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Optimization of Ambient Temperature Biodiesel Production from Palm Oil Mill Effluent by A Consortium of *Candida albicans* and *Pseudomonas Aeruginosa*

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A B S T R A C T

Palm oil mill effluent (POME) as a waste from palm oil production have potentials as alternative feed stock for biodiesel production. This study investigated the use of POME from Imo State, Nigeria, as feedstock for biodiesel production through a consortium of POME-isolated Pseudomonas aeruginosa and Candida albicans at room temperature (28±2°C). The POME samples were characterized using standard methods. Microbial population was also determined by standard methods. Biodiesel production was optimized following a 4X3 Box-Behken experimental design involving microbial consortium inocula sizes (0.1%v/v, 0.2%v/v, 0.3%v/v), pH 6, 7, 8 and ethanol/POME ratios: 2:1, 4:1, 6:1 (ml) and incubation time (12, 18, and 24 hours), and the resulting biodiesel was measured. There were significant variations ($p < 0.05$) in pH, free fatty acid (FFA) content and moisture content of the POME samples. Acid values were 13.06±0.03, 15.73±0.02, 40.46±0.05, and 20.06±0.02 mgKOH/mg for Umuagwo, Umuokanne, Naze, and ADAPALM samples, respectively. Saponification values were 145.98±0.12, 159.25±0.13, 88.12±0.05, and 141.08±0.12 mg/g, respectively. Microbial analysis showed abundant populations of lipolytic C. albicans and P. aeruginosa across POME samples, with Umuagwo and ADAPALM samples having the highest lipolytic Candida counts (103.60 × 107±20 and 95.00 × 107±20). C. albicans from Umuokanne and P. aeruginosa from Naze exhibited the highest lipolytic zones of 0.4 ± 0.08 and 0.7±0.14, respectively. The biodiesel yield under optimized conditions (Time: 24 hr; pH: 8; Inoculum size: 0.27ml; Ethanol/POME Ratio: 3.8v/v) was 42.83±0.76%, and within the predicted interval (0.3513, 0.4597). The acid value of the produced biodiesel, cetane rating, and kinematic viscosity were within commercial biodiesel standards (0.5 mg/KOH, 48-60, 1.9-6.0 mm^2/s at 40°C, respectively). This study demonstrated the feasibility of using Imo, Nigeria POME as an alternative biodiesel feedstock, highlighting the importance of optimization for maximum yields

Keywords: Palm oil mill effluent, green alternative, waste utilization, biodiesel production, microbial consortium

1. INTRODUCTION

Palm oil mill effluent (POME) is a major waste product of the palm oil industry that poses significant environmental challenges when indiscriminately dumped without remediation due to its high organic content and acidity (Ibegbulam-Njoku & Achi, 2014). However, POME also represents a potential feedstock for biodiesel production due to its high lipid content mostly free fatty acid (FFA) that show similar characteristics to jatropha oil (Primandari et al., 2013) which has been reported to yield between 63.90% to 82.51% biodiesel (Agus et al., 2020). Recent researches have explored the use of microbial consortia (such as yeast and microalgae; yeast and bacterium) for concomitant lipid/biodiesel production and bioremediation of POME (Ignatia et al., 2023; Ahasanul et al., 2021) with a focus on optimizing the process parameters. For example, Dhany and Dianursantim (2022) optimized the ratio of two microalgae consortium *Chlorella vulgaris* and *Spirulina plantesis* and nitrogen content of the growth medium. They reported a lipid content increase of up to 36% compared to Chlorella vulgaris at a ratio of 3:2 for *Chlorella vulgaris* - *Spirulina plantesis*. Ignatia et al., (2023) co-cultured a yeast *Rhodotorula toruloides* and a microalgae *Ankistrodesmus falcatus* and reported fold increases of 2 and 1, respectively for lipid production and COD in POME reduction. Ahasanul et al., (2021) reported lipid production of 2.27 ± 0.10 g/L by a co-culture inoculum of a yeast (*Lipomyces starkeyi*) and bacterium (*Bacillus cereus*), which were substantially higher than those of the monocultures investigated.

Though, not having been investigated in the context of biodiesel production from POME, *Pseudomonas* and *Candida* spp, along with other soil lipolytic microorganisms are excellent lipid accumulators, especially triacylglycerols (TAGs) - the main substrates for biodiesel production (Cea et al., 2015; Halim et al., 2012). In the context of POME, certain strains of *Pseudomonas* spp have been identified for their proficiency in breaking down complex organic molecules, including phenols and fatty acids, present in POME (Ong et al., 2014). For example, Ong et al. (2014) reported on the efficient degradation of phenolic compounds in POME by *Pseudomonas citronellolis* and lipid accumulation by *Candida* tropicalis. *Candida*'s lipid-rich biomass serves as a valuable precursor for biodiesel synthesis (Halim et al., 2012).

The efficient degradation of organic matter by *Pseudomonas* spp is a fundamental step in the co-culture approach with *Candida* sp for biodiesel production. Thus, co-cultivation of *Pseudomonas* and *Candida* spp takes advantage of their complementary metabolic activities. While *Pseudomonas* sp degrades the complex organic compounds present in POME into simpler substrates such as fatty acids, *Candida* sp readily absorbs these fatty acids, converting them into high-quality lipids/ biodiesel. This symbiotic relationship optimizes the utilization of organic matter and enhances the overall efficiency of the biodiesel production process (Chin et al., 2017).

In addition, the composition of the growth medium and other process parameters optimization also play key roles in lipid accumulation. For example, Mattanna et al., (2014) investigated the effects of medium components and culture conditions on the lipid yields and lipid profiles of two (2) *Yarrowia lipolytica* strains, QU22 and QU137, using shake flasks. They reported the biomass, lipid yield, and lipid content reach of 6.75 g/L, 3.48 g/L, and 51.55% for *Y. lipolytica* QU22, and 6.85 g/L, 3.52 g/L, and 51.38% for *Y. lipolytica* QU137, respectively under optimized conditions (cultivation for 3 days at 25^{o}C and 150 rpm, using peptone as a nitrogen source and glucose as a carbon source). Ahasanul et al., (2021) used a co-culture of yeast (*Lipomyces starkeyi*) and bacterium (*Bacillus cereus*) to optimize lipid accumulation capability and simultaneous treatment of wastewater using palm oil mill effluent (POME) as a carbon source. Their reported Design of experiments (DoE) results suggested that the maximum lipid accumulation of 2.95 g/L and COD removal efficiency of 86.54% could be obtained while the inoculum composition, pH, temperature, and incubation time were 50:50, 6.50, 32.5 °C, and 90 h, respectively.

The fatty acid profile of the microbial lipids is crucial for determining biodiesel properties. Zayed et al., (2017) characterized the chemical components of waste oils using GC-MS, identifying their fatty acid methyl esters (FAMEs) and other obtained compounds by retention times and confirming them by comparing their mass fragmentation patterns with the GC-MS instrument library storage mass spectra.

While these studies demonstrate the potential of using microbial consortia for biodiesel production, challenges remain. Demonstrating ambient temperature biodiesel production from POME using a consortium of POME-isolated *Pseudomonas aeruginosa* and *Candida albicans* has not been documented. Further, lipid productivity improvement, large-scale cultivation systems optimization, production costs reduction needs to be investigated.

Therefore, the current energy crises in Nigeria has necessitated that optimizing the process parameters of biodiesel production at ambient temperature using a consortium of POME-isolated *Pseudomonas aeruginosa* and *Candida albicans* be investigated as this could lead to the development of an economically viable and environmentally friendly process for converting POME into biodiesel.

