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Substrate Specificity and Temperature Sensitivity of Crude Lipases from POME-Isolated *Pseudomonas Aeruginosa* and *Candida Albicans*

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A B S T R A C T

Microbial lipases have garnered attention in diverse industrial processes due to their availability, affordability, environmental benign nature, and potential for reuse when immobilized. This study partially profiled the characteristics of two lipases produced by POME-isolated Pseudomonas aeruginosa and Candida albicans. The POME samples were collected from three palm oil mills in Ohaji, and one in Naze, Owerri, Imo State, Nigeria. The physicochemical characterization of POME samples was carried out by adopting standard methods. Standard microbiological methods of determining lipolytic microbial population was adopted. The isolated lipolytic organisms were screened for lipase production on minimal salt medium containing 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, 0.5 (w/v) NaCl, 0.01 (w/v) CaCl2.2H2O and 2% bacteriological agar (pH 7) supplemented with 2% Tween-80. The two organisms that exhibited the highest lipolysis were used for lipase production in POME as a fermentation medium. The physicochemical characterization of POME samples from different locations revealed significant variations ($p < 0.05$) in parameters such as pH, free fatty acid content, and moisture content. Further, microbial analysis revealed abundant populations of lipolytic Candida and Pseudomonas species across the POME samples, with Umuagwo and ADAPALM samples having highest lipolytic Candida counts of 103.60 ×107 ±20 and 95.00 ×107±20 respectively while POME sample from Umuagwo had the highest lipolytic Pseudomonas count of 142.66×106 ±20 cfu/ml. C. albicans isolated from Umuokanne POME sample and P. aeruginosa isolated from Naze POME sample exhibited highest lipolysis zones of 0.4 ± 0.08 and 0.7±0.14 respectively among the isolates. The effects of metal ions, temperature, and pH on the relative enzyme activities of P. aeruginosa and C. albicans lipases showed significant differences and interaction effects ($p < 0.001$). Zn2+ and Na+ showed strong enhancement of activity for Candida lipase (273.7/319 RA) compared to Pseudomonas lipase (130.5/127.8 RA); Cu2+ inhibited both Pseudomonas lipase (101 RA) and Candida lipase (126.3 RA). Enzyme activity for P. aeruginosa/ C. albicans lipase increased from 30°C to 50°C (192-410 RA), with highest activity at 50°C and from 40°C to 60°C (448 RA), with maximum activity at 60°C for C. albicans lipase. Optimal activities for both enzymes were at pH 9 (200 and 280 RA for Pseudomonas and Candida respectively). Pseudomonas and Candida lipase showed the highest relative activity on hydrolysing sunflower oil (31.3 and 43.8 respectively), followed by soybean and coconut oils. Higher lipase activities were observed from Pseudomonas lipase on coconut oil (18.8) than from Candida lipase whilst Candida lipase hydrolysed soya oil (25) more than Pseudomonas lipase. The optimal activity of Pseudomonas aeruginosa and Candida albicans lipases at specific conditions suggests potential for industrial applications in sectors such as biofuel production, food processing, and detergent formulation. The ability of these lipases to effectively hydrolyze oils like sunflower and soybean highlights their utility in the production of biodiesel and other value-added products. Additionally, the enhancement of lipase activity by metal ions like Zn^{2+} and Na^{+} could inform the development of more efficient and cost-effective industrial processes.

Keywords: *Palm oil mill effluent (POME), Lipase production,POME-Isolated microbial lipase, fermentation medium, partial microbial lipase profiling*

INTRODUCTION

The palm oil industry generates a massive volume of effluent known as sludge palm oil or palm oil mill effluent (POME), a waste stream with the potential to cause significant environmental damage if left untreated (Jeong et al., 2014; Liew et al., 2015). However, POME also harbors a hidden opportunity – its triglyceride content as well as its abundant population of lipolytic organisms (Iyakndua et al., 2017). *Pseudomonas aeruginosa* and *Candida albicans* have been isolated and identified from POME as well as demonstrated their lipase production efficiencies (Nwuche et al., 2014; Soleimaninanadegani & Manshad, 2014; Islam et al., 2017).

Chemically known as triacylglycerol hydrolases (EC 3.1.1.3), lipases play a crucial role in the breakdown of triacyclglycerols, which are esters made up of glycerol and free fatty acids (Ilesanmi et al., 2020). Due to their remarkable stability in various organic solvents as well as diverse pH and temperature ranges, these biocatalysts effectively catalyze interactions in aqueous as well as nonaqueous conditions (Tan et al., 2018). Lipases find applications in numerous industrial processes such as the manufacture of saturated fatty acids from fats and oils hydrolysis (Balaji et al., 2020), bioremediation (Ktata et al., 2020), biodiesel production through esterification in the presence of shorter-chain alcohols (Cavalcante et al., 2021), the synthesis of fragrances, aromas, and flavors, (dos Santos et al., 2021) amongst others.

Lipases from fungal and bacterial sources have received a great deal of research attention because of their growing industrial applications. The major fungal genera involved in the synthesis of lipases include *Aspergillus*, *Rhizopus*, *Penicillium*, *Candida*, *Rhizomucor*, *Mucor*, *Geotrichum*, *Humicola*, *Lipomyces starkeyi,* and *Beauveria* (Chandra et al., 2020; Ahasanul et al., 2021). Biocatalysts (lipases) from these organisms and others such as *Bacillus* have been implicated in environmental cleanup of POME due to their ability to convert triglycerides into fatty acid methyl esters (FAMEs) under mild reaction conditions and their COD/BOD reductions (Ignatia et al., 2023; Ahasanul et al., 2021). Islam et al., (2017) investigated the use of a POME-isolated oleaginous yeast, *Lipomyces starkeyi* as a cost-effective microbial lipid through the bioremediation of POME.

