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TITLE OF MANUSCRIPT: ENHANCEMENT OF LIPOLYTIC CHARACTERISTICS OF A PALM-OIL MILL EFFLUENT-SOURCED *KODAMAEA OHMERI* PL2

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ABSTRACT

The hydrolysis of long-chain triglycerides and fats is catalyzed by lipases, important enzymes which have essential biotechnological, medical, and industrial roles. Fungi were isolated from palm-oil mill wastes and screened for their lipid-hydrolyzing potentials. Lipase production and activity characteristics were determined using different substrates. Sixteen, out of the 75 fungi isolated, exhibited lipolytic characteristics. Yeast isolate PL2 gave the highest zone of hydrolysis (44.5 mm) and was identified as *Kodamaea ohmeri*. This yeast was cultured under diverse media conditions for lipase production and the enzyme produced under each physicochemical condition was determined spectrophotometrically. The optimum parameters for lipase production (1% Tween-80, Ca2+, peptone, 37°C and pH 6) were used to produce lipase which was further purified through (NH4)2SO4 precipitation, membrane dialysis and gel chromatography. The activity of the partially purified lipase increased progressively with each purification stage. The lipase had optimum activity at 50oC and was alkalophilic with peak pH for enzymatic hydrolysis at 8.0. The lipase was supported by 0.5% concentrations of SDS and Triton X-100 (129.9 and 129.4%, respectively), and a 100-fold activity increase with 0.5% Tween-80. Lipase activity was augmented by Manganese and calcium ions (10 mM) but inhibited by NH4+, Fe3+ and Na+. The use of Tween-80 resulted in increased enzyme yield as well as better enzyme characteristics compared to other substrates. The knowledge obtained in this study may be harnessed for commercialization of *Kodamaea ohmeri* lipase.

Keywords: Palm-oil mill wastes, Fungal lipases, *Kodamaea ohmeri*, Enzyme characterization

INTRODUCTION

Lipases are hydrolytic enzymes which catalyze esterification reactions in lipids (water insoluble energy sources important to all life) to generate simpler products of lipid hydrolysis (**Das *et al*., 2016**). Over the years, the study of lipases has gained attention as it finds multipurpose applications in various sectors. Lipases possess outstanding economic properties such as the ability to function in micro-aqueous environment, catalyze aminolysis, acidolysis, esterification and transesterification reactions (**Joseph *et al*. 2008; Asih *et al*. 2014; Alami *et al.* 2017**). Unlike some other enzymes, lipases do not require cofactors to catalyze hydrolytic reactions and can remain active in the presence of organic solvents (**Liu and Kokare, 2017; Chandra *et al.* 2020**). Lipases hydrolyze long chain water-insoluble triglycerides into diglycerides, monoglycerides, glycerol and fatty acid (**Gilham and Lehner, 2005; Alami *et al.* 2017**) and hence, have attracted continued extensive research on their detection methods, production and optimization, assay, purification strategies and industrial applications (**Hasan *et al*. 2006, Hasan *et al*. 2009; Alabdalall *et al.* 2020**). Lipases have been numerously applied in the biodiesel industry, wastewater treatment, de-oiling lipid-clogged drains, the hydrolysis of milk and fat in the dairy industry, leather industry to remove lipids from hides and skin, as a component of detergent for the removal of oil and fat stains, in the textile industry, in pharmacy and medicine as diagnostic tools, (**Kumar *et al*., 2005; Alabdalall *et al.* 2020**). Lipases are widely distributed and present in animals, plants, and microorganisms (**Lascano-Demera *et al.* 2019**). Yeasts are vegetative budding unicellular eukaryotic organisms which are safe desirable producers of enzymes because of their lack of production of toxic secondary metabolites (**Pham *et al.* 2019**). For thousands of years, they have been employed in many traditional fermentative, industrial and biotechnological processes (**Turker, 2015; Lascano-Demera *et al.* 2019**), such as in the production of enzymes, pharmaceutical proteins, bioremediation, biocatalysts, to mention a few (**Turker, 2015; Alami *et al*. 2017; Lascano-Demera *et al*. 2019; Alabdalall *et al*. 2020**). Many fungi have been reported to produce lipase and the *Pichia, Candida, Saccharomyces, Yarrowia* and *Kodamaea* species isolated from a waste sample collected from a grease trap were documented to exhibit lipolytic activities (**Sakpuntoon, 2020**). Microorganisms abound in many organic wastes and the isolation of new microorganisms with unique characteristics from these wastes is highly probable. Nigeria has many cottages palm-oil processing farms which generate vast volumes of organic wastes from palm fruit processing. Palm-oil mill effluent (POME) is the voluminous liquid/wastewater obtained from palm-oil processing. The production ratio of this 'waste' to palm-oil is 5:2. When discharged, the organic POME, rich in organic matter, is a brown sludge and contains suspended particles with a pH range between 4 and 6 (**Asih *et al.* 2014; Setiadi *et al*. 2015**). The effluent contributes to environmental pollution in neighboring soil and water bodies resulting in increased demand for the total oxygen required to breakdown organic contaminants (**Setiadi *et al*. 2015**). The organic nature of the POME also makes it a rich source of microorganisms which would utilize the nutrients in the waste for growth and metabolism, hence a candidate for the isolation of lipolytic organisms.