2. METHODOLOGY

Study Area

Sampling locations were three (3) palm processing mills in Ohaji and 1 from Naze with the coordinates: ADAPALM 5.4591° N, 6.8374° E; DMS coordinate of Umuagwo 5°18'12.60" N 6°56'26.39" E. Naze 5° 26' 36.3300" N and 7° 3' 57.3696" E (Google maps, 2024). Ohaji and Naze are communities located in Imo State, Nigeria, known for their rich biodiversity (Egbuche et al., 2022) and significant contribution to oil palm production in the region. ADAPALM is a state government established palm oil processing company sited in Ohaji region, and is a well-known in the oil palm research and processing sector in Nigeria.

Sample collection

POME was collected aseptically from four (4) palm oil processing sites, three (3) in Ohaji, Imo State - ADAPALM, Umuagwo, and Umuokanne, Ohaji Imo State, and one (1) from Naze as described by (Opurum et al., 2017) but modified. Four (4) sterile five-liter (5L) containers were filled with POME samples and they were placed in two iceboxes before being transported to the laboratory where they were preserved at 4^{o}C.

Isolation and Enumeration of *Pseudomonas aeruginosa* and *C. albicans*

One millilitre (1ml) of each of the POME samples was serially diluted in 9 ml of sterile physiological saline in a 20 ml sterile test tube. After thorough vortexing, a 10- fold (v/v) serial dilution was made by transferring 1 ml of the stock solution into freshly prepared diluent (normal saline) to a range of 10^1, 10^2, 10^3 to 10^7 dilutions. Adopting spread plate technique, 0.1 ml of the diluted sample was inoculated onto sterile Cetrimide agar for the enumeration and visual isolation of *Pseudomonas aeruginosa* (Solberg et al., 1972), 0.1 ml of the diluted sample was also inoculated onto CHROMagar *Candida* (Murray et al., 2005) and SDA for the isolation of *Candida albicans,* and the enumeration of fungal population respectively. Inocula were uniformly spread with a sterile hockey stick spreader and plates were incubated at ambient temperature (28±2^{0}C) for 24-48 h for the cetrimide agar and 3-7 days for CHROMagar *Candida*/SDA.

Screening of *Pseudomonas* and *Candida* spp For Lipolytic Activity

The isolated microorganisms from Cetrimide agar and CHROMagar *Candida* were screened for lipase production according to (Salihu et al., 2011) but Tweeen-80 was used as a lipid source instead of olive oil. The incubation was 24h for the bacterium and 48h for the yeast.

Identification of Highest Lipolytic *Pseudomonas* and *Candida* spp

DNA Extraction

Bacterial and fungal genomic DNA extraction was done using a ZR fungal/bacterial DNA mini prep extraction kit supplied by Inqaba South Africa. A heavy growth of the pure culture of the bacterial and fungal isolates was suspended in 200 microliters of isotonic buffer into a ZR Bashing Bead Lysis

tubes, 750 microliters of lysis solution was added to the tube. The tubes were secured in a bead beater fitted with a 2 ml tube holder assembly and processed at maximum speed for 5 minutes. The ZR bashing bead lysis tube were then centrifuged at 10,000xg for 1 minute (ZYMO Research Instruction Manual, 2021).

DNA Quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer (Bunu, Otele, Alade & Dodoru, 2020). The software of the equipment was lunched by double clicking on the Nanodrop icon. The equipment was initialized with 2 µl sterile distilled water and blanked using normal saline. Two microliters of the extracted DNA were loaded onto the lower pedestal, the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the "measure" button.

16S rRNA Amplification

The 16S region of the rRNA gene of the isolate was amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R: 5'-CGGTTACCTTGTTACGACTT-3' primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microlitres for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV transilluminator.

Internal Transcribed Spacer (ITS) Amplification

The ITS region of the rRNA gene of the isolate was amplified using the ITS1F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3, primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 microliters for 35 cycles. The PCR mix included: the X2 Dream Taq Master mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 53ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 15 minutes and visualized on a UV transilluminator**.**

Sequencing of 16SRNA and ITS

Sequencing was done using the BigDye Terminator kit on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10 µl, the components included 0.25 µl BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10 µM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing conditions were as follows 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4min (Applied Biosysytems, 2010).

Phylogenetic Analysis

The obtained sequences were edited using the 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 & Nei, 1987). The bootstrap consensus tree inferred from 500 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes & Cantor 1969).

Characterization of POME

POME samples were preheated at 60ºC until they become homogenous. Then, they were filtered with triple layers of cheesecloth to remove debris (Opurum et al., 2017). Characteristics such as saponification and acid values, free fatty acid (FFA) and moisture contents were determined and fatty acids composition analyzed using GC/FID (Abdullah et al., 2013; Salihu et al., 2011).

Determination of Acid Value and FFA

A total of 50ml of freshly neutralised hot 95% ethanol (ethyl alcohol) and about two drops of phenolphthalein indicator (prepared by dissolving 1g of phenolphthalein in 100mL of ethyl alcohol) were transferred into 1.5 g of each oil sample contained in different conical flasks. The mixture was then boiled in a water bath for about fifteen minutes (75-80°C). After which, the mixture was titrated with 0.1 M NaOH with constant shaking for proper mixing until the endpoint indicated by a slight pink colour that persisted for 30s. The volume of titrant used was recorded (AOAC, 2005). The titration was in triplicates for each sample and acid value calculated as below:

$$Acid\ Value\ (mgKOH/g) = \frac{56.1 \times V \times N}{W} \qquad 1$$

Where,

V = Volume in mL of standard potassium hydroxide or sodium hydroxide used

N = Normality of the potassium hydroxide solution or Sodium hydroxide solution; and

W = Weight in gm of the sample

FFA will be calculated using either equation 2 below

FFA (as oleic acid) = $\frac{AV \times MW\ Oleic\ Acid}{10\ (\text{MW KOH})}$ (2)

Moisture Content Determination

The moisture content of oil was carried out by gravimetric method according to AOCS Ca 2c–25 (Bahadi, Japir, Salih & Salimon, 2016) modified for this study. A total of 10 g of each POME sample transferred into aluminum dishes was heated at 101 °C for half an hour. The heating was continued until a constant weight was achieved. Then, equation 3 was used to calculate the moisture content.

$Moisture\ Content\ (0/0) = \frac{Wb - Wa \times 100}{\text{Wb}}$

Where Wb=weight before drying

Wa=weight after drying

pH Determination

The pH of each homogenized POME sample was determined by using recently calibrated (Piyush & Narvdeshwa, 2022) HANNA pH meter. A total of 50ml of each of the homogenized POME samples were transferred into separate 100ml volumetric flasks. Then, the calibrated pH meter was directly inserted into each of the flasks and the content was stirred with the handle of an inoculation loop gently to ensure uniformity. After about 40 seconds, the pH reading displayed on the pH meter stabilized and was recorded.

Saponification Value Determination

The saponification value (SV) of crude palm oil was determined in line with British Standards BS 684 2.6.1977 (Abdullah et al., 2013). Firstly, 2 g of the oil sample was placed in a 100 mL round bottom flask. Afterward, 25 mL of 0.5 N ethanolic potassium hydroxide was added to the sample. The mixture was subsequently refluxed, boiled uniformly and repeatedly agitated for a duration of 1 hour. The warm mixture was then titrated against 0.5 N HCl in the presence of phenolphthalein (1%). To another flask, a blank was prepared by adding the same quality and quantity of the ethanolic potash but without the oil sample. The saponification value was calculated using equation (6) shown below.

$$SV = \frac{((V_b - V_s) \times (N)\text{of titrant}) \times (56.1)}{\text{weight of samples in grams}} \left(\frac{\text{mgKOH}}{\text{gsample}}\right), \quad (4)$$

where, Vb = volume (mL) of blank; Vs = volume (mL) of titrant.

