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Selection of *Enterobacter cloacae* Strain POPE6 for Fermentative Production of Extracellular Lipase on Palm Kernel Oil Processing Effluent

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Abstract: An *Enterobacter cloacae* strain was isolated from palm oil processing effluent and demonstrated ability to elaborate extracellular lipase on Tween 80-minimal medium using the rapid plate assay of precipitate formation. Peak productivity of the enzyme in submerged culture occurred at 72 h with lipase concentration of 95 µg/mL. Enzyme activity, monitored using different carbon-chain lengths of *p*-nitrophenol (*p*-NP)-triacylglycerol substrates, revealed that stearate was the most suitable substrate for the lipase. Preliminary optimization of major medium variables revealed highest lipase concentration in palm kernel oil processing effluent (Carbon source; P = 2.22E-05; Time, P = 1.99E-25; $P < 0.05$). Ammonium nitrate (NH_4NO_3) emerged as best nitrogen source with Na_2HPO_4/KH_2PO_4 (2:1) selected as phosphate combination with best buffering capacity. A fermentation medium incorporating the selected carbon, nitrogen and phosphorus sources, and supplemented with 2% (v/v) inoculum volume containing 10^8 cells/mL led to the production of 147.59 µg/mL lipase within 72 h of incubation. The lipase was found to possess highest activity of 2.031 U/mL at 35°C, pH 7 within 10 min, with stearate as substrate. One unit of lipase activity was defined as the amount of enzyme required to release 1 mM of *p*-nitrophenol per min from *p*-NP-stearate under the assay conditions of pH 7 and temperature of 35°C. The bacterium is recommended for lipase production on palm oil processing effluent as an oily waste management option.

1. Introduction

Lipases or triacylglycerol acylhydrolases: EC 3.1.1.3 are protein molecules belonging to the group of esterases that catalyze the hydrolytic cleavage and synthesis of esters from glycerol and long-chain fatty acid precursors **[16, 20].** They occur naturally in plants, animals, humans and in all microbial groups namely bacteria, molds, yeasts and microscopic algae **[4, 10, 17].** However, microbial lipases have been widely exploited for sundry applications owing to their robustness, ease of production on a large scale and low cost of final product **[17, 19-21**].

Considering the kind of reactions catalyzed by lipases, a number of applications of the enzyme including chemical processing, dairy industries for improvement of flavour, paper industries, pharmaceuticals, synthesis of surfactants, detergent industries, leather industries and polymer synthesis are readily discernible **[11, 12**]. In recent times lipases have been employed as effective biocatalysts to synthesize optically pure compounds like cyclohexane **[14**].

Fermentative production of lipases is largely dependent on the nature of producing organism, fermentation medium composition and recovery techniques. Medium composition tilts heavily to the nature of carbon, nitrogen and phosphorus sources that are available first for cellular metabolism by the producing organism and secondly to direct the synthesis of the metabolite of interest. A number of hydrophobic organic substrates have been investigated for the provision of carbon skeleton for the producing microorganism and for lipase. Triacylglycerol substrates of different carbon chain lengths have been investigated in their pure forms **[19**] and as oils or their processing wastes **[2, 6, 15].**

In the present study, we report the use of an uncommon bacterial species, *Enterobacter cloacae* strain isolated from palm oil processing effluent from a palm oil processing factory in Southern Calabar, Nigeria for the production of lipase using palm kernel oil processing effluent; a waste that had constituted an environmental menace to the peasant locals of the region for 18 years.

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2. Materials and Methods

2.1 Collection of pure bacterial cultures

Bacterial cultures were isolated from 10 environmental samples comprising 6 soil and 4 water samples, by the pour plate technique on nutrient agar. Morphologically-distinct bacteria were described in terms of colonial distinctions made in terms of form, pigmentation, margin, elevation and consistency and purified by repeated quadrant streak-plate technique.

2.2 Qualitative screening of bacteria for lipase production potential

Three hundred and fifty-five morphologically-distinct bacterial colonies were screened for lipase producing ability by the rapid precipitation technique **[19]** on Tween 80-minimal agar medium containing (g/L) NH_4Cl 1.0; $CaCl_2.2H_2O$ 0.1; $MgSO_4.7H_2O$ 0.5; K_2HPO_4 1.0; KH_2PO_4 1.0; Agar-agar 15; Tween 80 1% (v/v); Distilled water 1000 mL. The medium pH was adjusted to 7.0 with 1M HCl/1M NaOH and sterilization achieved by autoclaving at 121°C for 15 min. Upon cooling and solidification in 8.5 mm Petri dishes, pure bacterial isolates were inoculated by single-line streak technique in triplicates. Plates were incubated in the ambient conditions of the laboratory, specifically room temperature (28 ± 2°C) for 36 h. *Pseudomonas fluorescens* UCCM 0005 served as positive control. Formation of white insoluble crystals (precipitates) around bacterial colonies indicated positive result.

2.3 Quantitative screening of bacteria for lipase production on solid medium

Qualitatively-positive lipolytic bacteria totaling 65 were screened by the rapid precipitation technique **[19]** once again on Tween 80-minimal agar medium with composition as described in section 2.3. Inoculation and incubation followed as described in the same section. *Pseudomonas fluorescens* UCCM 0005 served as positive control. Formation of precipitates around bacterial colonies indicated positive result. Diameters of precipitating zones were measured by means of a meter rule. Similarly, diameters of colonies of bacteria grown along the line of streak were also measured. Lipase production potentials were scored as *per cent* lipase using the equation by **[3]**

$$LPP\ (\%) = \frac{MTZD - MCD}{MTZD} \times 100 \qquad Eqn.\ 1$$

where LPP refers to lipase production potential; MTZD is the mean total zone diameter and MCD, the mean colonial diameter.

Only bacteria with precipitate-developed zone diameters greater than or equal to 20 mm were considered for further studies.

2.4 Lipase production potentials of bacteria in submerged culture

Six secondarily-selected lipase-positive bacteria were screened in submerged medium in 100 mL Erlenmeyer flasks. Medium was Tween 80-minimal broth medium containing (g/L) NH_4Cl 1.0; $MgSO_4.7H_2O$ 0.5; K_2HPO_4 1.0; KH_2PO_4 1.0; 1% (v/v) Tween 80; Distilled water 1000 mL. Calcium chloride dihydrate ($CaCl_2.2H_2O$) was intentionally removed from the medium because precipitation on solid medium was no longer desired. pH was adjusted to 7.0 with 1M HCl/1M NaOH and medium sterilization done by autoclaving at 121°C for 15 min. Each flask was inoculated upon cooling with an 18-h old tryptic broth culture of each of the six lipase positive bacteria at 2% (v/v). Flasks were incubated at room temperature (28 ± 2°C) on a rotary shaker agitating at 150 rpm for 72 h. *Pseudomonas fluorescens* UCCM 0005 served as positive control. Crude lipase solution was obtained by centrifugation of the 72 h broth at 8,000 rpm for 15 min, followed by filtrations using 4.5 µM and 0.22 µM. Sterile crude lipase solution was stored at 4°C in a refrigerator until required for further studies **[7].** Triplicate protein determinations were performed on sterile crude lipase solutions by the Bradford method described in **[3]** with bovine serum albumin as standard protein.