The production of enzymes has been reported to be influenced by diverse cultural conditions and their activities seen to be in response to the physicochemical changes they are exposed to during growth. The attributes of lipases necessitate continuous search for microbial sources which can contribute to meet the market demand (**Geoffry and Achur, 2018**), hence, much work is still required to understand the potentials of microorganisms in the production of valuable resources. Also, there is an ever-increasing demand for lipases in many industrial applications which encourages further search for new lipase-producing microorganisms from diverse sources. In this work, the characteristics of a lipolytic *Kodamaea ohmeri* PL2 strain, isolated from palm oil mill effluent, and the contributions of physicochemical amendments to cultivation media for enzyme production and lipase characteristics were investigated.

MATERIALS AND METHODS

Sample collection

Samples were collected from a cottage palm oil processing mill site in Igbo Oloyin, Ibadan, Nigeria. From the palm oil mill, different types of waste samples generated during palm-oil processing were obtained from different locations including the freshly released palm-oil processing effluent: A (7°32'35.724"N, 3°58'15.648"E), old/abandoned effluent site: Eo (7°32'39.564"N, 3°58'7.494"E), mud-like sludge generated from settling of the effluent over time: L (7°32'41.442"N, 3°58'11.58"E; 7°32'37.846"N, 3°58'15.972."E) and soil: S (7°32'33.648"N, 3°58'12.966"E) over which waste water had been discharged for a long period of time. These were collected in sterile Ziploc bags and analyzed microbiologically in the laboratory.

Isolation of microorganisms and screening for lipase producers

Using the pour plate method, 10 g samples were appropriately diluted in cooled sterile water to generate 10-fold dilutions. Aliquots (1 mL) of the dilutions were introduced into sterile, 9 cm disposable Petri dishes after which cooled, sterilized agar (Yeast Extract Agar and Potato Dextrose Agar) amended with 0.1g/L streptomycin were poured into the plates, swirled, and allowed to set. The Petri dishes were incubated at 27oC and examined for growth over 96 hours. Morphologically unique yeast and mold isolates were subjected to lipase plate screening assays by stab-culturing in 1% sterilized (a) olive oil medium and (b) Tween-80 medium both with basal medium constituents of 10 g/L Peptone; 5 g/L NaCl; 0.1 g/L CaCl2; 0.5 g/L Congo red; 20 g/L Agar-agar and 1 L distilled

water, while the Tween-80 medium additionally had 3 g/L Yeast extract. The inoculated plates were incubated at 27oC for 48 hours.

Culture selection and identification of lipolytic microbe

A detection of fungal lipolytic potential was confirmed by the formation of clear/pale halos around the point of fungal inoculation in the colored agar mat. The isolate with the highest diameter of clearance was selected for further work and identified based on morphological, fermentative, and molecular characteristics. The morphological characteristics of the colony of the selected isolate was observed to determine the size, color, elevation, opacity, texture, edge, pigmentation, and shape (**Yarrow, 1998, Alami *et al.*2017**). This was followed by wet mount microscopic morphological examination using lactophenol cotton blue stain under x40 and x100 magnification. Biochemically, the isolate was examined for its sugar fermentation patterns by inoculating 24 hours old culture in filter-sterilized sugars and steam-sterilized gelatinized starch in screw-capped tubes of sterile yeast extract-methyl red medium (g/L: yeast extract 23; methyl red 0.5, carbohydrate sugars 10) with inverted Durham tubes. The carbohydrates included glucose, lactose, maltose, arabinose, mannitol, sucrose, fructose galactose, glycerol, and starch. A medium in which no organism was inoculated served as control for the experiment and the tube was observed daily for color change and gas formation. For molecular characterization, the yeast genomic DNA was extracted, and the ITS universal primer set (ITS 1: 5' TCC GTA GGT GAA CCT GCG G 3'; ITS 4: 5' TCC TCC GCT TAT TGA TAT GC 3') was used (**Looke *et al.* 2011**). These were sequenced using a Genetic Analyzer 3130XL sequencer (Applied Biosystems) with BigDye Terminator V3.1 cycle sequencing kit following the manufacturers' manual. A Bio-Edit software and MEGA 6 was used for all genetic analysis.

Effect of physicochemical conditions on lipase production

Effect of lipid substrates as carbon sources on lipase production and optimization of production conditions: For enhanced production of the enzyme, a study of various parameters such as temperature, pH, carbon sources, nitrogen sources, metallic ions and incubation time on the production of lipase was done. The initial production medium composed of Peptone: 10 g/L, NaCl: 5 g/L, $CaCl_2$: 0.10 g/L, Olive oil: 10 mL, Distilled water: 1000 mL at a pH of 7. Each of these parameters were modified as shown below (**Musa and Adebayo-Tayo, 2012; Bakir and Metin, 2015**). A loopful of the chosen isolate was taken from a 24-hour old culture, into 0.85% NaCl and the suspension turbidity was then adjusted to an equivalent of No. 2 McFarland standard solution which then served as the seed inoculum for the fermentation studies.Various oils (castor oil, coconut oil, olive oil, melted shea butter and Tween-80) were enlisted as substitutes to olive oil in lipase production. These served as the sole carbon source in the fermentation medium used in the cultivation of the selected yeast and were compared to the effect which glucose exerted. Fifty milliliters each of the respective media were inoculated with 1% overnight culture, incubated for 24-120 hours, and after each day of incubation, samples were withdrawn from the production broth and assayed spectrophotometrically to determine lipase production (**Bakir and Metin, 2015**). The effects of increasing the substrate concentration of the best carbon source (1-5% v/v) was also determined to know what concentration would be continued with in subsequent lipase production studies.