Optimization of Biodiesel Production from POME using a co-culture of *Pseudomonas aeruginosa* and *Candida albicans*

Standardization of Inoculum

Three loopfuls each of 24 h culture of the identified lipolytic *Pseudomonas aeruginosa* and *Candida albicans* were inoculated onto previously sterilized nutrient broth and potato dextrose broth, respectively. Standardization of the inocula was performed using MacFarland 0.5 turbidity standard (prepared by adding 0.5 ml of 1.175% barium chloride to 99.5 ml of 1% H_2SO_4). The turbid suspension was viewed at 625nm and its density equivalent to a bacterial suspension of 1.5×10^8 cfu ml^{-1} was compared to the density of the newly prepared bacterial/fungal suspension.

Biodiesel Production

The optimum biodiesel production conditions at 28 ± 2^0C was determined using Box - Behken Design in RSM analysis at 3 levels and 27 sets of experiments for each set carried out in triplicates (Table 1). Agitation speed was maintained at 250 RPM (Ricca *et al*., 2013).

Table 1. Experimental Design of the Optimized Biodiesel Production

Time (hr)	pH	Inoculum Size (ml)	Ethanol/POME Ratio (ml)
18	7	0.3	6
18	7	0.2	4
18	7	0.1	2
24	6	0.2	4
24	7	0.3	4
24	7	0.3	4
12	7	0.2	6
18	6	0.3	4

12	8	0.2	4
18	8	0.1	4
18	7	0.2	4
12	7	0.2	6
18	7	0.3	6
18	6	0.2	6
12	7	0.1	4
18	6	0.1	4
12	7	0.3	4
24	7	0.2	2
18	7	0.1	6
12	7	0.1	4
18	8	0.2	2
18	7	0.2	4
12	7	0.3	4
12	7	0.2	2
18	8	0.3	4
12	7	0.2	6
18	6	0.2	2
18	7	0.2	4
18	7	0.2	4
18	7	0.1	6
18	7	0.2	4
18	6	0.2	2
18	8	0.3	4
18	8	0.2	6
24	8	0.2	4
18	7	0.1	2
24	7	0.2	6
12	6	0.2	4
18	8	0.2	6
12	8	0.2	4
18	6	0.2	2
24	7	0.2	2
18	8	0.2	6
12	6	0.2	4
24	8	0.2	4

24	7	0.1	4
18	7	0.1	2
18	7	0.2	4
12	7	0.3	4
24	7	0.2	2
18	7	0.3	6
18	6	0.1	4
18	8	0.1	4
24	7	0.1	4
18	6	0.3	4
12	7	0.2	2
18	7	0.1	6
18	6	0.1	4
24	7	0.1	4
18	8	0.3	4
12	8	0.2	4
18	7	0.3	2
24	6	0.2	4
24	6	0.2	4
18	7	0.3	2
18	8	0.2	2
18	6	0.2	6
12	7	0.1	4
12	7	0.2	2
18	6	0.3	4
18	7	0.2	4
18	7	0.2	4
24	7	0.3	4
18	7	0.3	2
18	6	0.2	6
24	8	0.2	4
24	7	0.2	6
18	8	0.2	2
12	6	0.2	4
18	8	0.1	4
24	7	0.2	6

Key: Ino= Inoculum size, E/P =Ethanol/POME Ratio

A total of three (3) sets of 100 ml of POME were each dispensed into three (3) 200 ml volumetric flasks. Phosphate buffers (pH 6, 7, and 8) were used separately to stabilize the previously homogenized POME and labelled. These flasks and their contents were sterilized. After cooling to about 40ºC, one (I) ml of the pH- stabilized POME samples (labelled, 6,7,8) were dispensed into eighty-one (81) sterile 20ml test tubes labelled according to the experimental design.

Thereafter, the inoculum size- Ethanol/ratio (catalyst-alcohol mixtures) as described in the above ratios were introduced into each of the pH- stabilized POME samples dispensed in the 81 labelled test tubes. These tubes were shaken for 12, 18, and 24hrs respectively, after which the volume of biodiesel produced were siphoned out with a one (1) ml syringe and measured. The produced biodiesel was washed with boiled water (50ºC) and allowed to settle in a separating funnel for 12h (Ishola *et al*., 2020).

Biodiesel yield

Biodiesel yield was calculated according to equation 5.

$$Biodiesel\ Yield\ (0/0) = \frac{VBP \times 100}{\text{VP+VA (ml)}} \qquad 5 \text{ (Aworanti et al., 2019)}$$

Where VBP=Volume of biodiesel produced (ml)

VP =Volume of POME used (ml)

VM= Volume of ethanol used (ml).

Flash point Determination

The flash point of the diesel was determined using closed cup tester by ASTM D92 standard (Thushari & Babel, 2018). The sample (30ml) was thoroughly mixed and brought to the specified test temperature (32.5°C). The closed cup containing the sample was then heated at a specified rate of 5°C to 6°C (9°F to 11°F) per minute using a controlled heat source. At intervals of every 0.5°C rise in temperature during the heating, a small volume of air was introduced into the cup by opening and closing a shutter or valve. At each introduction of air, a small flame was passed across the opening of the cup to check for the presence of flammable vapors. The temperature at which the flammable vapors from the sample briefly ignited and produced a distinct flash was recorded as the flash point.

Viscosity Determination

Viscosity was by using OFITE automatic viscometer D445 (Saeed et al., 2019). Ten (10) ml of the diesel fuel sample was thoroughly mixed and brought to the specified test temperature, 60°C. The sample was allowed to reach thermal equilibrium at the test temperature. The appropriate size viscometer capillary tube was selected based on the expected viscosity range of the diesel sample, as specified in the ASTM D445 standard. The selected viscometer capillary tube was thoroughly cleaned, dried, and mounted on the OFITE automatic viscometer. The viscometer was charged with the diesel sample by inverting it and allowing the sample to flow into the capillary tube. Then, the charged viscometer was placed in a temperature-controlled bath and allowed to equilibrate at the test temperature for 15 minutes. The OFITE automatic viscometer automatically tilted the viscometer to the horizontal position, allowing the diesel sample to flow through the capillary tube under the influence of gravity. The time required for the meniscus of the diesel sample to pass between two specified timing marks on the capillary tube was determined and the kinematic viscosity of the diesel sample was calculated by multiplying the measured flow time (in seconds) by the calibration constant of the specific viscometer capillary tube used.

Determination of Cetane Rating

Cetane rating was done using handheld Labgent octane/cetane meter ASTM D976 standard (Moser, 2009, Kaisan et al., 2020). A small amount (2 ml) of the prepared diesel fuel sample was injected into the combustion chamber of a newly calibrated octane/cetane meter. The meter measured the combustion characteristics of the fuel sample (the ignition delay time and the pressure-time curve). These parameters are related to the Cetane number through empirically derived correlations. Based on the combustion analysis data and the calibration curves, the meter calculated and displayed the Cetane number of the diesel fuel sample.