Several studies have documented the lipase production capabilities of *P. aeruginosa*. For instance, Mobarak-Qamsari et al., (2011) isolated a strain of *P. aeruginosa* KM110 from the wastewater of an oil processing plant that exhibited high lipase activity using tributyrin agar plates**.** Other studies have reported lipase production from *P. aeruginosa* using diverse substrates, including olive oil, soybean oil, and even palm oil mill effluent (POME) (Hermansyah et al., 2018). Similarly, studies have demonstrated successful lipase production from various strains of *C. albicans* using substrates such as olive oil, corn oil, and even tributyrin (Carrazco-Palafox et al., (2018).

While both *P. aeruginosa* and *C. albicans* are adept lipase producers, their respective enzymes exhibit distinct characteristics. Lipases from *P. aeruginosa* generally exhibit broader substrate specificity, effectively hydrolyzing various triglycerides and even displaying phospholipase activity (Madan & Mishra, 2010). In contrast, lipases from *C. albicans* often exhibit a preference towards shorter-chain triglycerides (Yao et al., 2021). Moreso, lipases from *P. aeruginosa* typically function optimally at moderate temperatures (30-40°C) and near-neutral pH (pH 6-8) (Zouaoui et al., 2012). Conversely, lipases from *C. albicans* can exhibit a wider range of temperature and pH optima, with some functioning effectively at slightly higher temperatures (37-45°C) and a broader pH range (pH 5-8) (Padhiar et al., 2011).

Crude lipases are unpurified enzyme preparations obtained from fermentation broth after cell removal. Despite their crudity, they offer several advantages such as reducing the often expensive and time-consuming purification processes for lipases, enhancing enzyme activity, stability, greater variability in activity, and selectivity due to other cellular components and their possible accessory proteins content (Filho et al., 2019; Chandra et al., 2020) which may be acting as chaperones, aiding proper folding or protecting lipases from harsh environments.

While lipases from other sources like *Thermomyces lanuginosus, Aspergillus niger,* and *Bacillus subtilis* have been successfully employed for POME-based biodiesel production and POME bioremediation, crude lipases from POME-isolated *Pseudomonas aeruginosa* and *Candida albicans* from Imo State, Nigeria, offer a cost-effective alternative to the remediation of improperly dumped POME.

METHODOLOGY

Study Area

Sampling locations were 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. Ohaji and Naze are both communities located in Imo State, Nigeria, known for their rich biodiversity and significant contribution to oil palm production in the region (Egbuche et al., 2022). ADAPALM is found in Ohaji and is 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) in 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 in a cold room at 4^{o}C.

Isolation and Enumeration of Heterotrophic Microorganisms

The total microbial population in the POME samples were determined by the spread plate techniques using Nutrient Agar (NA), SDA, CHROMagar *Candida*, Cetrimide Agar and observed after incubation at ambient temperature (28±2^{0}C) for 24-48 h for NA/Cetrimide Agar and 3-7 days for SDA.

Screening of Isolated *Pseudomonas* and *Candida* spp for Lipase Production

The isolated organisms from Cetrimide agar and CHROMagar *Candida* were screened for lipase production according to (Salihu et al., 2011; Gopinath & Anbu, 2005) but Tweeen-80 broth instead of olive oil was used as a lipid source. Positive lipolysis was confirmed with Sudan 11 dye spotting and measured with a mini meter rule.

Identification of lipolytic *Pseudomona*s and *Candida* spp

Molecular characterization of lipase-producing *Pseudomonas* and *Candida* spp

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/ 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 2ml 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 et al., 2020). The software of the equipment was lunched by double clicking on the Nanodrop icon. The equipment was initialized with 2 ul of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was 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 RRNA region of the rRNA genes of the isolates were 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 genes of the isolates were 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 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, 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 10ul, the components included 0.25 ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye sequencing buffer, 10uM 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

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) is taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes & Cantor 1969).

Lipase Production by the identified *Pseudomonas aeruginosa* and *Candida albicans.*

Standardization of Inoculum

Three (3) loopfuls each of 18 h culture of the selected identified lipolytic *Pseudomonas aeruginosa* and *Candida albicans* were standardized using MacFarland 0.5 turbidity standard and a density equivalent to a bacterial suspension of 1.5×10^8 CFU ml−1 was compared to the newly prepared bacterial/fungal suspension.

Lipase Production in *C. albicans* and *Pseudomonas aeruginosa*

Lipase was produced in 10 ml of POME at 1% TSS as basal medium containing nutrients (0.45% v/v peptone, 0.6% v/v Tween- 80, and 2.2% v/v of standardized inoculum). Initial pH was adjusted to 6.0 using 1M NaOH. Flasks were incubated for 48hr at 28±2ºC and centrifuged at 4000rpm for 30 min. Cell-free extract were used as lipase (Salihu et al., 2011).

Lipase Assay, Purification and Characterization

Lipase Assay

Lipase activity assay was determined by spectrophotometry using pNPP (p-nitrophenyl palmitate) hydrolysis method as described by Ghori et al., (2011) and Gupta et al., (2004). One (1) ml of isopropanol containing 3 mg of p-nitrophenyl palmitate was mixed with 9 ml of 0.05 M Tris–HCl buffer (pH 8.0), containing 40 mg Triton X-100 and 10 mg gum Arabic in a 20ml test tube. After complete dissolution of the constituents, a total amount of 2.5 ml of freshly prepared substrate solution and varying enzyme volumes (0.1ml, 0.2ml, 0.3ml) in different test tubes were incubated for 15 min at 37◦C. After incubation, the optical density at 410 nm was recorded against an enzyme free control. A progress curve was constructed and enzymatic activity (U/ml) calculated. One lipase unit (U) was defined as the amount of enzyme that liberated 1 mol p-nitrophenol per milliliter per minute under the standard assay conditions. Enzyme activity was calculated using Beer-Lambert law:

$$Enzyme\ Activity = \frac{\Delta A\ x\ Total\ volume}{(\varepsilon\ x\ Path\ length\ x\ Enzyme\ volume\ x\ Time)} \quad 1$$

at experimentally determined control OD

$$Enzyme\ Activity = \frac{\Delta A\ x\ Total\ volume}{(\varepsilon\ x\ Path\ length\ x\ Enzyme\ volume\ x\ Time)}$$

Where:

ε is the molar absorptivity of para-nitrophenol at 410 nm = 18,300 M-1cm-1

Path length = 1 cm

Total volume = 2.4 ml

Time = 15 mins = 0.25 h

Lipase purification by Ammonium Sulphate Fractionation.