Effect of metallic ions on lipase production: The contribution of metallic ions to lipase production was determined using chloride salts of Manganese, Zinc and Iron. These were used as substitute to the Ca^{2+} in the control production medium described above and for each of the reaction, 0.01% of the metal to be varied was supplied in the reaction medium. These were sterilized and each conical flask was seeded with 1% of the inoculum and incubated for 120 hours at 27°C. The lipase assay was carried out at 24 hours intervals.

Effect of different nitrogen sources: The effect of selected organic and inorganic nitrogen sources (Peptone, Yeast extract, Urea and Tryptone; $(NH_4)_2SO_4$, NH_4Cl, NH_4NO_3 and $NaNO_3$,) on the enzyme production was determined over five days. The reaction medium contained 1% w/v of the nitrogen compound which was inoculated and incubated as described above.

Effect of temperature: The cultivation temperature was varied in order to obtain the optimum incubation temperature that favored the production of the lipase enzyme. The fermentation medium was inoculated with the yeast and incubated at 27°C, 37°C, 40°C and 50°C for five days.

Effect of media pH on lipase production: The optimum pH for the enzyme production was determined by incubating the selected yeast in culture medium whose pH was adjusted between 3 and 10. Three different buffers were used to attain the ranges of pH enlisted: pH 3-5 (Sodium-citrate), pH 6-8 (Potassium Phosphate) and pH 9-10 (Carbonate-Bicarbonate) (**Bakir and Metin, 2015**). The fermentation medium was prepared with the buffers at the different pH ranges and sterilized. On cooling, the medium was inoculated and incubated at 27°C for 120 hours. Samples were assayed to determine lipase production at 24-hour intervals.

Assay for lipase activity: Using *p*-nitrophenyl palmitate (*p*NPP) as substrate and the method of **Sumarsih *et al.* (2019)**, assay of the lipase product in broth was carried out by determining the activity of the lipase using to produce *p*-nitrophenol. Following each incubation time, the enzyme solution was obtained by centrifugation at 4000 rpm for 30 minutes. A reaction mixture was formulated which consisted of 0.1 mL of the enzyme solution, 0.8 mL 50 mM Phosphate buffer (pH 8.0) and 0.1 mL 10 mM *p*NPP dissolved in ethanol. The reaction mixture was incubated at 30°C for 30 minutes. After incubation, 0.25 mL of 100 mM Na_2CO_3 was added to stop the reaction. The mixture was centrifuged at 4,000 rpm for 15 minutes and read spectrophotometrically at 410 nm. One unit of lipase activity (U/mL) was defined as the amount of lipase that caused the release of 1µmol of *p*-nitrophenol from *p*-nitrophenyl palmitate (*p*NPP) in one minute.

Production and characterization of lipase

Lipase was produced by submerged fermentation using the optimum culture parameters from the various culture conditions above. The medium was prepared, sterilized, cooled, inoculated, and incubated at 37◦C for 48 hours. After production, the reaction medium was centrifuged at 4,000 rpm for 30 minutes to obtain the cell-free supernatant which was used as enzyme solution for characterization studies.

Effect of temperature on the lipase activity: The lipase was maintained at different temperatures (27, 35, 40 and 50°C) for different time intervals (15, 30, 45 and 60 minutes) and then used in reaction mixtures containing 0.1 M phosphate buffer (pH 8.0) and the enzyme solution and afterwards, an assay was carried out using the standard assay procedure earlier described.

Effect of pH on lipase activity: Lipase activity was determined at various pH ranging from 3.0 - 10.0. Each of the adjusted pH buffers was introduced, using equal volumes, into test tubes containing the enzyme and incubated for 30 minutes at 27°C. Afterwards, standard assay procedure was used to quantify the activity of the lipase.

Effect of surfactants on lipase activity: Surfactants (Tween-80, SDS and Trixton-X 100) were used at 0.05%, 0.1%, 0.5% concentrations by mixing equal volumes of the enzyme and surfactants, then allowing the mixture to incubate for 30 minutes, and then assayed.

Effect of cations on the lipase activity: Varying concentrations (10 mM, 25 mM, 50 mM) of Mn^{2+}, Ca^{2+}, Na^{+}, Fe^{3+}, and NH_4^{+} salts were introduced into test tubes containing an equal corresponding volume of enzyme. These were incubated for 30 minutes and quantified using the standard lipase assay.

Effect of increasing concentration of reaction substrate on the lipase activity: Increasing concentrations of pNPP (0.1, 0.25, 0.5% w/v) dissolved in 0.1 M potassium phosphate buffer (pH 8.0) were added to a corresponding equal volume of enzyme solution and left to incubate for 30 minutes. The released P-nitrophenol was monitored spectrophotometrically.