2.5 Time course and bacterial productivity determination for lipase production

The experiment in section 2.4 was repeated in 250 mL Erlenmeyer flasks with two lipase-positive bacteria, POPE5 and POPE6. Medium composition, inoculation, incubation and analyses were as described in section 2.4 except that triplicate determinations of lipase concentrations were performed every 12 h. Data obtained were analyzed statistically and used to calculate bacterial productivity. Productivity was defined as amount of enzyme per unit time (µg/mL/h). Incubation at room temperature (28 ± 2°C) on a rotary shaker agitating at 150 rpm for 96 h. Crude lipase solution was obtained by centrifugation of the 96 h broth at 8,000 rpm for 15 min, followed by filtrations using 4.5 µM and 0.22 µM. Sterile crude lipase solution was stored at 4°C in a refrigerator until required for further studies **[7].** Triplicate protein determinations made every 12 h. A two-way analysis of variance (ANOVA) was employed to ascertain the influences of time and nature of bacterium on lipase production. Second-order polynomial was employed to fit regression models for the specific effect of time on final lipase concentration.

2.6 Lipase assay

Lipolytic activity was determined by spectrophotometric methods measuring the release of *p*-nitrophenol using *p*-NP-palmitate (C16) and *p*-NP-stearate (C18) as substrates **[19].** The substrate mixture contained 1% (v/v) of each substrate in methanol, 50 mM Tris-HCl buffer (pH 8) and 0.1% Triton X-100. The standard assay mixture contained

4.5 mL of substrate mixture and 0.5 mL of the crude enzyme supernatants from the 2 bacterial isolates, POPE5 and POPE6. The standard mixture was incubated at 30°C for 5 min. Absorbance of reaction was measured with Helios-α spectrophotometer equipped with a thermostatic cell holder (Unicam, Cambridge, UK) at 405 nm wavelength using curvettes of 1 cm path length. The assay was conducted in triplicates. One unit of lipase activity was defined as the amount of enzyme required to release 1 mM of *p*-nitrophenol per min under the assay conditions. Activity was calculated using the equation derived from Beer-Lambert's law as follows

$$EA = \Delta A \times V \times df \div \varepsilon \times t \times v \qquad Eqn.2$$

where EA = enzyme activity (U/mL); ΔA = change in absorbance over time; V = total volume of reaction mixture (mL); df = dilution factor; ε = molar extinction coefficient at 405 nm (/mM/cm); t = incubation time (min); v = volume of enzyme in the assay mixture (mL). The molar coefficient of extinction (ε) of *p*-nitrophenol (*p*-NP), under the conditions described, was determined from the absorbance at λ = 405 nm of standard solutions of *p*-NP from 0.01 to 0.16 mM/mL as 17.85.

2.7 Identification of lipase-positive isolate POPE6

Colonial morphological characterizations including consistency, elevation, form, margin and pigmentation; microscopic morphology including cell shape, size and arrangement; physiological and biochemical characterizations **[13]** were employed for this purpose. The tentative identity of the isolate obtained from the tests above was confirmed by 16S rRNA sequencing.

The molecular methodology was based on PCR and Sanger sequencing analysis. DNA extraction and bio-informatic analysis of sequences performed at the Molecular Laboratory Services Division of Teddy & Thaddeus Nig. Co., Akoka, Lagos, Nigeria. Sequencing analysis was done at Inqaba Biotechnology Pty, South Africa. DNA extraction and purification were done using ZR Fungal/Bacterial DNA MiniPrep™50 Preps. Model D6005 (Zymo Research, California, USA).

The PCR reaction was performed on the extracted DNA samples using universal degenerate primers 27F.1 Forward 5´AGRGTTTGATCMTGGCTCAG 3´ and 1492R reverse 5´ GGTTACCTTGTTACGACTT 3´ that amplifies the entire 16S variable region at annealing temperature of 58°C. Each PCR reaction contained 5 µL of 10 × Taq buffer, 2 mM $MgCl_2$, 1.5 U Super-Therm DNA Polymerase (Southern Cross), 0.25 mM dNTPs, 0.1 µM of each primer, 1 µL of extracted DNA and Nuclease Free Water (NFW) up to the final reaction volume of 50 µL. The PCR cycle started with an initial denaturation step at 94°C for 10 min. This was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 5 min that was then followed by cooling to 4°C. DNA sequencing was performed by Sanger (dideoxy) sequencing technique to determine the nucleotide sequence of the specific microorganism isolated using automated PCR cycle- Sanger Sequencer™ 3730/3730XL DNA Analyzers from Applied Biosystems. Results were obtained as nucleotides. Sequence analysis from resultant nucleotide base pairs was performed by BLAST analysis using MEGA5 software. The resultant top hits with minimum E-score for the BLAST result showing species name was used to name the specific organism.

2.8 Selection of appropriate carbon source for lipase production

The carbon sources screened for lipase production by the selected bacterium included palm oil processing effluent (POPE), palm kernel oil processing effluent (PKOPE), olive oil, sunflower oil, waste frying oil (WFO) and Tween 80. Fermentation medium contained (g/L) NH_4Cl 1.0 $MgSO_4.7H_2O$ 0.5; KH_2PO_4 1.0; K_2HPO_4 0.5; $FeSO_4.7H_2O$ 0.01; distilled water 1000 mL. pH was adjusted to 7.0 with 1M HCl and 1M NaOH. The medium was supplemented with each carbon source at 1% (v/v) (Ekpenyong *et al*., 2017). Sterility was secured by autoclaving at 121°C for 15 min. Fermentation mode was submerged in 250 mL Erlenmeyer flasks containing 1/5 reaction volume. Each flask was inoculated with 2% (v/v) 18 h-old tryptic broth culture of test bacterium, POPE6. Flasks were incubated at room temperature on a rotary shaker agitating at 150 rpm for 96 h. Periodic (every 12 h) withdrawal of 5 mL fermentation broth and centrifugation at 8,000 rpm for 15 min for determination of lipase concentration **[3]**. *Pseudomonas fluorescens* UCCM 0005 served as positive control while Coomassie brilliant blue (protein reagent) was the negative control. A two-way analysis of variance was employed to ascertain the dual influences of time and carbon source on lipase production by the isolate.