The enzyme was purified by ammonium sulphate saturation (70%) of the cell-free extract as described by Sharma et al., (2017) but with slight modification. A total of 5.15 grams of ammonium sulfate was slowly added to ten (10) ml of the crude enzyme (cell-free extract) in a 50ml volumetric flask while stirring at 4°C. The mixture was incubated for about 30 minutes to ensure complete precipitation of proteins. Then, cold centrifugation of the mixture at 4000 rpm for 3hr followed. Thereafter, the supernatant was carefully decanted and the pellets containing the partially purified lipase were re-dissolved in 5 ml of Tris-HCl buffer (pH 6.8) and dialyzed overnight against 2 l of the same buffer. The eluted lipase (enzyme) solution was used for studying the effects of temperature, pH, metal ions, and substrate specificity.

Effect of Temperature on Enzyme Activity

The optimum temperature for enzyme stability was determined by separately incubating 0.1ml of the crude enzymes with 0.1ml of the pNPP substrate in Tris- HCl buffer (pH 8) in 10ml test tubes in a water bath at different temperatures, i.e., 30°C, 40°C, 50°C, 60°C, and 70°C for 30min as described by Bussamara et al., (2010) and Ghori et al. (2011). The reaction was stopped by the addition of one (1) ml of 1M Na_2CO_3 and the absorbance of the reaction mixtures which corresponded to the release of para-nitrophenolpalmitate (pNPP) was read at 405 nm using a UNISPEC Spectrophotometer. Enzyme activity was expressed as the amount of pNPP produced per minute and calculated using equation 2.

$$\text{Enzyme activity(units/ml)} = \frac{Absorbance\ @\ 405nm}{\text{Time (min)} \times \text{Volume (ml)}} \qquad 2a$$

The obtained activities at different temperatures were compared in a graph of enzyme activity against temperature and the temperature at which the enzyme activity was highest was recorded as the optimal temperature for the enzyme's activity.

Effect of pH on Enzyme Activity

Similarly, the optimum pH for enzyme stability was determined by incubating 0.1ml of the partially purified crude enzymes with the same volume of pNPP prepared in in 0.8 ml of 0.1M acetate buffer (pH 5.0), 0.1M phosphate buffer (pH 6-7), and 0.1M Tris- HCl buffer (pH 8-9) at different pH in 20ml test tubes at 50°C for 30min. At the end of the incubation, the reaction was stopped by adding 1 ml of 1 M sodium carbonate and the absorbance read at 405 nm using a UNISPEC Spectrophotometer. Enzyme activity was expressed as the amount of pNPP produced per minute and calculated using equation 2a.

Effect of Metal ions on Enzyme Activity

Lipase activity was determined in the presence of metal ions by incubating 0.1ml of the enzymes separately in different 20ml test tubes with 0.1ml of 1mM solution of each salt, i.e., ZnCl2, MgCl2, Fe3+, K+, Na+, $CuSO_4$ and CaCl2 in 0.8ml of Tris-HCl buffer (pH 8.0) at 50°C for 30min. This was done in triplicates and the relative enzymatic activity calculated as described above.

The results of the lipase characterization experiments were expressed as Residual Lipolytic Activity (RLA (%)), in which the value obtained in the analysis (LAassay) was divided by the maximum activity determined in the optimal condition of pH and temperature tested (LAcontrol), as follows:

$$Relative\ \text{activity}(0/0) = \frac{LA\ assay}{\text{LA control}} \qquad 2b$$

(Defranceschi Oliveira, Fernandes & André Bellin, 2014)

Substrate Specificity of the Lipase

Three substrates - olive oil, coconut oil, and sunflower oil were used in a titrimetic method according to Niyonzima and More, (2014). A total of 2.5 ml of substrate, 2.5 g gum acacia and 50 ml of 0.1 M Tris-HCl buffer (pH 8.5) were mixed with a magnetic stirrer in a 100ml test tube for 10 min to get homogeneous emulsion. Then, 1 ml of the partially purified produced lipase (50 mg/ml) was added to the substrate solution (the emulsion) and incubated for 30 min at 30◦C with shaking at 100 rpm. The reaction was stopped by the addition of 4 ml ethanol/acetone (1:1). 2 drops of phenolphthalein were added to the produced fatty acids which were then titrated to an end point of pH 10.0 with 0.05 M NaOH solution. One ml of enzyme denatured at 95°C for 10 min was used for a blank titration. One lipolytic unit (U) of a lipase corresponded to the amount of enzyme which liberated 1 µmol of fatty acids per min per ml under the assay conditions.