Lipase purification and protein determination: The lipase present in the crude cell-free extract (100 mL) was partially purified by ammonium sulphate precipitation, allowed to stay overnight at 4°C (**Dixon and Webb, 1971**). The precipitate recovered by centrifuging this initial mixture at 10,000 rpm for 10 minutes using a cold centrifuge (Eppendorf centrifuge 5427R) was used for dialysis and column chromatography using a pre-activated dialysis bag submerged in 0.1M phosphate buffer solution of pH 8.0 overnight. A 10 mL solution of the partially purified enzyme was eluted in a column packed with solution of Sephadex G-100 (in PBS solution). The samples were eluted with the same buffer at a flow rate of 75 mL/hour and the eluted fractions were used for determination of the enzyme activity and protein concentration using bovine serum albumin as the protein standard. The protein concentration readings were taken in duplicates in tubes containing 0.1- 1.0 mL of 0.2 mg of protein stock solution (2 mg/mL bovine serum albumin) and brought up to 1mL with distilled water. For the test mixture, 0.5 mL of Phosphate buffer saline pH 7.0 was added to 0.5 mL of the crude enzyme. To the reaction and test mixtures, 5 mL of bovine serum albumin solution was then added, and the mixture was allowed to stand for 10 minutes. Dilute Folin ciocalteu reagent (0.5 mL) was added, followed by the thorough mixing of the solution which was then allowed to stand for 25 minutes at room temperature. The absorbance was read at 660 nm and the protein concentration was determined afterwards (**Lowry *et al.*, 1951**).

Data were subjected to descriptive statistics.

RESULTS

Isolation of, screening for and identification of lipolytic fungi from palm-oil mill waste samples

The palm-oil containing samples recorded a total fungal count between 1.4 x 10^3 and 3.0 x 10^5 per gram. A total of 75 fungi were isolated from the palm-oil mill

wastes. Lipase screening, using olive oil and Tween-80 as substrates showed that 16 isolates (13 yeasts and 3 mold) had zones of hydrolysis (Table 1). The production of zones served as a confirmation of the lipase producing potentials of the isolates obtained. Isolates with high clear zones (in reducing order), indicated by a color change of the chromogenic oil-containing medium were isolates PL2, YA3, YA1, with 44.5 mm, 42 mm and 26.5 mm zones of hydrolysis, respectively, at 72 hours. Also, six of the lipolytic organisms (37.5%) originated from the sludge sample (PL1, PL2, PL3, PSSL1, YL1 and YSSL3) while YA1 and YA3 originated from freshly discharged watery mill effluent component.

Table 1 Zone of lipid hydrolysis by lipolytic fungi on Tween-80 and olive oil screening medium

Fungal Isolate	Zone of hydrolysis (mm)	
	Tween-80	Olive oil
YA1	4	26.5
PL1	3	15
PE2	5	24
YA3	9	42
PL2	9	44.5
PA2	1.5	21
YSSL3	6	10
PEO2	3	14.5
PEO3	3	15
PSSL1	12.5	8.5
YL1	6	6
PA4	18.5	18.5
YA2	0	8.5
PE1	4	21.5
PS3	3.5	20.5
PL3	1.5	8.5

Among these active isolates, PL2 was selected for further lipase production studies. The selected yeast strain PL2 was morphologically cream colored, raised, entire, circular, glistening. Microscopically, the spores were spherically shaped and budding. The yeast was able to ferment glycerol, fructose, sucrose, glucose, and maltose and produced gas with glucose and was presumptively identified as *Pichia pastoris* PL2. Molecular analysis confirmed the presumptive *Pichia* yeast strain as *Kodamaea ohmeri* PL2 with the assigned accession number MW192826 and a phylogenetic tree to show the relatedness of *Kodamaea ohmeri* PL2 to some other selected organisms (Fig 1).

Figure 1 Phylogenetic tree of *Kodamaea ohmeri* PL2 in relation to other microbial strains

Lipase production by *Kodamaea ohmeri* PL2 in modified media

The highest quantity of lipase (262 U/mL) was produced by *K. ohmeri* PL2 at the fourth day of incubation in a medium containing Tween 80 (Figure 2a). Other production media containing castor oil and olive oil also recorded high enzyme activity at this time. At 24 hours (Day 1), the best lipase production by *Kodamaea ohmeri* PL2 also occurred in the Tween-80 medium (166.6 U/mL), closely followed by that in the Olive oil containing medium (160 U/mL) while the least was produced in the glucose medium. Hence, Tween-80 was selected as the substrate for lipase production in subsequent studies. Varying the concentrations of Tween-80 in the production medium, lipase production was not significantly different between 1-5% (P value 0.0977), hence, 1% Tween-80 concentration was continued with (Figure 2b).

From Figure 2c, Calcium ion stimulated the highest lipase production. The other cations also supported the production of lipase especially at 96 hours. On the effect of different sources of organic and inorganic nitrogen, it was observed that peptone had the highest stimulatory effect on lipase production (342 U/mL) compared to the other sources (Figure 2d). In addition, the ammonium-containing inorganic compounds (especially ammonium sulphate and ammonium chloride) also supported enzyme production, but with lower yields. The medium in which urea was utilized gave the least enzyme yield. From Figure 2e, 37°C favored the optimum yield of the lipase throughout the incubation period with the highest yield observed at 24 hours (148.5 U/mL). The lipase yield increased gradually with increase in the pH of the production medium until it peaked at pH 6 with the highest lipase yield (117.53 U/mL) realized at 96 hours (Figure 2f). The values obtained at pH 7 and 8 were close, but lower, presenting the same pattern over the incubation days. The least enzyme quantity was obtained at pH 10.