3. RESULTS AND DISCUSSIONS

The acidic pH of POME has been widely documented given that it is rich in free fatty acids (Leela et al., 2018; Soleimaninanadegani & Manshad, 2014; Primandari et al., 2013). The very high acid and free fatty acid levels observed in the Naze sample (Table 2) align with studies noting issues with uncontrolled hydrolysis of palm oil at some mills (Lam & Lee, 2011). This high acidity indicates excessive release of free fatty acids during milling and explains why the pH of POME is adjusted with NaOH before lipase production is initiated as reported by Ricca et al., (2013). Also, the current finding agrees with the works of Chinyere et a., (2018) who reported a significant decrease in acidity on POME polluted soils in Abia State. The present study differed from the reported pH of Anwana and Ogbemudia, (2015) from Akwa Ibom (5.5, 5.6, 6.0) from three sampling locations. These pH differences could be attributed to the fact that the sampling sites were palm oil selling sites where different species of oil pam nut milled are spilled. The results of other physicochemical properties of the POME samples investigated revealed that all locations were significantly different from each other for all parameters ($p < 0.05$) except: Saponification Value where no difference was noticed between Umuagwo and ADAPALM; pH where no difference between Okanne and Adapalm was registered. The FFA acid content reported for ADAPALM sample (56.64) agrees with the value Ricca et al. (2013)

reported (51.64) for a sludge palm oil sample they investigated. Further, the saponification values were 145.98±0.12, 159. 25±0.13, 88. 12±0.05, and 141. 08±0.12 mg/g respectively for POME collected from the four investigated sites. These values were far lower than the reported work of Ricca et al. (2013) which was 113.17mg KOH/mg, an acid value so high that it doesn't favor alkaline transesterification. Moisture determination revealed the sample from Naze at 77.92% as highest. Others were at 66.87%, 59.82%, and 71.90% respectively for Umuagwo, Umuokanne, and ADAPALM. Similar findings were reported by Leela, Nur, Yandri & Ariati, (2018), who found palm oil mills influenced pH, moisture content, and other effluent properties. The positive relationships between mill location and effluent parameters concur with research by Leela et al (2018). The significant impact of sample location highlights the importance of characterizing POME at individual mills rather than relying on generic averages (Lam & Lee, 2011).

Table 2. Physicochemical characterization of POME Samples across the different Locations

Parameters	Location			
	Umuagwo	Umokanne	Naze	ADAPALM
AV(mgKOH/g)	13.06 ± 0.03^{a}	15.70 ± 0.02^{b}	40.46 ± 0.05^{c}	20.03 ± 0.02^{d}
FFA	36.88^{e}	43.00^{f}	114.25^{g}	56.64^{h}
SV (mg/g)	145.98 ± 0.12^{c}	159.25 ± 0.13^{a}	88.12 ± 0.05^{b}	141.08 ± 0.12^{c}
pH	4.10^{e}	3.20^{d}	3.90^{f}	3.50^{d}
Moisture (%)	66.87^{a}	59.82^{b}	77.92^{c}	71.90^{d}

*AV acid value, FFA free fatty acid value, SV saponification value. All analysis were done in triplicates. The experimental values of the parameters with different superscripts across the locations are significantly different from each other at P<0.13105. Values are represented as mean ± STD

From Table 3 and Figure 1, stearic acid, palmitic acid, and myristic acid were the most abundant found in the analysed POME, with 9.323 (20.31%w/w), 7.294 (15.89%w/w), and 6.573 ppm (14.32%w/w) respectively.

Table 3. Fatty Acid profile of the POME

Names of Fatty Acids	**Amount (PPM)**
Myristic Acid C14	6.573
Palmitic Acid C16	7.294
Stearic Acid C18	9.323
Oleic Acid C18:1	1.294
Linoleic Acid C18:2	1.062
Linolenic acid C18:3	3.617
Eicosanoic Acid C20	5.416
Mead Acid C20:3	4.26518
Arachidonic Acid C20:4	2.321
Eicosapentaenoic Acid C20:5	3.95078
Docosatetraenoic Acid C22:4	4.036
Docosapentaenoic Acid C22:5	1.171
Cervonic Acid C22:6	2.947
Total	45.884

Matinja et al., (2019) reported Palmitic and oleic acid as occurring highest from their extracted POME oil with 43.5% w/w and 40.0% w/w respectively while Nasaruddin et al., (2014) recorded 42.12% palmitic acid and 40.31% oleic respectively, a difference which could be accounted for by the geographical and climatic situations of the sampling locations. The presence of stearic acid, palmitic acid, and myristic acid, however, in substantial quantities is advantageous for the transesterification process, which is the primary reaction involved in biodiesel production. Stearic acid (C18:0), palmitic acid (C16:0), and myristic acid (C14:0) are long-chain saturated fatty acids, suitable feedstocks for biodiesel production because their long-chain structures and lack of double bonds make them more stable and less prone to oxidation compared to unsaturated fatty acids. Several studies have explored the use of POME as a feedstock for biodiesel production due to its high lipid content and the potential for valorizing this waste stream (Matinja et al., 2019; Primandari et al., 2013). For example, a study by Chong et al. (2019) investigated the production of biodiesel from POME using a

two-step acid-base catalyzed transesterification process. They found that the fatty acid composition of the POME sample was suitable for biodiesel production, with palmitic acid being the most abundant fatty acid.