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 (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 1.5g of each homogenized POME 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 0.1M 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 International, 2005). The titration was triplicated for each sample and acid value calculated as below:

$$\text{Acid value (mg/KOH)} = \frac{56.1 \times V \times N}{W} \qquad 3$$

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 was calculated using equation 4

$$\text{FFA} = \frac{AV \times MW\,oleic\ acid}{10\ (\text{MW KOH})} \qquad 4$$

Moisture Content Determination

The moisture content of each homogenized POME sample was determined by gravimetric method (Bahadi et al., 2016) modified for this study. A total of 10g each of the homogenized POME samples transferred into separate previously weighed aluminum dishes was heated at 105 °C for half an hour. Then repeated until a constant weight was achieved. Then, equation 5 was used to calculate the moisture content.

$$\text{Moisture Content} = \frac{Wb - Wa \times 100}{Wb} \qquad 5$$

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 the POME 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%). A blank prepared by adding the same quality and quantity of the ethanolic potash but without the oil sample was also titrated. The saponification value was calculated using equation (6) shown below.

$$\text{Saponification value} = \frac{Vb - Va \times N\ titrant \times 56.1\ (mg\ KOH)}{\text{Weight of sample (g)}} \qquad (6)$$

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

RESULTS AND DISCUSSIONS

The result in Table 1 showed that the mean heterotrophic bacteria count ranged from 1.12×10^9 to 2.74×10^9 cfu/ml with Umuagwo recording the highest population at $2.74 \times 10^9 \pm 20$ cfu/ml. These counts were within the typical ranges reported in other studies isolating bacteria from palm oil mill effluent. For example, a study by Nwuche et al., (2014) found plate enumeration counts between 2.4×10^6 cfu/mL (1) and reported identifying bacteria of the genera, *Pseudomonas, Flavobacterium, Micrococcus*, and *Bacillus.*

Table 1. Total Microbial Population Isolated from the POME Samples across Different Locations

Location	Microbial Group (Mean ± SEM)			
	Bacteria No.	Fungal No.	*Pseudomonas* No.	*Candida* No.
ADAPALM	$203.67 \pm 15\times10^{7a}$	$3.8 \pm 0.6 \times10^{6b}$	$105.33\pm20\times10^{7c}$	$95.0\pm20\times10^{6d}$
NAZE	$157.67 \pm 15\times10^{7a}$	$5.1\pm0.3 \times10^{6b}$	$102.66\pm15\times10^{7a}$	$79.6\pm20\times10^{6c}$
OKANNE	$112.00 \pm 10 \times10^{7a}$	$4.9 \pm0.8\times10^{6b}$	$77.33 \pm12\times10^{7c}$	$81.3\pm15\times10^{6d}$
UMUAGWO	$274.00 \pm 20\times \times10^{7a}$	$2.3 \pm0.3\times10^{6b}$	$142.66\pm20\times10^{7c}$	$103.6\pm20\times10^{6d}$

* Across the rows, values with dissimilar superscripts are significantly different.

Soleimaninanadegani and Manshad, (2014) also isolated *Staphylococcus aureus* from POME as its lipolytic activity has been previously demonstrated (Narihiro et al., 2014). Bala et al., (2018) reported THB in the range of $9.5 \times 10^5 – 7.9 \times 10^6$ cfu/ml and further identified *Micrococcus luteus* 101PB, *Stenotrophomonas maltophilia* 102PB, *Bacillus cereus* 103PB, *Providencia vermicola* 104PB, *Klebsiella pneumoniae* 105PB, and *Bacillus subtilis* 106PB.

In addition, the total fungal population ranged from $2.30\pm0.3 \times 10^6$ to $5.10\pm 0.3 \times 10^6$ and comprised of organisms like *Candida, Aspergillus,* and *Mucor*. The mean *Candida* spp counts ranged from $79.6 - 103.6 \times 10^6$ cfu/ml.

The comparative statistics showed a wider range and higher mean for bacterial populations compared to fungal populations, indicating a more significant presence of bacteria in the POME samples. The fungal populations, while lower, show less variability across the locations. It is noteworthy that the heterotrophic bacterial and *Pseudomonas* population had a strong positive correlation (0.972) whilst the correlation between the mean bacterial and fungal populations across the locations is negative (-0.943*). Candida* population and heterotrophic fungal population had a strong negative correlation (-0.983).

Further, the abundant populations of lipolytic yeasts recorded in the present study is expected given the oil-rich nature of POME. However, some functional differences between the locations 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 including potential sources of lipids, organic matter availability, and microbial interactions. Similar studies have reported abundant lipolytic *Candida,* other yeasts and fungi counts. For example, Islam et al., (2017) investigated the use of a POME-isolated oleaginous yeast, *Lipomyces starkeyi* as a cost-effective microbial lipid 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.* Nwuche and Ogbonna (2011) identified twelve fungal lipase producing strains belonging to *Aspergillus, Penicillium, Trichoderma* and *Mucor* genera that they isolated from a POME-dumpsite in Nsukka, and reported that *Aspergillus* sp were more frequent. The absence of *Candida* in their work may have been due to overcrowding by mould.

Also, the differences in *Candida* and *Pseudomonas* counts in the POME samples have several implications: given the notoriety of *Pseudomonas* species in organic pollutants degradation, locations with higher *Pseudomonas* counts might have more efficient natural degradation capabilities. Conversely, elevated *Candida* counts, especially lipolytic strains, might indicate a high level of organic pollutants. This could suggest a need for more stringent treatment processes in such POME-dumped areas to prevent potential environmental contamination. Further, certain strains of *Pseudomonas* and *Candida* can be pathogenic. Therefore, higher counts in POME- dump sites could pose health risks to workers and nearby populations, particularly where aerosolization of particles occurs. This necessitates adequate protective measures and monitoring to prevent disease transmission. Okwute et al.,(2015) evaluated the biodegradation of POME and lipase activity by *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Candida albicans* treatment. Their reported highest microbial count (8.30×10^9 cfu/ml) during biodegradation of POME was that of *P. aeruginosa*. 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.

Supporting the POME biodegradation potential of *P. aeruginosa* and *C. albicans,* the lipolytic screening of the isolates showed that the overall zone diameters for *Candida* fell within 0.2-0.4cm with Umuokanne-isolated *Candida* demonstrating the most lipolysis while those of *Pseudomonas* were within the 0.4 - 0.7 cm range after 48h incubation, with Naze demonstrating the most lipolysis and that from Umuagwo the least. The differences in inhibition zones between organisms across sample locations in the present study could indicate generally consistent lipolytic microbial profiles in this effluent. The evolutionary tree in fig 1 showed 100% relatedness of the two highest lipolytic isolates to *C. albicans* and *P. aeruginosa.* 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). Carrazco- Palafox et al (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. Thus, the size of inhibition zones is highly dependent on the organism, culture conditions, assay methods, and other factors (Carrazco-Palafox et al., 2018).