Characterization of *Kodamaea ohmeri* PL2 lipase

The effect of various physicochemical conditions on the activity of the *Kodamaea ohmeri* PL2 lipase is shown in figures 3a-d. Figure 3a shows the effect of enzyme incubation at increasing temperatures and incubation period on the activity of the PL2 lipase. The highest activity was observed at 50°C with increasing activities between 15 minutes to a peak at 30 minutes (160.62%) and a slight reduction thereafter at 45 and 60 minutes, respectively, (160.26 and 158.08%). At 40 °C a slight increase in the enzyme activity was recorded at 15 and 30 minutes. The lipase therefore was temperature stable over one hour. The lipolytic activity was neutral to slightly alkaline and peaked at pH 8 (Figure 3b). The lipase activity increased steadily up till pH 8, where the maximum activity was observed with a relative lipolytic activity of 163.05%. The values obtained at pH 7 and 9 were slightly less (with 35 and 21% increases, respectively), making the lipase an alkaliphilic enzyme. Figure 3c shows the effect of surfactants on the activity of the *Kodamaea ohmeri* PL2 enzyme. The lipase activity increased consistently with increasing Tween-80 concentration. Tween-80 stimulated lipase activity giving an activity of 130, 147 and 210% at 0.05, 0.10 and at 0.50 mM Tween-80 concentration, respectively, relative to the untreated enzyme. TritonX-100 and SDS augmented lipase activity at their highest concentration (0.5mM).

The effect of various cations on the activity of the enzyme is shown in figure 3d. From the observations, Calcium ion and Manganese ion had a 24% stimulatory effect on the lipase activity at 10 mM concentration. However, Fe^{3+}, NH_4^+ and Na^+ suppressed lipase activity. In most of the cations, the enzyme activity reduced with increasing cation concentration.

When a fixed volume of lipase was reacted with increasing *p*NPP concentrations, the highest activity of the *Kodamaea ohmeri* PL2 lipase was recorded at the 10 mM *p*NPP concentration (96.43 U/mL). The lipase activity thereafter reduced, and the least activity (49.5 U/mL) was observed using 50 mM concentration (results not shown).

From the purification of the *Kodamaea ohmeri* lipase, the maximum specific activity recovered from chromatographic fraction was 3.0 U/mg with a purification fold of 2.01 and lipase activity of 83.9 U/mL (Table 2).