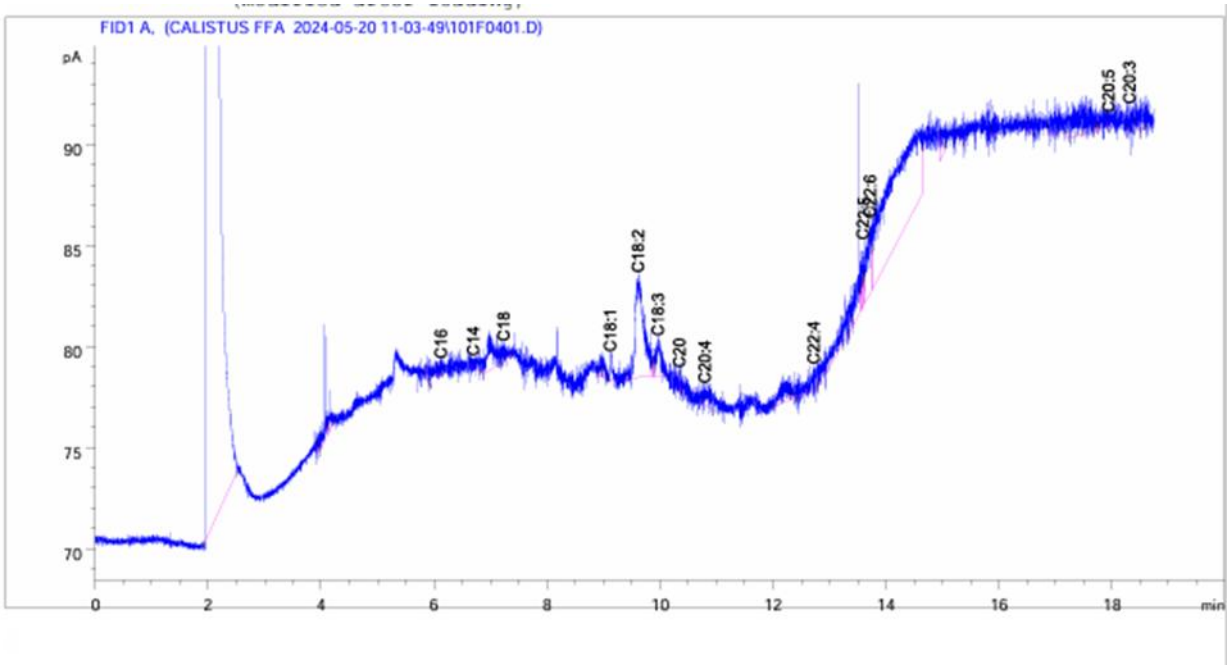

Figure 1: POME chromatograph

The abundant populations of lipolytic yeasts recorded in the present study (Table 4) is expected given the oil-rich nature of palm oil mill effluent. However, some functional differences may exist between locations the locations. These differences suggested that Umuagwo and ADAPALM POME may provide a more favorable environment for the growth and proliferation of lipolytic *Candida* species. The specific factors contributing to this disparity could include differences in soil composition, nutrient availability, temperature, moisture levels, and other environmental variables and ecological conditions including potential sources of lipids, organic matter availability, and microbial interactions. Similar studies have reported abundant lipolytic *Candida*, other yeasts and fungi counts. Islam et al., (2017) investigated the use of a POME-isolated oleaginous yeast, *Lipomyces starkeyi* as a cost-effective microbial lipid use through the bioremediation of POME. Nwuche et al., (2014) recorded fungal count of 1.8×10^3 colonies/ml and identified fungal genera included *Aspergillus, Penicillium, Trichoderma,* and *Mucor*. Bala et al, (2018) also recorded a population of POME-degrading fungi ranging from $2.8 \times 10^3 – 4.7 \times 10^4$ cfu/ml. The total heterotrophic fungi (THF) ranged from $2.1 \times 10^4 – 6.4 \times 10^4$ cfu/mL.

Further, as *Candid*a species with lipolytic activity have the potential to play a role in bioremediation processes, breaking down lipids and contributing to the degradation of organic pollutants in soil and water, the higher *Candida* count in POME samples from Umuagwo and ADAPALM could imply a greater capacity for lipid degradation in that area.

Table 4. Total lipolytic *C. albicans and P. aeruginosa* obtained after 48h incubation at 28±°C (cfu/ml) and 37°C respectively

Location	Pseudomonas spp No (Mean± SEM)	*Candida* spp No. (Mean± SEM)
ADAPALM	$105.33±20×10^{7c}$	$95.0±20×10^{6d}$
NAZE	$102.66±15×10^{7a}$	$79.6±20×10^{6c}$
OKANNNE	$77.33 ±12×10^{7c}$	$81.3±15×10^{6d}$
UMUAGWO	$142.66±20×10^{7c}$	$103.6±20×10^{6d}$

Further, as *Candid*a species with lipolytic activity have the potential to play a role in bioremediation processes, breaking down lipids and contributing to the degradation of organic pollutants in soil and water, the higher *Candida* count in POME samples from Umuagwo and ADAPALM could imply a greater capacity for lipid degradation in that area.

Also. in addition, the results demonstrated that POME from Umuagwo supported the most *Pseudomonas* growth. This finding contributes to the broader understanding of microbial diversity in different geographical regions. Okwute et al., (2015) evaluated the biodegradation of palm oil mill effluent and lipase activity by *Pseudomonas aeruginosa, Bacillus subtilis* and *Candida albicans* treatment. The total viable counts of the microbial isolates during biodegradation of POME revealed the highest colony counts as that of *P. aeruginosa* ($8.30×10^9$ cfu/ml). Their findings and that of the current investigation underscore the fact that the *Pseudomonas* isolated from the present study could well be employed in POME bioremediation.

Table 5. Demonstration of lipolysis in *Candida albicans* and *Pseudomonas aeruginosa*

Location	***C. albicans Mean Zone of Inhibition (cm) ± SEM)***	***P. aeruginosa Mean Zone of Inhibition (cm) ± SEM)***
Umuagwo	0.2± 0.05	0.4 ± 0.08
Umuokanne	0.4± 0.08	0.6 ± 0.12
Naze	0.3 ±0.06	0.7 ± 0.14
ADAPALM	0.3± 0.06	0.5 ± 0.10

Highest lipolytic *Candida* sp and *Pseudomonas* spp (Table 5) were identified and showed 100 percent relatedness to *Candida albicans* and *Pseudomonas aeruginosa* (figs 2 &3). Carrazco-Palafox, Rivera-Chavira, Ramírez-Baca, Manzanares-Papayanopoulos and Nevárez-Moorillón, (2018) reported a large clear zone of 2.02 cm with *P. aeruginosa* when 5.0 mM magnesium was added to tributyrin agar (mTBA) and left for 72h, an increment of 38% more zone of inhibition more than without the addition of ions.

The differences in inhibition zones between organisms across sample locations in the present study could indicate generally consistent lipolytic microbial profiles in this effluent. Other studies have found higher variability in lipolytic activity between isolation sources, likely due to differences in effluent composition, treatment methods, etc (Carrazco-Palafox et al., 2018). Thus, the size of inhibition zones is highly dependent on the organism, culture conditions, assay methods, and other factors (Carrazco-Palafox et al., 2018).

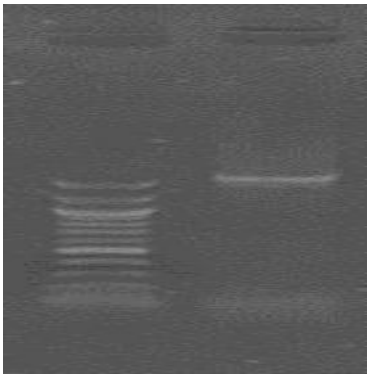

Fig 2 Agarose gel electrophoresis showing the amplified 16srRNA. Lanes L1 represents the amplified 16srRNA at 1500bp while lane L represents the 100bp DNA ladder.

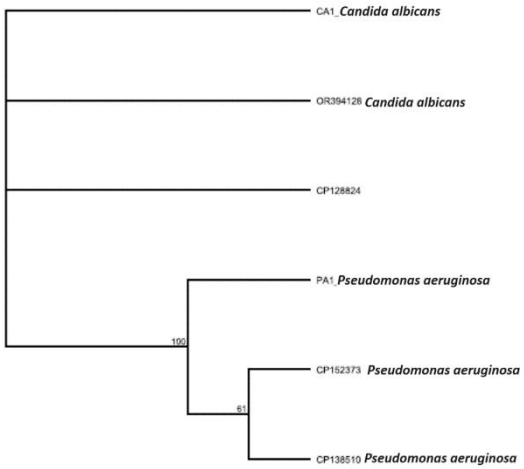

Fig 3 Phylogenetic tree showing the evolutionary distance between the fungal and bacterial isolates

Figure 4 showed a series of peaks labeled with different carbon chain lengths (e.g., C18:2, C18:3, C20, C22, etc.), indicating the presence of various fatty acid methyl esters (FAMEs) commonly found in biodiesel derived from palm oil. Both chromatograms (Figs 4 & 5) displayed multiple peaks,

indicating the presence of hydrocarbons and fatty acid derivatives in both samples, which is expected for biofuels and fossil fuels. The retention times and peak distributions differed significantly between the two chromatograms. This is due to the distinct chemical compositions of biodiesel derived from renewable sources (e.g., vegetable oils or animal fats) and fossil diesel derived from crude oil. In the biodiesel chromatogram the peaks were more defined and concentrated within a specific retention time range (around 8-14 minutes). This pattern is characteristic of fatty acid methyl esters (FAMEs), which are the main components of biodiesel fuels whereas in the fossil diesel chromatogram, the peaks were more spread out across a broader retention time range, reflecting the complex mixture of hydrocarbons and other compounds present in fossil-based diesel fuels.

Further, the intensity and relative peak heights differed between the two chromatograms, suggesting variations in the concentrations of specific components between the biodiesel and fossil diesel samples. Zayed, Abd El-Kareem, and Zaky (2017) similarly characterized the chemical components of waste oils using GC-MS, identifying their fatty acid, methyl esters (FAMEs) and other obtained compounds by retention times and confirming them by comparing their mass fragmentation patterns with the GC-MS instrument library storage mass spectra. Musharraf, Ahmed, and Zehra (2015) indicated that the developed GC-MS/MS method applied in their investigation effectively separated and quantified FAMEs in complex samples of biodiesel–diesel blends.