2.9 Selection of appropriate nitrogen source for lipase production

The nitrogen sources screened for lipase production by the selected bacterium included ammonium chloride, ammonium nitrate, ammonium sulphate, potassium nitrate, sodium nitrate and urea. Fermentation medium contained (g/L) $MgSO_4.7H_2O$ 0.5; KH_2PO_4 1.0; K_2HPO_4 0.5; $FeSO_4.7H_2O$ 0.01; distilled water 1000 mL. pH was adjusted to 7.0 with

1M HCl and 1M NaOH. Nitrogen sources were each supplemented at 1% (w/v) before sterilization except urea. Flasks were sterilized as described earlier. Upon cooling, the flask labeled for urea-N was supplemented with filter-sterilized urea (0.2 µM Millipore) at 1% (w/v) **[8]**. Other conditions like inoculation, incubation, protein determination, controls and statistical analysis were as described in section 2.8.

2.10 Selection of appropriate phosphate combinations for lipase production

Four phosphate combinations including K_2HPO_4/KH_2PO_4, Na_2HPO_4/NaH_2PO_4, Na_2HPO_4/KH_2PO_4 and K_2HPO_4/NaH_2PO_4 were screened for appropriateness for mediating lipase biosynthesis. Medium contained (g/L) $MgSO_4.7H_2O$ 0.5; $FeSO_4.7H_2O$ 0.01; NH_4NO_3 1% (w/v); distilled water 1000 mL. pH was adjusted to 7.0 with 1M HCl and 1M NaOH. The different phosphate combinations were added at 1% (w/v) to the fermentation medium. Other conditions like inoculation, incubation, protein determination, controls and statistical analysis were as described in section 2.8.

2.11 Selection of appropriate inoculum size for lipase production

Medium containing (g/L) NH_4NO_3 1.0; Na_2HPO_4/KH_2PO_4 1.0; $MgSO_4.7H_2O$ 0.5; $FeSO_4.7H_2O$ 0.01; distilled water 1000 mL, supplemented with palm kernel oil processing effluent (PKOPE) at 1% (v/v) was inoculated with 18 h-old Luria broth cultures of selected bacterium. Other conditions like inoculation, incubation, protein determination, controls and statistical analysis were as described in section 2.8.

2.12 Determination of optimum temperature and pH for lipase activity

Enzyme assay was conducted as described in section 2.6 but at varying temperatures ranging from 30 to 50°C with 5°C increments. Substrate was *p*-NP-stearate (C18); pH 8.0. Stability of lipase at various temperatures was obtained by estimating enzyme activity after incubation for 2.5, 5, 7.5, 10 and 12.5 min from spectrophotometric readings taken at a wavelength of 405 nm. A two-factor analysis of variance was employed to test the significance or otherwise of dual effects of temperature and time on lipase activity. Similarly, enzyme assay was conducted at 35°C using different buffers at pH levels ranging from 3 to 12. For pH 3 to 6; sodium-phosphate buffer was used; Tris-HCl buffer was used for pH 7 to 8; potassium phosphate buffer for pH 9 to 10 and bicarbonate-phosphate (sodium) buffer for pH 11 to 12. Substrate was *p*-NP-stearate (C18). Stability of lipase at various pH was obtained by estimating enzyme activity after incubation of 0.2 mL of enzyme with 1.8 mL of the substrate/buffers for 10 min. A single-factor analysis of variance was employed to test the significance or otherwise of pH effect on lipase activity.

3 Results

Results of the qualitative screening of 355 morphologically-distinct colonies of bacteria for lipase producing potentials are presented in Table 1. The results show that only 65 (18.31%) of total bacteria had such potential and that palm oil processing effluent sample had the highest percentage of lipase producing bacteria of 38.89%. Quite interestingly, higher percentages of lipase-positive bacteria occurred in lipidic samples than any other, however, analysis of variance of data revealed that there was no significant difference ($P = 0.99 > 0.05$) among samples in number of lipase-positive bacteria.

Results of quantitative screen test on solid medium revealed that only 6 isolates had ≥ 20 mm precipitate zone diameters with > 90% *per cent* lipase production (Table 2). However, in the submerged method, only isolates POPE5 and POPE6 could produce lipase in amounts detectable by the Bradford method of protein determination.

In the experiments set up to determine the time-course of lipase production and productivities of the 2 bacteria, results show that both bacteria gradually increased their lipase concentrations over time until 72 h when lipase concentration dropped. This result is presented in Figure 1. The productivity plot of lipase production by the isolates is presented as Figure 2. The more productive bacterium of the two was selected as POPE6.

Table 3 is a presentation of activity potentials of isolates POPE5 and POPE6 using two triacylglycerols. The table reveals that isolate POPE6 lipase had a higher activity (1.147 U/mL) than POPE5 which activity was 0.549 U/mL when stearate served as triacylglycerol.

Results of the characterization and identification experiments of best lipase-producing bacterium are presented in Table 4 and reveal that isolate POPE6 was a strain of *Enterobacter cloacae* with a 99% sequence homology with *Enterobacter cloacae*, Accession Number CP022148.1. The bacterium was defined as a catalase-positive, oxidase-negative facultatively anaerobic Gram-negative rod-shaped bacterium.

Figure 3 is a presentation of the experiments leading to the selection of appropriate carbon source for lipase production by *Enterobacter cloacae* strain POPE6. The figure shows that palm kernel oil

processing effluent (PKOPE) was the best carbon source as it mediated the production of 79.83 µg/mL of lipase by 72 h. Waste frying (sunflower) oil emerged second best carbon source by mediating the synthesis of 68.02 µg/mL at 72 h. A two-factor analysis of variance revealed that nature of carbon source and fermentation time significantly influenced (Carbon source; P = 2.22E-05; Time, P = 1.99E-25; $P < 0.05$) lipase concentration.