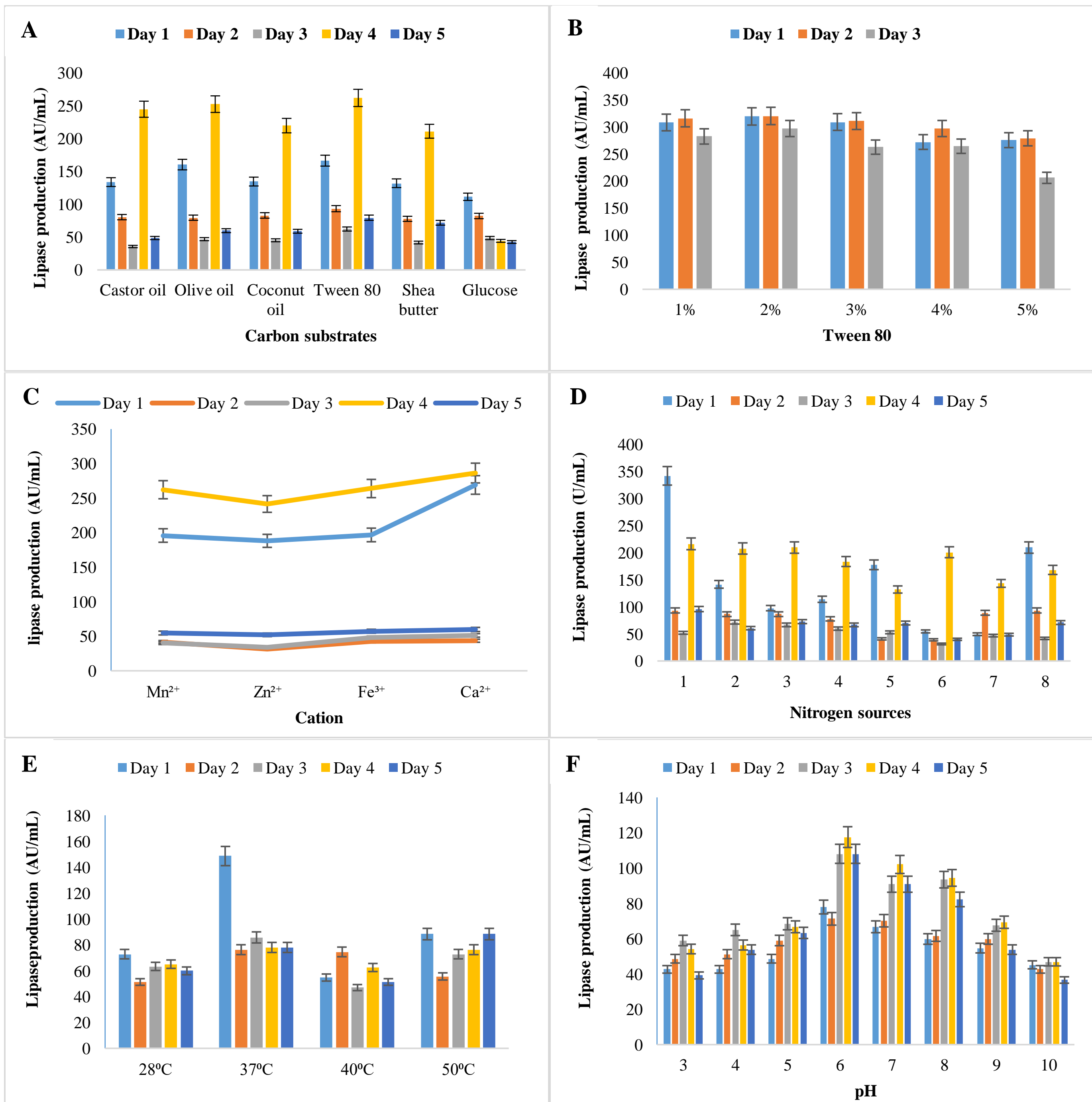

Figure 2 The effect of (a) various oil-based carbon sources, (b) increasing Tween 80 concentrations, (c) diverse metal ions, (d) organic and inorganic nitrogen sources [1: Peptone; 2: (NH4)2SO4; 3: NH4Cl; 4:NaNO3; 5: Yeast extract; 6: Urea; 7: Tryptone; 8: NH4NO3], (e) culture cultivation temperatures and (f) effect of modifications of cultivation medium pH on lipase production by *Kodamaea ohmeri* PL2 with increasing incubation days

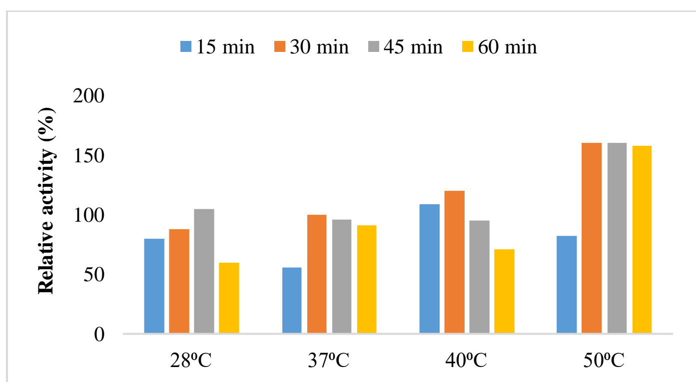

Figure 3a Effects of increasing temperature with corresponding increasing enzyme incubation time on the lipolytic activities *Kodamaea ohmeri* PL2

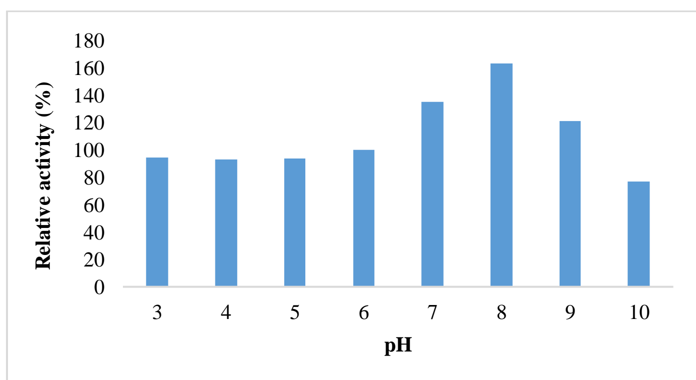

Figure 3b Effects of increasing pH regimes on the characteristics of the *Kodamaea ohmeri* PL2 lipase

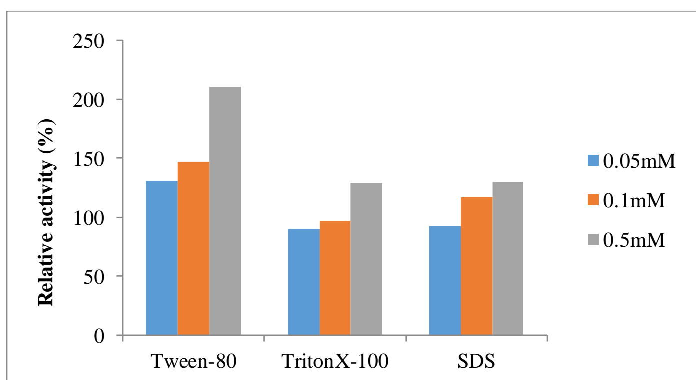

Figure 3c Effects of increasing concentrations of different surfactants on the lipolytic activities *Kodamaea ohmeri* PL2

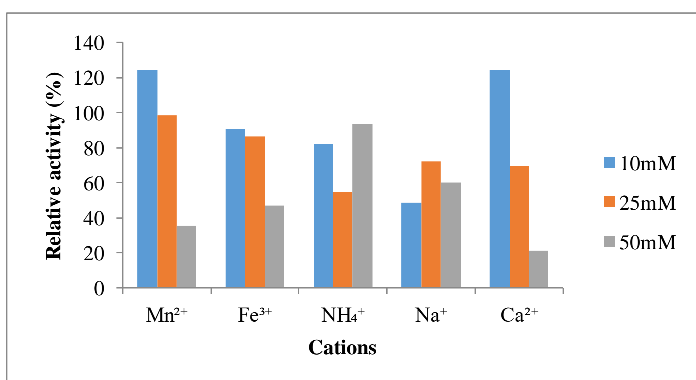

Figure 3d Effect of increasing cation concentrations on the activity of lipase produced by *Kodamaea ohmeri* PL2

Table 2 Protein Content and the effect of purification on Lipase produced by *Kodamaea ohmeri* PL2