Table 2 Demonstration of lipolysis in *Candida albicans* and *Pseudomonas aeruginosa*

Location	*Microbial Group (Mean ± SEM)*	
	Candida No	*Pseudomonas No.*
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**

Further, the physicochemical properties of the POME samples investigated showed that all locations were significantly different from each other for all parameters ($p < 0.05$) except for saponification value (SV) where no difference was noticed between Umuagwo and ADAPALM and in pH where no difference between Okanne and Adapalm was registered.

Table 3 Physicochemical characterization of POME Samples across the different Locations

1.	**Parameter**	**Location**			
2.		**Umuagwo**	**Okanne**	**Naze**	**ADAPALM**
3.	**AV(mg KOH/g)**	**13.06 ±0.03^{a}**	**15.73± 0.02^{b}**	**40.46± 0.05^{c}**	**20.03± 0.02^{d}**
4.					
5.	**FFA**	**36.88^{e}**	**43.00^{f}**	**114.25^{g}**	**56.64^{h}**
6.					
7.	**SV (mg/g)**	**145.98±0.12^{c}**	**159. 25±0.13^{a}**	**88. 12±0.05^{b}**	**141.08±0.12^{c}**
8.					
9.	**pH**	**4.1^{e}**	**3.2^{d}**	**3.9^{f}**	**3.5^{d}**
10.					
11.	**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 in each column are significantly different from each other at P<0.13105. Values are represented as mean ± STD

The FFA acid content reported for ADAPALM sample was 56.64 which agrees with the value Ricca et al., (2013) reported (51.64) for a sludge palm oil sample they investigated. The reported acid values for the four samples were 13.06±0.03^{a}, 15.73±0.02^{b}, 40.46±0.05^{c}, and 20.06±0.02^{d} mgKOH/mg for Umuagwo, Umuokanne, Naze, and ADAPALM samples respectively. 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. 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 discourages alkaline transesterification. Moisture determination demonstrated that the sample from Naze at 77.92% was highest. Others were at 66.87%, 59.82%, and 71.90% respectively for Umuagwo, Umuokanne, and ADAPALM. Similar findings were reported by Leela et al., (2018), who found that palm oil mills influenced pH, moisture content, and other effluent properties. The positive relationships between mill location and effluent parameters concurred with research by Leela et al (2018). They attributed the differences in POME properties to disparities in palm oil processing techniques, sterilization temperature, clarification efficiency, and water use between mills. The significant impact of sample location highlighted the importance of characterizing POME at individual mills rather than relying on generic averages (Lam & Lee, 2011).

Further, the lipase activity measurement of the *Pseudomonas* lipase was 30.00 µmol/min/ml (3U) of enzyme volume whilst for *Candida*, the lipase activity was approximately 690.00 µmol/min/ml (6.90 U). These findings were high, demonstrating their potential in producing commercial lipases as well as in remediating POME. Adetunji & Olaniran, (2018) observed lipase activity of 264.02 ± 1.94 (U/ml) from *Bacillus aryabhattai* SE3-PB. Okwute, Stephen and Anyanwu (2015) evaluated the biodegradation of POME and lipase activity by *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Candida albicans* treatment. The highest lipase activity was recorded on the 8th day by *C. albicans* as (29.83±1.40 U/ml) followed by *P. aeruginosa* (29.02±1.02 U/ml), thus supporting the high lipase activities of the enzymes from the *Pseudomonas* isolates.

Relative enzyme activity for *Pseudomonas* lipase increased from 192 to 410 from 30°C to 50°C, with highest activity at 50°C. It then decreased to 404 at 60°C. A further decrease from 404 to 292 was evident from 60°C to 70°C and at 70°C, seemed to outperform *C. albicans* lipase. For *Candida* lipase, enzyme activity was lowest at 30°C (158). It increased gradually from 40°C to 60°C (192 to 448), with maximum activity at 60°C. A steep decrease became evident from 60°C to 70°C (248). The results of the two-way ANOVA showed that there was a significant main effect of temperature ($p < 0.001$) and a significant main effect of organism ($p < 0.001$). There was also a significant interaction effect between temperature and organism ($p <$

0.001). This means that the relative lipase activity of the two organisms was significantly different at different temperatures, and that the effect of temperature on the relative lipase activity was different for the two organisms. Subsequently, the flip in relative activity at 70°C, where *Pseudomonas aeruginosa* outperformed *Candida albicans* lipase again might be indicative of a secondary optimal temperature or resilience to high temperatures for *Pseudomonas aeruginosa ('s)* lipase.

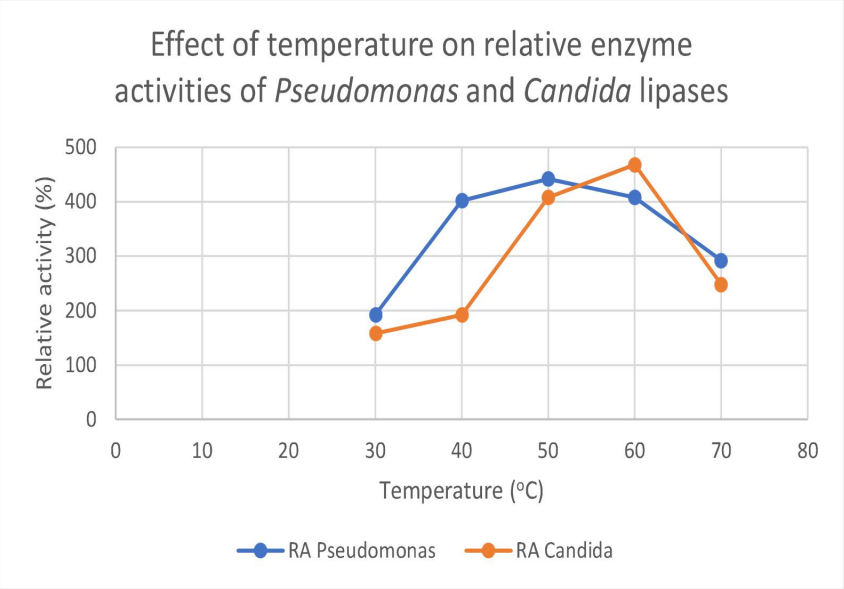

Fig 2 Effect of Temperature on relative enzyme activities of *P. aeruginosa* and *C. albican* lipases