Table 1 Primary screening of bacteria for lipolytic activity

S/N	Sample code	Number of distinct colonies	Number of lipase-positive bacteria	*Per cent* (%) lipase-positive bacteria per sample
1	ANW	52	5	9.61
2	EWDS	38	4	10.53
3	HTW	47	5	10.64
4	MWDS	42	6	14.29
5	MWSA	21	7	33.33
6	MWSB	32	8	25.00
7	PKOPE	29	9	31.03
8	PKOPS	42	7	16.67
9	POPE	18	7	38.89
10	POPS	34	7	20.59
	TOTAL	355	65	18.31

ANW-Anantigha water; EWDS-Ekorinim market waste-dump soil; HTW-Henshaw Town water; MWDS-Marian market waste-dump soil; MWSA-Mechanic workshop soil A; MWSB-Mechanic workshop soil B; PKOPE-Palm kernel oil processing effluent; PKOPS-Palm kernel oil processing soil; POPE-Palm oil processing effluent; POPS-Palm oil processing soil; S/N-Serial Number

Table 2 Quantitative screening for lipase-positive bacteria in solid medium

S/N	Isolate code	Mean total zone diameter (mm)	Mean colonial diameter (mm)	*Per cent* (%) lipase production
1	POPE5	24.5±0.97	1.2±0.14	95.10
2	POPE6	21.3±1.15	1.6±0.24	92.49
3	PKOPE3	27.9±1.28	1.1±0.32	96.06
4	PKOPE5	28.6±1.34	1.3±0.14	95.45
5	PKOPS4	21.4±0.63	1.5±0.24	92.99
6	POPS6	22.5±0.82	1.3±0.27	94.22

POPE-Palm oil processing effluent; PKOPE-Palm kernel oil processing effluent; PKOPS-Palm kernel oil processing soil; POPS-Palm oil processing soil ; S/N-Serial number

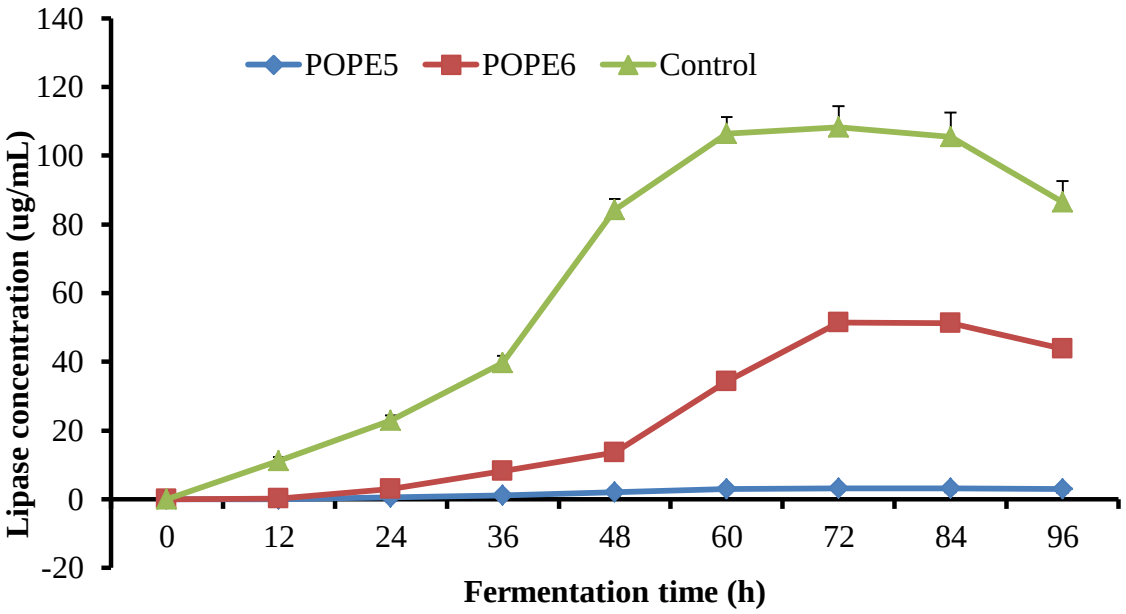

Fig.1: Time-course of lipase production by select bacteria.
POPE-Palm oil processing effluent

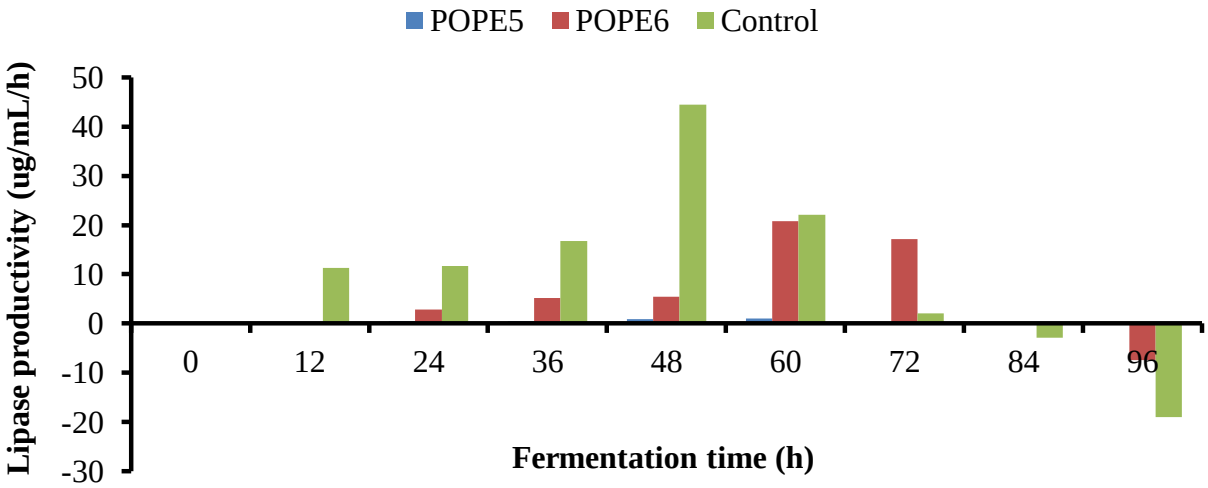

Fig. 2: Productivity of lipase-positive bacteria.
POPE-Palm oil processing effluent

Table 3 Activities of bacterial lipases on palmitate and stearate

S/N	Substrates	Absorbance (405 nm)		Activity (U/mL)	
		POPE5	POPE6	POPE5	POPE6
1	Palmitate (C16)	1.197 (±0.31)	1.845 (±0.32)	0.593 (±0.02)	0.956 (±0.08)
2	Stearate (C18)	1.118 (±0.18)	2.185 (±0.42)	0.549 (±0.01)	1.147 (±0.11)
3	Blank	0.138 (±0.01)			

POPE-Palm oil processing effluent

Table 4 Characterization for identification of *Enterobacter cloacae* strain POPE6

