Ueno, 2007). Several scientific reports have identified yeast as being environmentally friendly and ecologically diverse.

Conclusion and Recommendation

Palm oil processing will remain a major economic driver in the southern part of Nigeria and the effluent associated with these activities both at cottage and industrial scale will remain a major source of concern to both the scientific and entrepreneurial enthusiast until a cheap and feasible treatment and management protocol is established to meet these teaming concerns. The habitat and niche occupied by a number of ecological drivers such as yeast isolates have been identified in the present study. The Geotechnical and physicochemical properties of soil samples impacted by the palm oil mill process have been reported to have created a shift in the soil texture and profile. The microbial population of the soil samples evaluated was in tandem with already posited literature. The biodiversity of the soil has been identified to have narrowed the yeast flora observed during the study. Biosurfactant (Sophorolipid) production potentials was observed on the isolates obtained from the study. There was a significant surface tension, crude oil biodegradation potential, CTAB, emulsification and oil spread activities which suggested a major qualitative and quantitative potential of the yeast isolates. Molecular studies showed the presence of *Candida haemulonis*, *Candida parapsilosis*, *Pichia kudriavzevii* and *Saccharomyces cerevisiae* as the major microflora of the POME-impacted soil in Emohua Local Government area of Rivers State. This study was able to identify and agree with a number of peer review articles on the potency of these yeast isolates as ecofriendly and also as bioemulsifiers in their ecological niches. This investigation further recommends the potency of several impacted environmental matrices as archives and reservoirs of ecologically friendly yeast isolates whose potentials could be harnessed in a number of biotechnological processes. The academia at all level must synergize in the use of these prolific and ecologically diverse flora in the production of high value industrial and environmental technology and promote the application of indigenous microbes in diverse white biotechnological advancements.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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