Purification Step	Total lipase activity (U/mL)	Total Protein (mg/mL)	Specific Activity (U/mg)	Purification Fold	Yield (%)
Crude Lipase	202.5	132.7	1.53	1	100
$(NH_4)_2SO_4$ Precipitation	148.6	85.7	1.73	1.14	73.4
Dialysis	94.7	34.1	2.78	1.82	46.8
Gel Chromatography	83.9	27.9	3.00	2.01	41.4

DISCUSSION

An abundance of fungi was obtained from the different palm-oil mill wastes sampled and 21.3% of lipid-degrading organisms were present in the lipid-rich samples. When screening for lipolytic organisms and in lipase production, samples rich in oil and fatty carbon are essential (**Alabdalall *et al.*, 2020**). The highest number of lipolytic microorganisms was recorded in the sludge-like effluent of palm-oil processing area. This observation was in line with the work of **Salihu *et al.* (2011)** and **Asih *et al.* (2014)**, who respectively isolated lipolytic *Candida cylindracea* from oil effluent and produced lipase from a palm-oil mill waste. The fungal isolates in this work exhibited zones of hydrolysis on the olive-oil and the Tween-80 screening medium (shown as clear halos around the producing isolates and a color change of the medium from red to yellow). The use of olive oil and Tween-80 as screening substrates was reported in the work of **Zouaoui *et al.* (2012).** Even though the biochemical tests suggested that best lipolytic isolate, PL2, was probably *Pichia pastoris,* it was molecularly confirmed to be *Kodamaea ohmeri* PL2. Some members of the genus *Pichia* were reclassified as belonging to the genus *Kodamaea* (class: *Ascomycetae*; family: *Saccharomycetaeae*) based on the mating types and formation of spores having both spherical and hat shapes. **Corbu *et al.* (2019)** also reported the occurrence of an isolate CMGB-ST19 which was conventionally identified as *Pichia ohmeri* but reported using ITS-PCR as *Kodamaea ohmeri* CMGB-ST19. *Kodamaea ohmeri* has been reported in association with traditional fermentation of dairy products (**El-Sharoud *et al.*, 2009**). Strains of *Kodamaea ohmeri* have been documented to have possible biotechnological applications and produce D-arabitol (**Zhu *et al.*, 2010**) A xylose-producing antimicrobial *Kodamaea ohmeri* was described by **Corbu *et al.* (2019)** and two strains of *Kodamaea ohmeri* were reported to successfully control rice seedling rot disease (**Limtong *et al.*, 2020**).
The production of fungal lipases and their lipolytic characteristics have been documented by many authors and the sources of carbon are reported to considerably impact enzyme production. In this work, *Kodamaea ohmeri* PL2 successfully utilized all the oils supplied as carbon sources and produced lipases which yielded titers higher than what was obtained when glucose was used as controlled substrate. Out of the six sources of carbon tested, Tween-80 supported the best lipase production, followed closely by olive oil. This is in line with the work of **Geoffry and Achur, (2018)** who reported that the highest lipase activity was obtained in the presence of Tween-80 as a carbon source in their research. Cell permeability is increased by the addition of surfactants such as Tween-80 to the medium, hence, enhancing the release of various molecules across the cell membrane. Although Tween-80 is a surfactant, it can serve as the sole carbon source in a medium for lipase production because it contains fatty acids. When surfactants are added to an oil-containing production medium, they increase the lipase activity (**Ramani *et al.*, 2010; Geoffry and Achur, 2018**). As opposed to olive oil being the second-best carbon source for lipase production after Tween-80 as observed in this study, **Zouaoui *et al.* (2012)**, reported that olive oil supported the maximum lipase production for all the isolates used, although with values close to those in the Tween-80 medium. **Das *et al.* (2016)** reported the stimulation of lipase using vegetable oils. Also, the medium with glucose had the least lipase production, since it did not contain fatty acids which would induce lipase production, a finding supported by **Asih *et al.* (2014)**. The increasing concentrations (1-5% v/v) of Tween-80 did not translate to increased lipase production and 1% Tween-80 concentration was continued with. **Kutyla *et al.* (2021)** used Tween 80 as sole carbon source in optimized production for lipase by a *Chrysosporium pannorum*A-1 and reported highest lipolytic activity on day 4 of the culture using 0.4–0.9% Tween-80. The work by **Das *et al.* (2016)** reported no appreciable variation in the activity of lipase produced in a coconut oil-based fermentation setup at 0.1-1.5% v/v.
The presence of trace quantities of mineral salts in the production medium either stimulates or inhibits enzyme synthesis depending on the nature of the enzyme under study. Calcium ions supported the highest lipase production, an observation like the findings of **Ramani *et al.* (2010)**. According to **Celligoi *et***

al. (2016), calcium ions enhance the specificity of ion binding process on an enzyme's active site.

From the diversity of nitrogen sources used, both the organic and inorganic nitrogen sources stimulated lipase production with the highest lipase titre in a medium containing peptone. These findings were like the reports of **Kumar *et al*. (2011)** and **Sharma *et al.* (2016)** when studying the effect of nitrogen sources on lipase production.

The incubation temperature affects lipase production probably by modulating the growth of the cell and thus its metabolic activities (Gupta *et al*., 2004). *Kodamaea ohmeri* PL2 had its highest lipase production at 37ºC. **Sooch and Kauldhar, (2013)** and **Rasmey *et al*. (2017)**, reported best lipase production by some fungi at the mesophilic range (30-40ºC). The considering the source of isolate (POME sludge), it is likely that the environmental adaptation of *Kodamaea ohmeri* PL2 to a mesophilic condition also contributed to this finding.