S/N	TEST	RESULT
	Colonial morphology	
1	Consistency	Butyrous
2	Elevation	Raised
3	Form	Round
4	Margin	Entire
5	Pigmentation	None
	Microscopic morphology	
6	Gram reaction	-
7	Cell shape	Rod
8	Cell size	0.35µM
9	Cell arrangement	Singles & in pairs
10	Flagella	+
11	Capsule	-
	Sugar Fermentation	
12	Oxidative-Fermentation (O-F)	F (Fermentative)
13	Adonitol	-
14	Arabinose	+
15	Cellobiose	+
16	D-mannitol	+
17	D-mannose	+
18	Glucose	+
19	Maltose	+
20	Melibiose	+
21	Rhamnose	+
22	Sorbitol	+
23	Sucrose	+
24	Xylose	+
	Enzymatic characterization	
25	Arginine decarboxylation	+
26	DNase activity	-
27	Gelatin liquefaction	-
28	Lysine decarboxylation	-
29	Nitrate reduction	+
30	Ornithine decarboxylation	+
31	Phenylalanine deamination	-
	Biochemical characterizations	
32	Catalase	+
33	Citrate utilization	+
34	Growth in potassium cyanate (KCN)	+
35	Hydrogen sulphide (H_2S) production	-
36	Indole production	-
37	Methyl Red (MR)	-
38	Motility	+
39	ONPG (β-galactosidase)	+
40	Oxidase	-
41	Voges Proskauer (VP)	+
	Molecular characterization	
42	16S rRNA sequence identity	*Enterobacter cloacae* Accession Number CP022148.1.

Results of the experiments for the selection of appropriate nitrogen for lipase productions are presented as Figure 4. The figure shows that ammonium nitrate was the most appropriate nitrogen source for lipase production by *Enterobacter cloacae* strain POPE6 by releasing 95.86 µg/mL at 72 h. This was followed by 81.83 µg/mL, 79.83 µg/mL and 79.23 µg/mL of lipase for sodium nitrate, ammonium chloride and potassium nitrate respectively. Analysis of variance of data revealed that nitrogen sources and fermentation time significantly (Nitrogen source, P = 1.35E-06; Time, 1.57E-32 < 0.05) influenced lipase production by the bacterium.

Figure 5 presents the results of experiments for selecting appropriate phosphorus source for lipase production by the test bacterium. The phosphorus sources were also to serve as buffers during the fermentation. The results presented show that 102.53 µg/mL was the highest amount of lipase attained and this occurred at 72 h when Na_2HPO_4/KH_2PO_4 served as phosphorus source and buffer. Performances of other phosphate combinations were (in descending order) K_2HPO_4/KH_2PO_4-95.86 µg/mL> Na_2HPO_4/NaH_2PO_4-84.67 µg/mL> K_2HPO_4/NaH_2PO_4-79.43 µg/mL. Analysis of variance of data revealed that both phosphorus source and fermentation time were respectively significant (Phosphorus source, P = 5.01E-05; Time, 7.45E-22 < 0.05) in their influences on lipase concentration.

The results of experiments for selecting appropriate inoculum size for fermentative lipase production are presented in Figure 6. The figure reveals that for maximum lipase concentration of 147.59 µg/mL achieved at 72 h, the starting inoculum size was 10^8 cells/mL. Performances of other inoculum sizes were (in descending order) 10^7 cells/mL-119.04 µg/mL>10^6 cells/mL-88.48 µg/mL>10^5 cells/mL-83.23 µg/mL>10^9 cells/mL-67.83 µg/mL. Analysis of variance of data revealed that both inoculums size and fermentation time were respectively significant (Inoculum size, P = 0.0039; Time, 2.2E-13 < 0.05) in their influences on lipase concentration.

Results of influences of temperature and pH on lipase activity are presented in Figures 7 and 8. Figure 7 reveals that optimal temperature for lipase activity from *Enterobacter cloacae* strain POPE6 was 35°C where lipase activity was 2.024 U/mL at 10 min. Lipase activities at other temperatures were (in descending order) 40°C-1.703 U/mL in 5 min>30°C-1.622 U/mL in 10 min>50°C-1.578 U/mL in 7.5 min>45°C-1.414 U/mL in 7.5 min. A two-factor analysis of variance of data revealed significant (P = 0.039 <0.05) influence of temperature but a non-significant (P = 0.277 > 0.05) influence of time on lipase activity. Figure 8 reveals that optimum pH for test bacterial lipase activity was 7.0 with an activity of 2.031 U/mL at 35°C for 10 min.