The predicted diesel yield is shown in Fig 6. The model predicted a diesel yield of about 40.55%. The prediction interval was wider than the confidence interval, which is expected as it accounts for both the uncertainty in the mean estimate and the variability of individual observations.

Table 5. A comparison of the Fatty acid profiles of the biodiesel and EMPIRE Energy Fossil diesel

Name of Fatty Acid	**Amount (ppm)**	
	Biodiesel	Empire Energy Fossil Diesel
Lauric acid C12	-	0.401
Myristic Acid C14	4.750	1.228
Palmitic Acid C16	4.993	-
Stearic acid C18	7.623	1.422
Oleic Acid C18:1	6.432	7.057
Linoleic Acid C18:2	5.446	18.820
Linolenic Acid C18:3	17.374	14.690
Eicosanoic Acid C20	1.983	-
Arachidonic Acid C20:4	3.772	3.012
Docosanoic Acid C22	1.975	1.503
Docosatetraenoic Acid C22:4	3.149	5.409
Docosapentaenoic Acid C22:5	6.809	-
Docosahexaenoic acid (DHA) C22:6	2.257	-
TOTAL	66.568	53.984

Table 6. Comparing biodiesel and Empire fossil diesel characteristics with Commercial and ASTM standards

Property/Units	**Commercial B100**	**ASTMD6751-02**	**Biodiesel**	**Fossil diesel**
Flash point (ºC)/min	130	130	75	69
Acid value (mgKOH/g)	0.50	0.50	0.54	0.5
Cetane rating	48-60	47>	51	55
KinematicViscosity (mm^2/s at 40°C)	1.9-6.0	1.9-6.0	2.6	3.8

Biodiesel production from POME using whole cell organisms or co-cultures have been investigated. Athoillah and Farah (2022) reported better lipid extraction efficiency with *Aspergillus niger* demonstrating the highest lipid yield from the cultivation on POME, fuel properties within the limits of biodiesel standards as well as up to 70% COD removal. Ignatia et al. (2023) co-cultured a yeast *Rhodotorula toruloides* and a microalgae *Ankistrodesmus falcatus* and reported fold increases of 2 and 1, respectively for lipid production and COD in POME reduction. Ahasanul et al. (2021)

reported lipid production of 2.27 ± 0.10 g/L by a co-culture inoculum of a yeast (*Lipomyces starkeyi*) and bacterium (*Bacillus cereus*), which were substantially higher than those of the monocultures investigated.