These significant main effects of temperature and organism on relative lipase activity are widely reported in the literature. Temperature is a critical factor influencing enzyme activity, as it affects the kinetic energy of the molecules and the stability of the enzyme structure (Salihu, et al., 2011; Tan et al., 2003). Different organisms produce lipases with varying temperature optima and stability profiles, which can be attributed to their evolutionary adaptations to specific environmental conditions. The interaction effect between temperature and organism is also consistent with previous findings. Many studies have shown that the temperature dependence of lipase activity can vary across different microbial sources, reflecting differences in the enzyme structures and catalytic mechanisms. A study by Ghori et al. (2011) found that the lipase from *Pseudomonas aeruginosa* showed a rapid decline in activity above 50°C, while the lipase from *Candida antarctica* retained significant activity up to 70°C. This trend is consistent with the present observation that *Candida albicans* lipase had higher relative activity than *Pseudomonas aeruginosa* lipase at higher temperatures (60°C). In general, enzyme activity increased with rise in temperature due to increased kinetic energy and molecular collisions. Each enzyme showed an optimum temperature at which maximum catalytic activity was observed. The gradual decrease in enzyme activity from the optimum temperature to 70°C indicated thermal denaturation of the enzymes at higher temperatures. *Pseudomonas* lipase seemed more susceptible to thermal denaturation as its activity dropped sharply from 60°C to 70°C. This difference in temperature optima and stability can be attributed to various factors, such as the amino acid composition, tertiary and quaternary structure, and the presence of stabilizing factors like disulfide bridges or ion interactions in the enzymes from different organisms.

These findings have practical implications in selecting appropriate lipase sources for industrial or biotechnological applications that require specific temperature conditions. Additionally, understanding the temperature-activity profiles can guide enzyme engineering efforts to improve the stability and catalytic efficiency of lipases for a wider range of temperature conditions.

Presented in Fig 3 is the effect of pH on the relative lipase activities of the lipases from *P. aeruginosa* and *C. albicans.* At acidic pH 5, *Candida* lipase activity was inhibited (66.7) whilst at pH 6, *Candida* lipase showed slightly higher activity (113.3) than *Pseudomonas* lipase (86.7). While both enzymes were most active in alkaline conditions, *Candida* lipase demonstrated somewhat higher acid stability and alkali resistance (246.7 to 280 against *P. aeruginosa*'s 200).

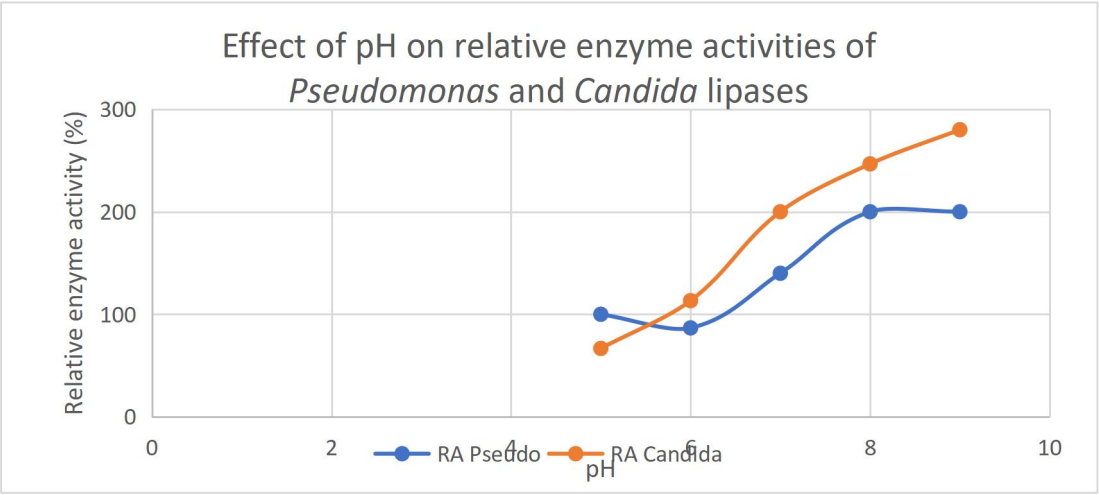

Fig 3 Effect of pH on the relative lipase activities of *Pseudomonas and Candida* lipases

The pH optima were slightly different between the two enzymes as well. The two-way ANOVA results indicated that both pH and lipase type have a significant effect ($p < 0.005$) on relative enzyme activity, and there is also a significant interaction between these two factors. This suggested that the effect of pH on enzyme activity differed between *Pseudomonas* lipase and *Candida* lipase. The post-hoc tests revealed that the optimal pH range for *Pseudomonas aeruginosa* lipase was around 8-9, while for *Candida albicans* lipase, it was slightly higher at around 9. These findings are consistent with several studies reporting the optimal pH range for lipases from different sources (Zouaoui, Bouziane and Ghalem, 2012; Noormohamadi, Tabandeh, Shariati, and Otadi, 2013; Ogunniran, Odeleye, & Femi-Ola, 2023). In contrast, *Pseudomonas putida* 922 was reported to have exhibited maximal growth and enzyme production at pH 10 and 30°C, with the enzyme being stable in the pH range of 5-11 and temperature range of 30-40°C (Padhiar, Das & Bhattacharya, 2011).