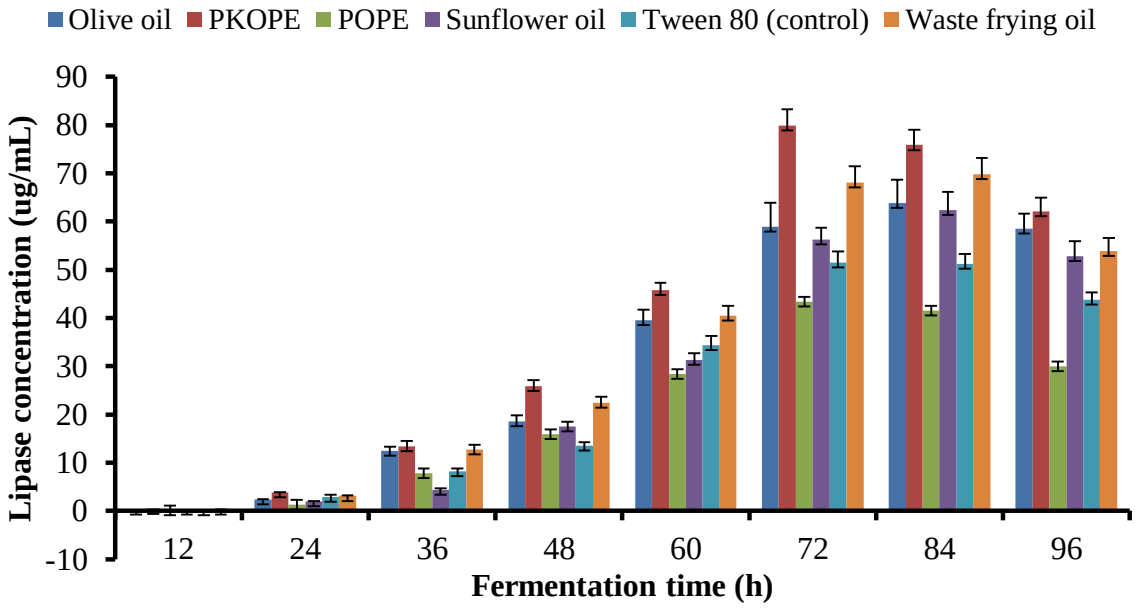

Fig.3 Effect of carbon source on lipase production

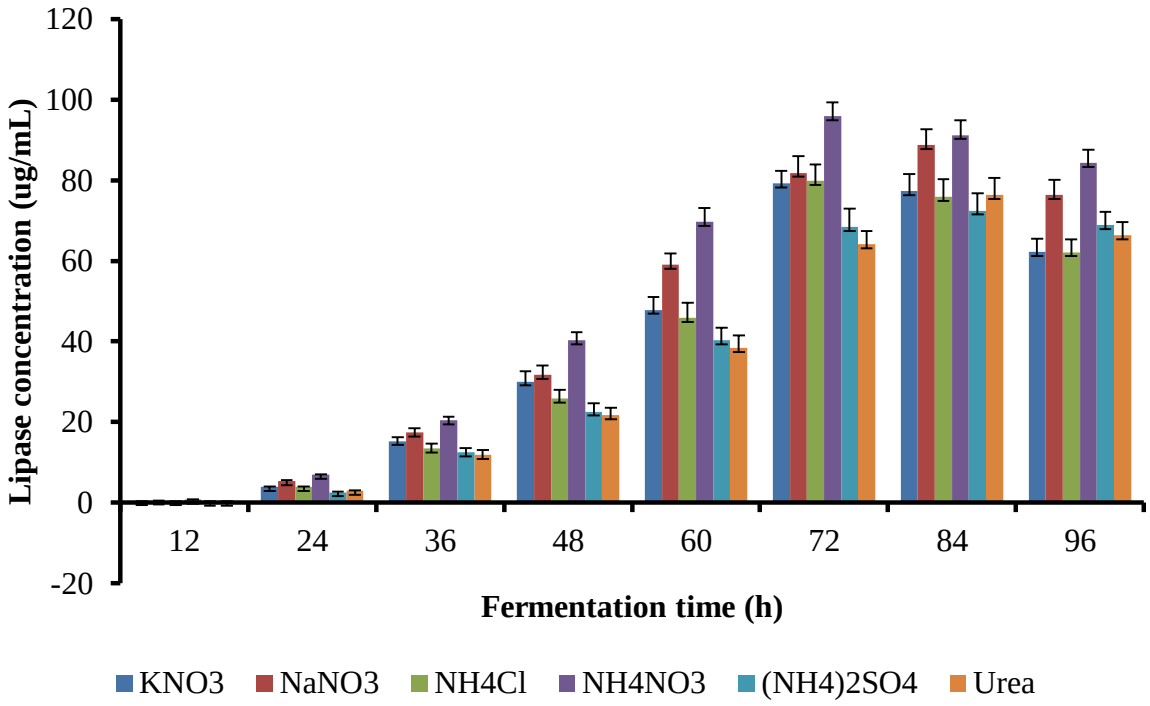

Fig.4 Effect of nitrogen source on lipase production
PKOPE-Palm kernel oil processing effluent; POPE-Palm oil processing effluent