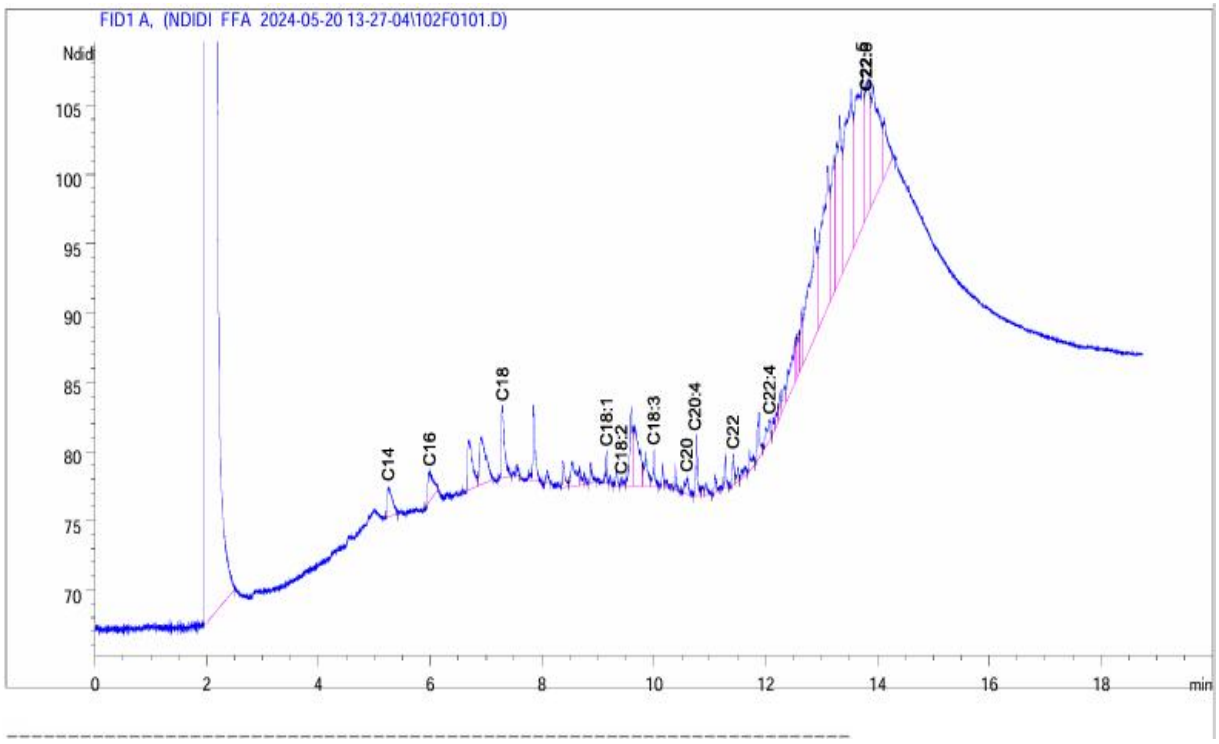

Fig 4 Produced Biodiesel chromatograph

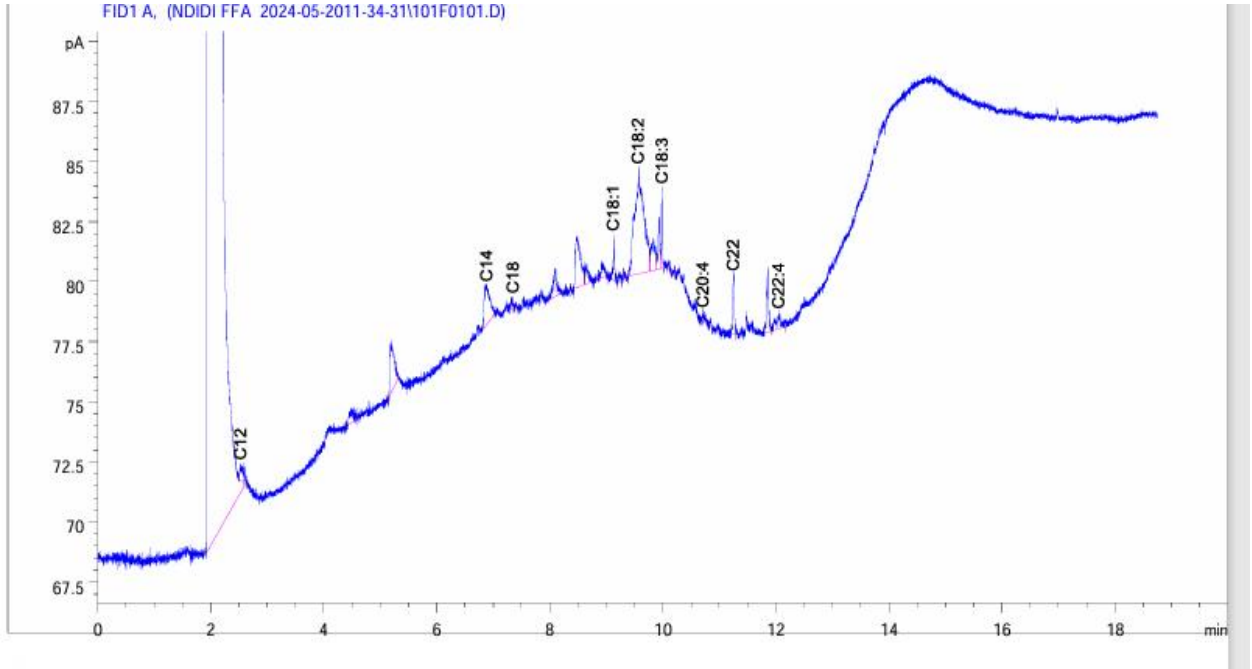

Fig 5 Fossil Diesel chromatograph

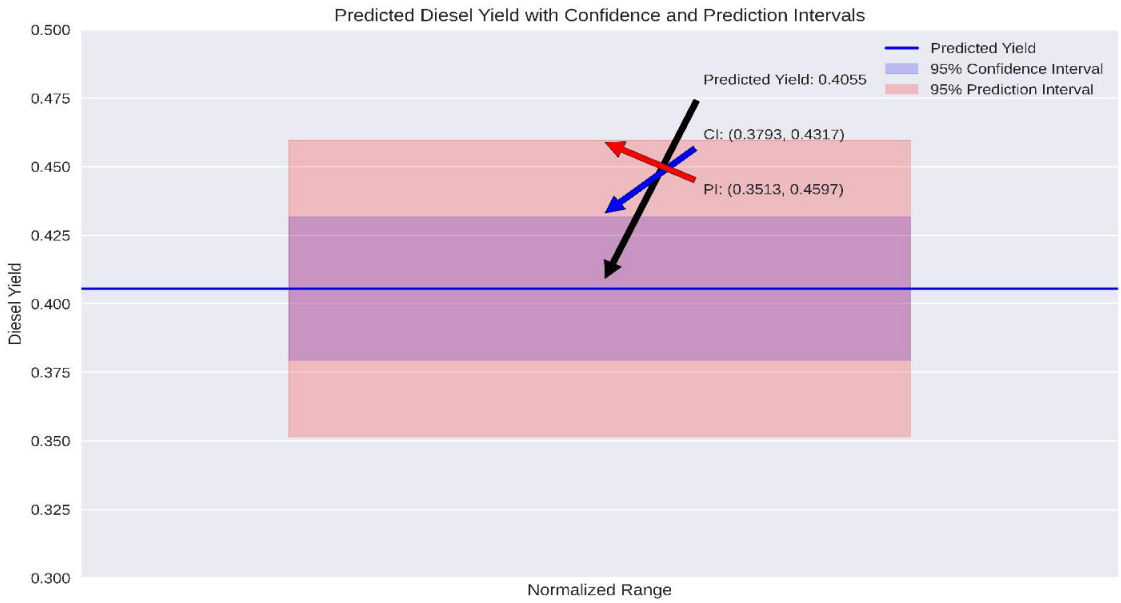

FIG 6 Predicted diesel yield by a consortium of *P. aeruginosa* and *C. albicans*

Multiple Regression Analysis of FAME is represented below

Diesel Yield = -0.012 + 0.0012 Time - 0.0042 pH + 0.611 Ino size + 0.0708 E/POME Ratio

- 0.000154 Time*Time - 0.00347 pH*pH - 2.639 Ino size*Ino size

- 0.00920 Ethanol /POME Ratio*Ethanol /POME Ratio + 0.00208 Time*pH

+ 0.0208 Time*Ino size - 0.000694 Time*Ethanol /POME Ratio

+ 0.0417 pH*Ino size + 0.00208 pH*Ethanol /POME Ratio

+ 0.0000 Ino size*Ethanol /POME Ratio……(6)

In the current study, as seen in Fig 7, the highest biodiesel yields were observed with the longest times (around 24) and the highest inoculum sizes (around 0.2-0.28). The highest yield region was observed at an ethanol/POME ratio of around 4 to 5, at inoculum size of 0.21 to 0.27. also from Fig 8, higher ethanol/POME ratios (6) led to higher yields across all pH levels, with pH 8 being the most favorable. Previous studies have demonstrated that the co-cultivation of *Pseudomonas* and *Candida* takes advantage of their complementary metabolic activities in optimizing the utilization of organic matter and enhances the overall efficiency of the biodiesel production process (Chin et al., 2017). From the current study, the model estimated 40.55% yield using a combo of *C. albicans* and *P. aeruginosa*. However, the obtained diesel yield at optimized condition (time: 24 hr; pH: 8; Inoculum size: 0.27ml; Ethanol/POME Ratio: 3.8v/v) was slightly higher at 42.85% (Fig 6). Rachmadona (2017) obtained a biodiesel yield of 81.87%% under the following conditions: lipase content of 0.3%, a methanol/oil molar ratio of 6:1, a water content of 4.7%, a stirring speed of 500 rpm, a reaction temperature of 30 °C and a reaction time of 24 hour using *Thermomyces lanuginosus* lipase. Rachmadona, Quayson, Amoah, and Ogino (2021) reported an ester content of 97.52 ± 0.21% w/w after 96 h using immobilized *Aspergillus oryzae* whole cells expressing *Candida antartica* lipase B (r-CALB) in the POME transesterification into biodiesel. Previously, Ricca et al., 2013 had reported a maximum yield of biodiesel of 62.3% (w/w SPO) at optimum levels of ethanol-to-SPO molar ratio, enzyme loading, reaction temperature, mixing speed and reaction time were 4:1, 10 U, 40°C, 250 rpm and 24 h, respectively with *Candida cylindracea* lipase.