From the present study, both *P. aeruginosa* and *C. albicans* lipases exhibited a strong positive correlation between pH and activity, indicated by the high Pearson's correlation coefficients (r) 0.9691, p-value = 0.0064 (significant positive correlation), r = 0.9915, p-value = 0.0002 for *P. aeruginosa* and *C. albicans* respectively. This is consistent with numerous studies reporting that lipase activity is often pH-dependent. For example, Ibegbulam-Njoku & Achi, (2014) and Pereira, De Castro, De Moraes and Zanin (2001) indicated that for *Candida albicans*, optimal lipase production occurred at pH 5.2 and 30°C, while *Candida rugosa* displayed maximum activity at pH 7.0 and 37°C. These findings highlighted the importance of pH and temperature optimization in maximizing lipase production by *Pseudomonas* and *Candida* species for various industrial applications. The two-way ANOVA and post-hoc tests highlighted the influence of lipase source (bacterial vs. yeast) on optimal pH and reinforced the concept of source-dependent optimal pH for lipases. These distinctions can guide selection and usage of the appropriate lipase enzyme for various biocatalytic applications. For both enzymes, the activity increased gradually from pH 5 to pH 8.

Shown in fig 4 is the effect of metal ions on the relative lipase activity of *Pseudomonas aeruginosa* and *C. albicans* lipases. Zinc ions (Zn^{2+}) showed strong enhancement of activity for *Candida* lipase (273.7 RA) compared to *Pseudomonas* lipase (130.5 RA). Sodium ion (Na^{+}) selectively enhanced *Candida* lipase activity (319 RA) but not *Pseudomonas* lipase (127.8 RA). Copper ions (Cu^{2+}) inhibited both *Pseudomonas* lipase (101 RA) and *Candida* lipase (126.3 RA). Calcium ions (Ca^{2+}) had negligible effects on the activity of both enzymes.

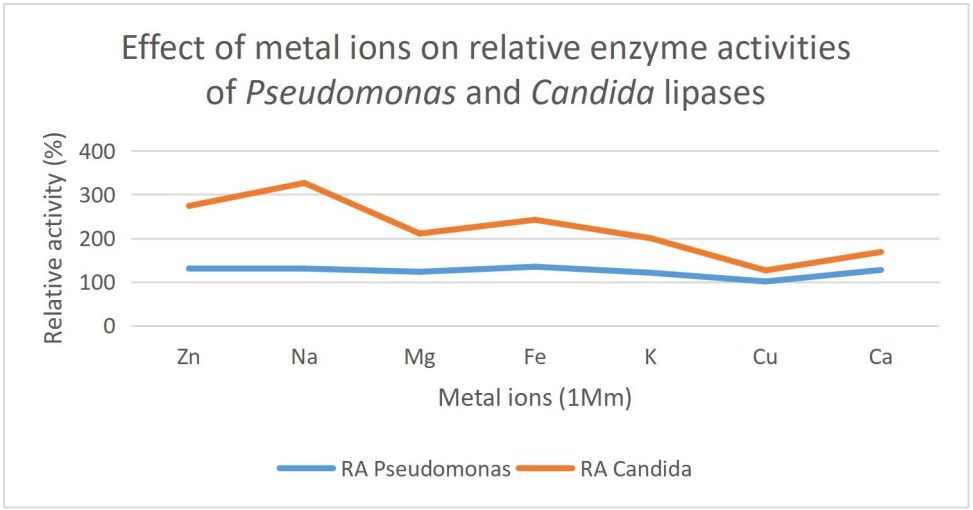

Fig 4 Effect of metal ions *on relative enzyme activities of P. aeruginosa* and *C. albican lipases*

The Assay statistics of *P. aeruginosa* lipase was 'Z'= 0.57 whilst that of *C. albicans* was Z'= 0.53. Both enzymes had excellent Z'-factors ((>0.5) demonstrating that these were robust activity assays suitable for screening. Further, correlation analysis of the lipases (Pearson's) r = 0.912 indicated a strong linear relationship between the relative activity profiles of the two enzymes across the different metal ion conditions, suggesting the metal ions impacted the enzymes in a similar manner. However, the two sample t-test revealed a statistically significant difference (p = 0.001) between the overall mean relative activities of *Pseudomonas* and *Candida* lipase. This indicated that there was a distinct quantitative difference in their reactivities in the presence of metal ions. The ANOVA (Metal ion: F=10.23, p<0.001; Enzyme: F=143.9, p<0.001; Interaction: F=1.46, p=0.207) showed that metal ion type and enzyme type both significantly influenced the relative activity. However, there was no significant interaction effect between the two factors. This suggested that while metal ions affected enzyme activity, their mechanism of action was independent and not specific for each enzyme. The post-hoc comparisons indicated that all metal ions showed significant difference between enzymes (p<0.01) except Ca^{2+} (p=0.059).

Thus, the relative lipase activities of *Candida albicans* against zinc ions (at Zn^{2+}), sodium ions (Na^{+}), copper 11 ions (Cu^{2+}), calcium ions (Ca^{2+}), Iron 11 ions (Fe^{2+}) and Potassium ions (K^{+}) (273.7, 326.3, 126.3, 168.4, 242.1, 200.0%) were significantly higher than those of *Pseudomonas aeruginosa* (130.5,130.5,101.0,127.3,134.7,121.0 %). However, for magnesium ions (Mg^{2+}), the relative lipase activity of *Pseudomonas aeruginosa* (223.1%) was significantly higher (p < 0.001) than that of *Candida albicans (*210.5%*)*. That means that Na^{+} selectively enhanced *Candida* lipase activity (319) but not *Pseudomonas* lipase (127.8), indicating specific activation of *Candida* enzyme. Fe^{2+}, Zn^{2+}, K^{+} caused moderate enhancement of *Candida* lipase activity but had minimal effects on *Pseudomonas* lipase.