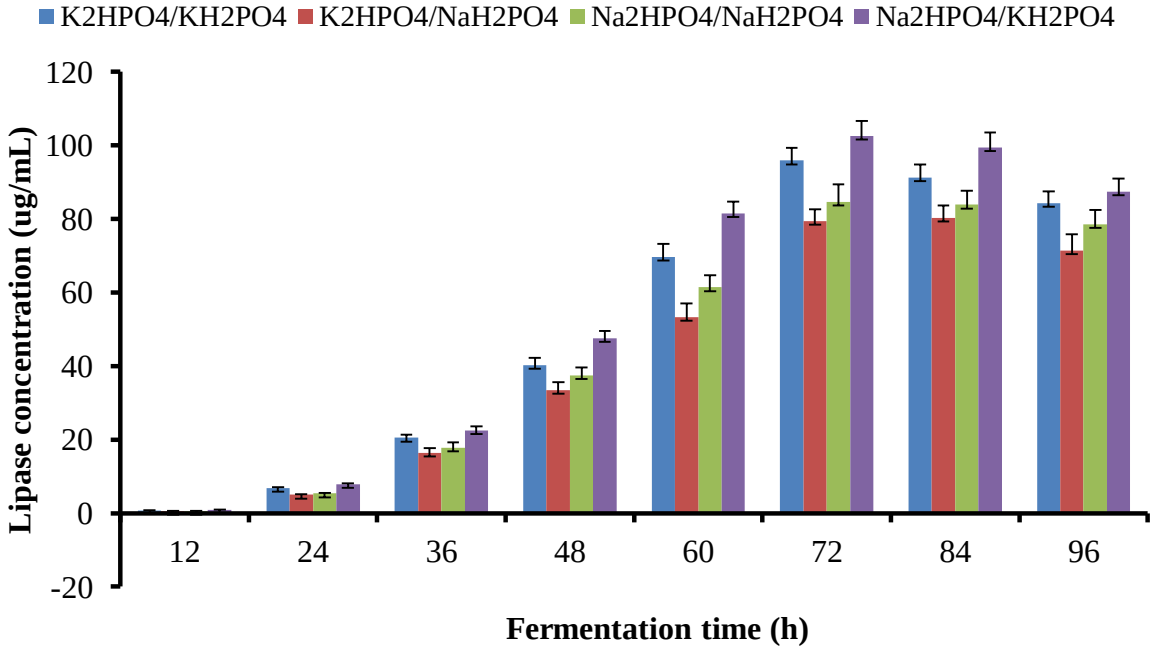

Fig.5 Effect of phosphate combinations on lipase production

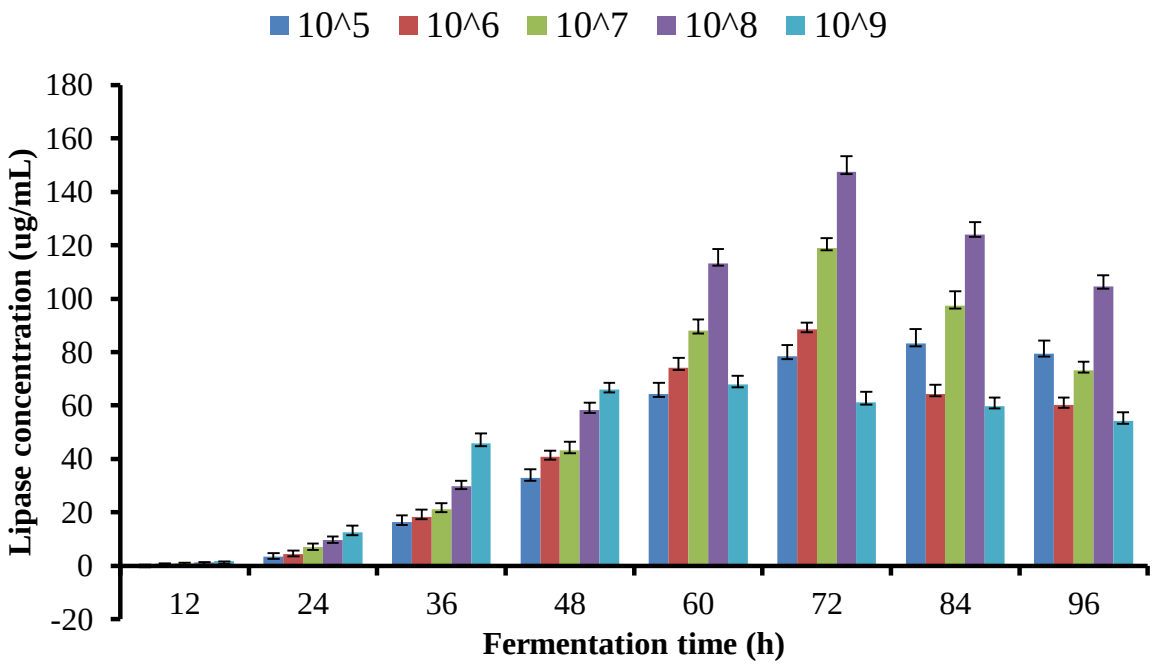

Fig.6 Effect of inoculum size on lipase production

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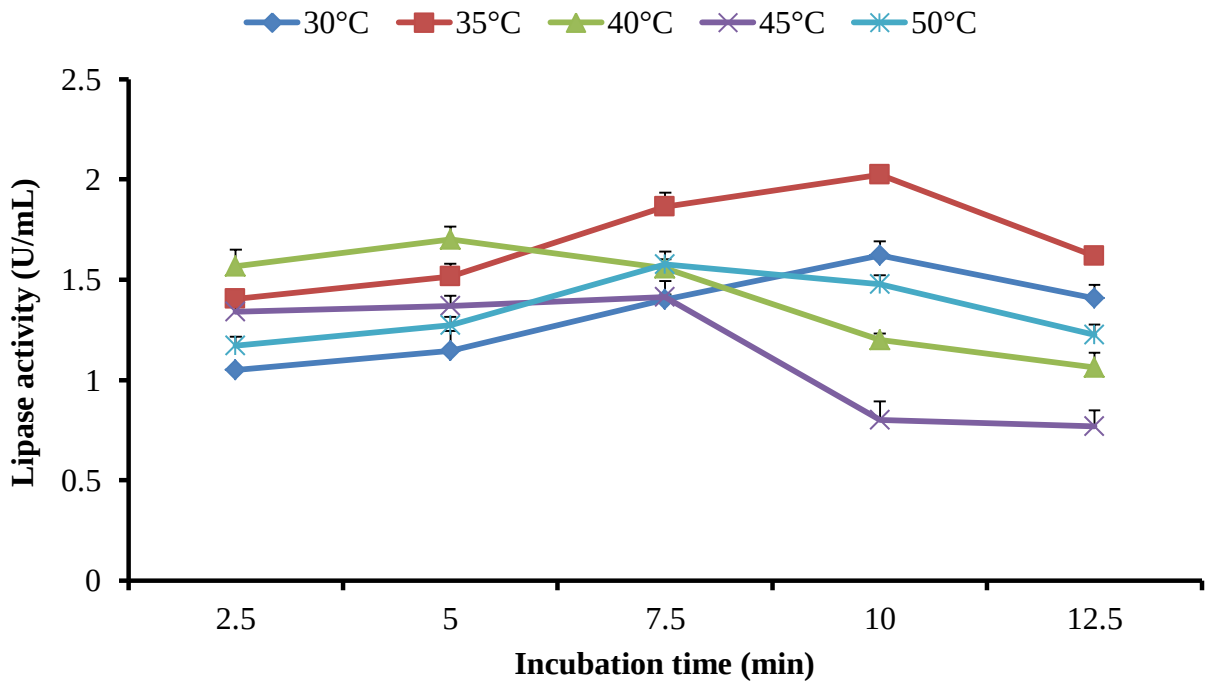

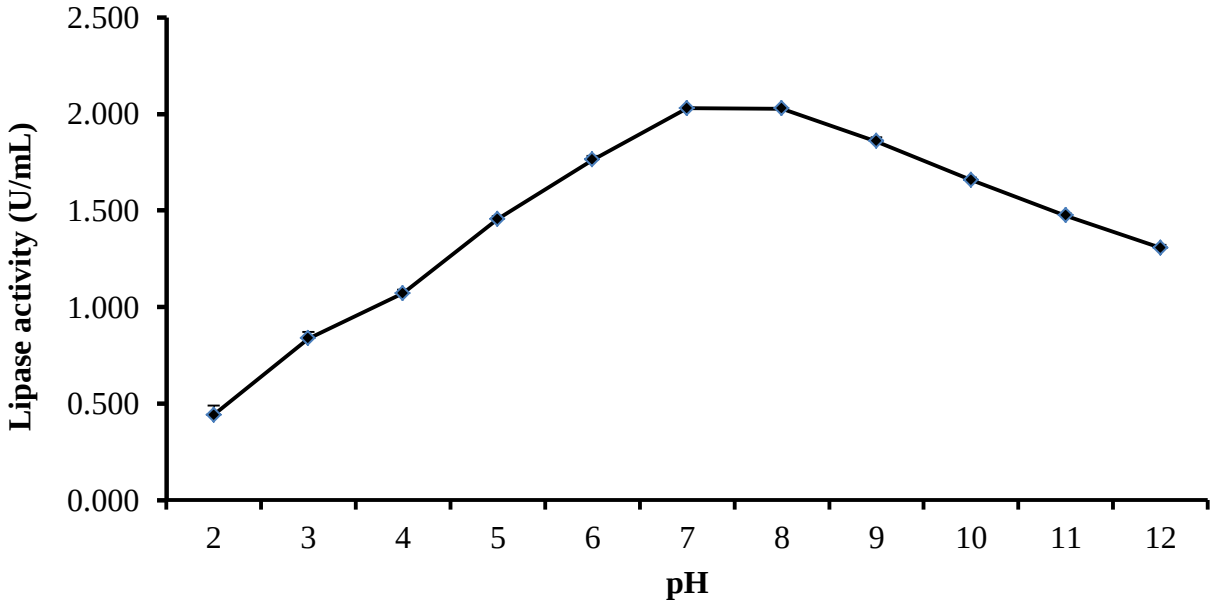

Discussion

A major concern of a biotechnological venture is production economics and pivotal to the economics of production is the producing organism. Of special interest to the biotechnologist is the quantity of metabolite produced per unit time (productivity). Because of the economic and legal complexities arising from use of genetically-engineered microorganisms in production, microbial biotechnologists hold bio-prospecting exercises in high regard. A large number of microorganisms from diverse groups, especially, bacteria and actinomycetes, yeasts and molds, pooled from different samples and environments are usually screened using high through-put techniques with the singular objective of selecting only those with demonstrable industrial potentials.