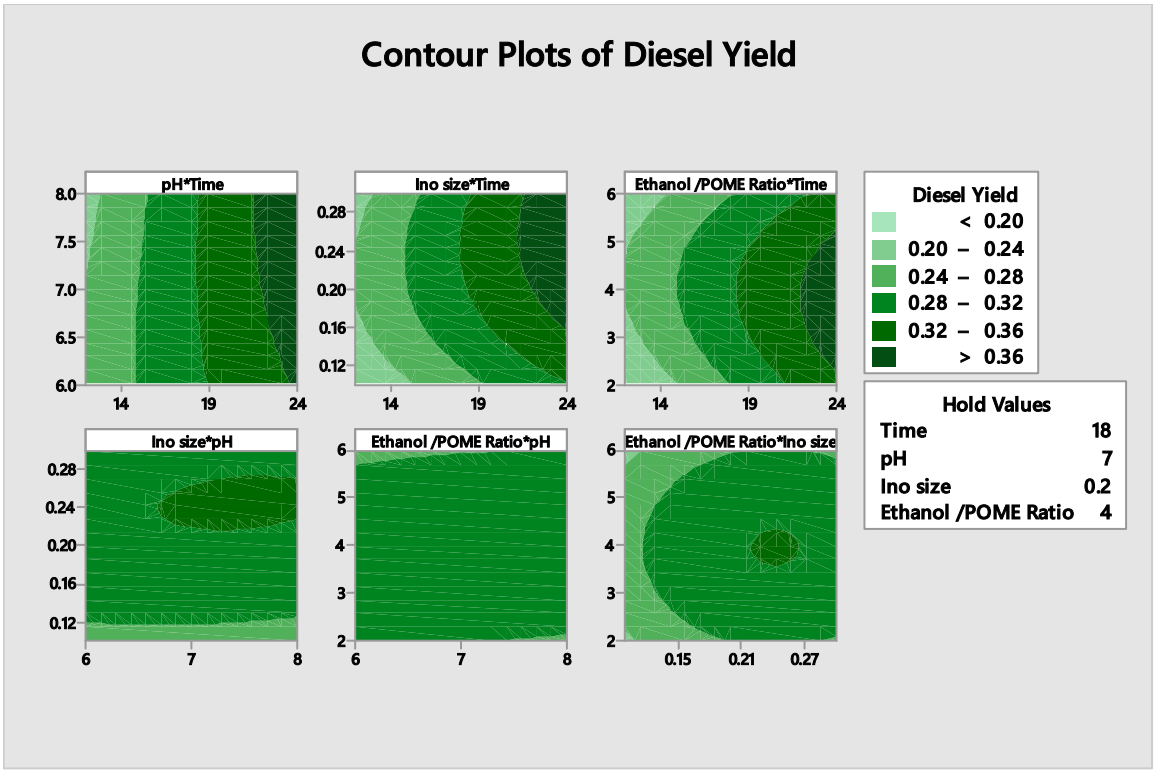

Fig 7 Contour plots of biodiesel yield using a consortium of *C. albicans* and *P. aeruginosa*

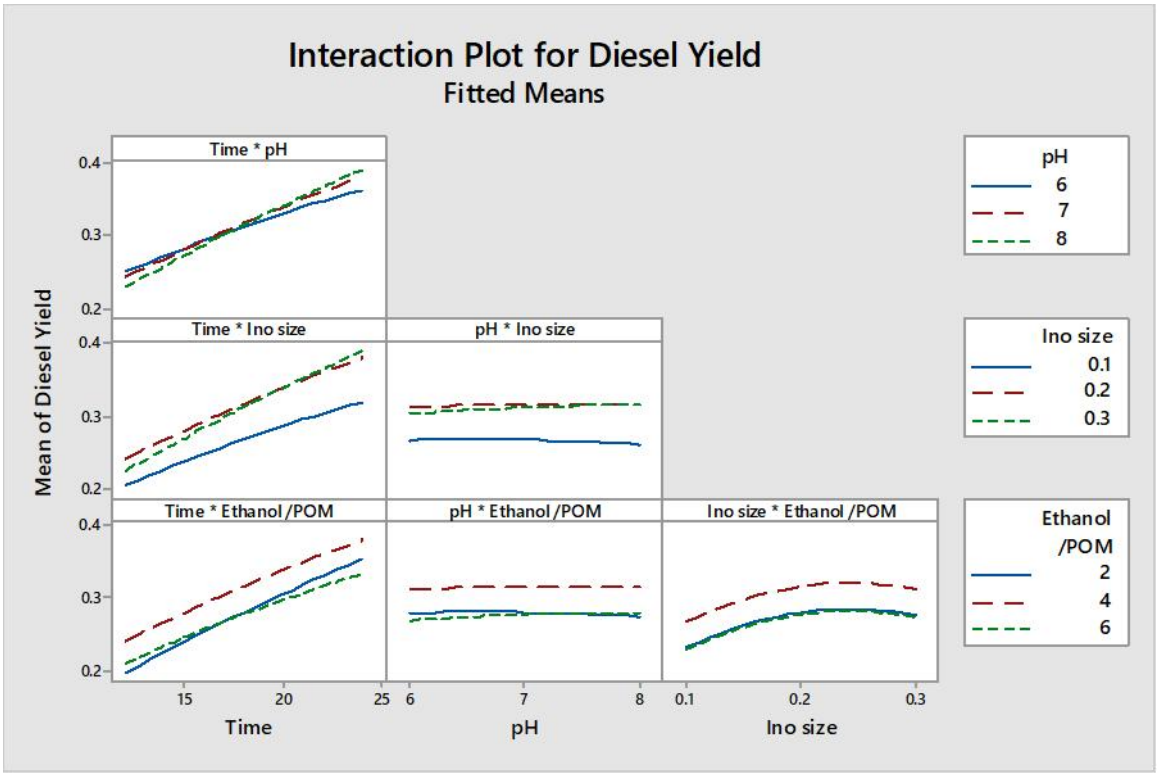

Fig 8 Interaction plot for biodiesel yield using a consortium of *C. albicans* and *P. aeruginosa*

The biodiesel from POME contained significant amounts of saturated fatty acids (Table 5) like myristic acid (C14: 4.750 ppm), palmitic acid (C16: 4.993 ppm), and stearic acid (C18: 7.623 ppm). This fatty acid profile is consistent with studies that have utilized POME as a feedstock for biodiesel production. For instance, Chong et al. (2019) reported that the major fatty acids present in their POME sample were palmitic acid (40.5%), oleic acid (38.3%), and stearic acid (4.9%).

Also, the POME-derived biodiesel had a moderate amount of oleic acid (C18:1: 6.432 ppm), a monounsaturated fatty acid commonly found in palm oil. This observation aligns with the findings of studies that have utilized POME as a feedstock, as palm oil is a primary component of POME.

Further, the biodiesel exhibited a higher concentration of Polyunsaturated fatty acids (PUFAs) like linoleic acid (C18:2: 5.446 ppm), linolenic acid (C18:3: 17.374 ppm), arachidonic acid (C20:4: 3.772 ppm), docosatetraenoic acid (C22:4: 3.149 ppm), docosapentaenoic acid (C22:5: 6.809 ppm), and C22:6 (2.257 ppm) compared to typical palm oil or fossil diesel. While POME is primarily derived from palm oil, which is relatively low in PUFAs, the presence of these PUFAs in the biodiesel could be attributed to other lipid sources present in POME feedstock, *C. albicans/P. aeruginosa* consortium or other biomass components. A study by Lam and Lee (2011) reported the presence of various microalgae species in POME, which are known to be rich sources of PUFAs. Additionally, Salihu et al. (2015) found that POME contained significant amounts of linoleic and linolenic acids, which could contribute to the PUFA content observed in the biodiesel sample.

The higher flash point of biodiesel (75°C) compared to fossil diesel (69°C) is in line with other reported values (Table 6). For example, a study by Fattah et al. (2014) found the flash point of POME-derived biodiesel to be 176°C, much higher than that of fossil diesel (60-80°C).

In addition, the slightly higher acid value of biodiesel (0.54 ppm) compared to fossil diesel (0.5 ppm) is consistent with findings from other studies. Ong et al. (2019) reported an acid value of 0.48 mg KOH/g for POME-derived biodiesel, while Fattah et al. (2014) found it to be 0.56 mg KOH/g.

Also, the lower cetane rating of biodiesel (51) compared to fossil diesel (55) is also in agreement with previous studies. Fattah et al. (2014) reported a cetane index of 47.5 for POME-derived biodiesel, while Ong et al. (2019) found it to be 51.2.

The lower viscosity of biodiesel (2.6 mm^2/s at 40°C) compared to fossil diesel (3.8 mm^2/s at 40°C) is consistent with other findings. Ong et al. (2019) reported a viscosity of 4.2 mm^2/s at 40°C for POME-derived biodiesel.

CONCLUSION

This study successfully demonstrated that palm oil mill effluent (POME) from Imo State, Nigeria, can be used as a viable feedstock for biodiesel production through the microbial activities of *Pseudomonas aeruginosa* and *Candida albicans*. Optimization of reaction conditions, including pH, inoculum size, and ethanol/POME ratio, led to significant biodiesel yields. The biodiesel produced met commercial standards, containing beneficial fatty acids and comparable properties to fossil diesel. These findings offer valuable insights into waste utilization for renewable energy production while underscoring the importance of site-specific analysis and optimization in biodiesel production.

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