The pH of a production medium determines microbial growth and the enzyme yield since a change in the extracellular pH will impact the intracellular pH equilibrium, pH can thus affect the mechanism of enzyme synthesis. From this study, the optimum pH for lipase production was reached at pH 6. **Gupta *et al.* (2004), Sooch and Kauldhar (2013), Celligoi *et al*. (2016), Liu and Kokare, (2017)** and **Rasmey *et al*. (2017)** all reported highest lipase production between pH 6 and 9.

Investigating the effect of incubation time on the production of enzyme revealed that the lipase production was highest at 24 and 96 while lower values were obtained at days 2 and 3. The longer an organism is incubated with its substrate, the greater the amount of product, however, the rate of product formation is not usually a simple linear function of time of incubation as the enzyme could suffer denaturation and loose a significant amount of their activity during the period of incubation. In a study by **Massadeh and Sabra (2011)** it was observed that the maximum lipase activity was obtained after 24 hours.

The lipase of *Kodamaea ohmeri* PL2 had its highest activity at 50ºC and was thermostable over 60 minutes with activities higher than what obtained at 15 minutes. **Islam *et al*. (2008)** reported a lipase active between 30-60ºC and **Tripathi and Thakur (2014)** also supported the temperature activity findings of this research signifying that lipase could be moderately thermo-active. The activity of this lipase peaked at pH 8 but values recorded at pH 7 and 9 were higher than those recorded at other pH regimes.

Tween-80 successfully augmented the catalytic activity of the *Kodamaea ohmeri* PL2 with a 110% increase at its 0.5% concentration. This is because of the reaction occurring at the aqueous/organic interface. **Liu and Kokare, (2017)** also reported a Tween-80 augmented lipolytic activity. Unlike the suggestion of **Sharma and Goswami (2020)** that a reduction in catalysis likely occurs as surfactant concentration increases, the three concentrations enlisted in this research (all below 1.0%) resulted in increasing lipase activity, mostly profound in Tween-80. At times, divergent/mixed responses may be recorded by a particular lipase upon different non-ionic surfactants. Hence, the lower activity seen in this work using Triton X-100 (29% increase) compared to Tween-80. It is therefore possible to apply this lipase in the catalysis of various industrial processes and product formation. **Borkar *et al*. (2009)** reported a lipase stable in the non-ionic Tween-80 along with a reduced activity using Trixton-X-100.

In the presence of Ca^{2+}, the maximum hydrolytic activity of the *Kodamaea ohmeri* PL2 was enhanced. **Islam *et al*. (2008)** reported the best activity using 10 mM Ca^{2+} concentration. **Amanda *et al*. (2001)** reported that Ca^{2+} may help in the structure formation of active enzymes leading to a higher enzyme activity. Lipases have been documented to possess a Ca^{2+} binding site **(Salameh and Wiegel, 2007; Liu and Kokare, (2017)** and this could have been responsible for the high lipase activity recorded in this work. The Ca^{2+} probably featured as ligands thereby facilitating the linkage to adjacent residues and in the formation of improved stability in lipase structure. Manganese also enhanced lipase activities and could also contribute to enzyme thermostability. Other cations (Fe^{3+}, NH_4^+ and Na^+) had inhibitory effects on the *Kodamaea ohmeri* PL2 lipase. Also, **Salameh and Wiegel, 2007** reported that cations were found necessary for lipase activities and supported the thermostability of lipases.

The highest specific lipase activity was recorded in the lipase subjected to gel chromatographic analysis, an indication that the specific activity improved with increasing purification steps. **Lukong *et al*. (2007)** suggested that the increase in the activity of enzyme after purification might be due to the removal of more impurities at each step of purification. Hence, the level of purification achieved can be measured by the increase in enzyme activity.

There was a reduction in lipolytic activity of the *Kodamaea ohmeri* PL2 lipase with pNPP concentrations above 10mM. This reduction may be because of the high starting concentrations employed in this study (10 mM) since generally, the rate of a reaction increases when higher concentrations of the substrate is made available to the reaction mixture until a certain peak after which the rate reduces. **Sumarsih *et al.* (2019)** who used 0.5-1.75 mM concentrations of p-nitrophenyl palmitate recorded highest lipase activity at a 1.5 mM pNPP concentration.

CONCLUSION

The importance of lipase cannot be overemphasized as it is one of the top three most commercialized enzymes, relevant in several industries in which it finds various applications. It is therefore important that cost effective methods should be harnessed as well as microorganisms with effective lipolytic abilities, this will allow for the increased production and application of this essential enzyme. In this research, the lipolytic potential of fungi isolated from waste sites of a local palm fruit processing factory was reported. The selected lipolytic yeast, molecularly confirmed to be *Kodamaea ohmeri* PL2, was able to utilize a variety of lipid substrates for lipase production. The study also outlined optimum parameters for lipase yield which included cationic additions, especially calcium ion, 1.0% Tween-80 concentration, peptone and ammonium nitrate, pH 6 and a culture incubation temperature of 37ºC. The moderately thermoactive *Kodamaea ohmeri* PL2 lipase was enhanced by a pH range of 7-9, stable at 50ºC for 60 minutes and had improved specific activity with increasing lipase purification steps. These optimum conditions can thus be explored for large-scale/industrial applications and commercialization of *Kodamaea ohmeri* PL2 lipase.

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