Metal ions play a crucial role in modulating the relative enzyme activity of lipases from different sources. Studies have shown that various metal ions like Ca^{2+}, Cr^{3+}, Co^{2+}, and Cu^{2+} can significantly impact the hydrolytic and transesterification activities of lipases, with different ions showing varying effects on enzyme kinetics and activation energies (Qu et al., 2018; Samaei-Nouroozi et al., 2015; Madhu & Amjad, 2013). Qu et al., (2018) reported on the effects of copper ions on the conformation and activity of *Candida rugosa* lipase (CRL) indicating that copper compacts the proteins peptide bond structure, changing its secondary structure and that it opened the "lid" of the CRL and entered the active center, which consequently changed the conformation of the CRL molecule. Katiyar and Ali (2013) reported that maximum enhancement in hydrolytic activity of *Candida rugosa* Lipase was observed by Ca^{+2}, and in transesterification activity by Cr^{+3} and Co^{+2}. Zouaoui, Bouziane and Ghalem, (2012) indicated that the enzyme activity of *P. aeruginosa* lipase was enhanced by Ca^{2+} and Mg^{2+} but strongly inhibited by heavy metals such as Zn^{2+}, Cu^{2+} and Mn^{2+}. Thus, in the present study, metal ions demonstrated selective activation and inhibition effects on the two lipase enzymes. *Candida* lipase showed greater enhancement by Zn^{2+}, Na^{+} indicating the presence of specific binding sites. Cu^{2+} non-selectively inhibited both enzymes. The distinct response profiles provided insights into the unique metal interactions for each lipase enzyme.

The substrate specificities of the *Pseudomonas aeruginosa* and *C. albicans* lipases (Table 4) showed that sunflower oil resulted to more enzyme activity. However, statistical comparison of the two lipases revealed a Pearson's r = 0.824 (high positive correlation); p = 0.127 (no significant difference between mean RAs); F(substrate)=143.2, p<0.001 (which significantly impacted RA, F(enzyme)=3.14, p=0.17 (no significant effect). Further, Interaction effect showed enzyme & substrate were dependent (Interaction: F=4.61, p=0.031). Post-hoc comparisons revealed that on sunflower oil, enzyme difference was significant (p = 0.043) while for coconut oil, p was (0.177). *Pseudomonas* lipase displayed broader substrate specificity and higher catalytic efficiency than *Candida* lipase on the oils tested.

Table 4 Substrate specificity of olive oil, sunflower oil, coconut oil on relative lipase activity of *C. albicans* and *P. aeruginosa*

Substrate	**RA *P. aeruginosa* (%)**	**RA *C. albicans* (%)**
Sunflower oil	**31.3^{a}**	**43.8^{b}**
Coconut oil	**18.8**	**12.5**
Soya oil	**12.5**	**25.0**

Key: RA Relative activity; the substrates values are in ml and values with dissimilar superscripts are statistically different.

The high positive correlation between *Pseudomonas* and *Candida* lipase activities (Pearson's r = 0.824) indicated that there was a strong linear relationship between the relative activities of the two enzymes across the different substrates suggesting that their substrate preference profiles were quite similar. However, enzyme type alone does not significantly influence activity. Importantly, the ANOVA showed a significant interaction effect between enzyme and substrate factors. This indicated that the effect of substrate on activity is dependent on the enzyme, and vice versa. The post-hoc tests showed that, specifically for sunflower oil, there was a statistically significant difference between *Pseudomonas* and *Candida* lipase activities. The reported high positive correlation between *Pseudomonas* and *Candida* lipase activities (Pearson's r = 0.824) aligns with Gupta, Gupta & Rathi (2004**),** who reported strong correlations for several microbial lipases tested across triglyceride substrates. This pointed to conservation of substrate specificity. Post-hoc comparisons revealed that on sunflower oil, enzyme difference was significant (p = 0.043) while for coconut oil, p was (0.177), signifying no statistical difference.

The superior activity of *Candida* lipase on sunflower oil compared to *Pseudomonas* lipase parallels Saxena, Sheoran, Giri & Davidson (2003), where sunflower oil was the best substrate for a *Candida rugosa* lipase, while a *Pseudomonas* lipase preferred olive oil. Also, Adetunji & Olaniran, (2018) reported that sunflower oil was found to induce max-lipase production in *Bacillus aryabhattai* SE3-PB at temperature (40 ^{o}C), pH (7.6), inoculum volume (2.8%, v/v), agitation (193 rpm) and inducer oil concentration (2%, v/v), giving an enzyme activity of 264.02 ± 1.94 U/ml.

The lack of significant difference ($p = 0.127$) between the activities of the two enzymes in the present study on coconut and soya oil is consistent with studies that have found little variation between microbial lipases in their action on shorter, saturated triglycerides like coconut oil (Vishnoi et al., 2020; Chandra et al., 2020). Both enzymes showed the highest relative activity on sunflower oil, followed by soya oil and coconut oil. *Pseudomonas* lipase had a relatively higher activity than *Candida* lipase on all substrates. *Pseudomonas* lipase displayed broader substrate specificity and higher catalytic efficiency than *Candida* lipase on the oils tested.

CONCLUSION

The study has demonstrated that optimal activity of *Pseudomonas aeruginosa* and *Candida albicans* lipases at specific conditions position them as crude catalysts for industrial applications in sectors such as biofuel production, food processing, and detergent formulation. The ability of these lipases to effectively hydrolyze oils like sunflower and soybean highlighted their utility in the production of biodiesel and other value-added products. Additionally, the enhancement of lipase activity by metal ions like Zn^{2+} and Na^{+} could inform the development of more efficient and cost-effective industrial processes.

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