In this study, 18.31% of total bacteria isolated from all samples demonstrated abilities to produce lipase on solid medium. Significantly, the sample with lowest number of morphologically-distinct colonies was palm oil processing effluent (18 isolates), yet 38.89% of these were lipolytic. This underlines the concept of natural genetic engineering in which

microorganisms adapt, over time, to environments chronically impacted with specific substrates as source of carbon and energy. Lipidic substrates naturally induce synthesis of lipolytic enzymes; esterases or lipases; therefore the result presented in this section was not at all surprising. Although six isolates made it out of the secondary screening on solid medium, only two could demonstrate lipase production in submerged culture. This supposes that these two bacteria produced lipase in amounts large enough to be detected by the Bradford method of protein determination, indicating that their concentrations were so large that their dilute solutions left sufficient amounts capable of driving the needed catalysis of triacylglycerol hydrolytic cleavage. Abilities of bacteria that did not meet the cut-off to be considered for submerged culture screening were nevertheless tested in the submerged method. As expected, none of these isolates could produce lipase in sufficient micro amounts to be detected by the method adopted in this study.

A consideration of the productivities of the isolates revealed that the amount of lipase per unit time varied between the bacteria. Productivity studies are conducted to guide the selection of a feasible duration of fermentation for a particular metabolite by a specific microorganism. The productivity plot (Figure 2) revealed that peak lipase productivity by isolate POPE6 occurred at 60 h as compared to the 48 h of the control bacterium. This suggests that (i) production of lipase decreases after this period (ii) degradation of already produced lipase begins (iii) Fermentation time for lipase production by this isolate is 60 h under the study conditions.

Enzyme concentration is not nearly as important to the microbial biotechnologist as its activity. The concentration of an active enzyme is always a better consideration even though the activity increases with concentration until all binding sites on substrate have been saturated. The activities of lipases produced by the test isolates were evaluated using two different substrates; palmitate (C_{16}) and stearate (C_{18}). Short chain fatty acids including acetate (C_2) and butyrate (C_4) were also included in the assay (data not shown) to establish that the triacylglycerol hydrolase so produced was actually a lipase and not an esterase **[21**]. The results showed that enzymes from the two isolates were lipases and not esterases (Table 3). The substrate preferences of the lipases could be explained by the phenomenon of induced-fit-model which claims that the substrate has the ability to cause substantial transformation in the three-dimensional link of the amino acids at the active site of the enzyme and these transformations in protein structure, directed by the substrate, will bring the catalytic groups of the enzyme into an orientation suitable for reaction **[16]**.

The bacterium was identified as a strain of *Enterobacter cloacae* commonly involved in nosocomial infections like bacteremia, lower respiratory tract, urinary tract, skin and soft-tissue infections a well as endocarditis and meningitis. This would presuppose that the bacterium is not an industrial organism because, although isolated from palm oil processing effluent in this study, the bacterium has its origin commonly in feces of animals and man, and in sewage. The pathogenic status of most strains of this bacterium combined with the documented natural habitats understandably cast doubt on its suitability; in terms of safety during fermentation operations; for industrial production of lipase. Results of this study indicate that lipase is an important virulence factor of this strain of *Enterobacter cloacae* which might be involved in the breakdown of lipid membrane barriers between cells/tissues and the environment. In spite of the presumed pathogenicity status of species of this genus, **[18**] studied the production of lipase by a strain of *Enterobacter aerogenes* and obtained commendable results. Our study bacterium could therefore be given the benefit of the doubt, at least until further studies on it and the safety of its lipase are concluded.

A common method of improving microbial metabolite concentration during fermentation is the use of appropriate major medium components especially carbon source, nitrogen source and phosphorus source, which also doubles as a buffer to resist drastic changes in pH during microbial metabolism. This aspect of fermentation is preliminary to optimization methods which deal with actual amounts of selected materials. Results of experiments leading to the selection of palm kernel oil processing effluent (PKOPE) as best carbon source for lipase production by this bacterium, suggest that nature of carbon source influences amount and activity of lipase. Analysis of variance of data revealed that duration of fermentation was also significantly influenced by nature of carbon source seeing that different carbon sources mediated lipase productivity differently. Palm kernel oil processing effluent reduced fermentation time with lipase productivity reaching its peak very early. This can be attributed to the shortening of time required for maximum production of cell protoplasm resulting in early copious lipase production **[5].** Preference of palm kernel oil processing effluent remarkably offers an approach to ridding the environment of this waste while producing a value-added metabolite at reduced cost.

The single-factor-at-a-time approach for the selection of appropriate nitrogen source revealed that ammonium nitrate was the best carbon source. This confirms the result of characterization test (Table 3) that the bacterial strain is a nitrate reducer. Reduction of nitrate increases the amount of available nitrogen in the system leading to increased protein synthesis and cellular metabolism that drives significant increase in lipase production.

The search for appropriate phosphorus source led to the selection of disodium hydrogen phosphate/potassium dihydrogen phosphate. Ekpenyong *et al*. **[8]** observed that the combination of Na_2HPO_4/KH_2PO_4 (2:1) was most appropriate for biosurfactant production; a metabolite very similar in activity with lipases, particularly emulsion formation. They theorized that there could be a requirement for sodium ions (Na^+) either for metabolite formation or their release. Moreover, a better buffering activity might be provided by the combination than with others following increased cellular metabolism which could have led to drastic changes in pH, perhaps towards acidity, with resultant impairment of lipase activity.

Microbial metabolite biosynthesis depends a whole lot on the starting inoculum size. Very often, this parameter is confused with inoculum volume in literature. While inoculum size concerns itself with the number of actively dividing (viable) cells per unit volume of inoculum **[8],** inoculum volume refers to the specific volume of an inoculum size used in a fermentation experiment and is often expressed as % (v/v) relative to the reaction volume in the reaction vessel. A 2% (v/v) inoculum volume containing inoculum size of say 2.34 x 10^4cells/mL and that containing 2.34 x 10^8 cells/mL are not the same if the fermentation vessels and the final reaction volumes are the same. Understandably, the vessel with the higher inoculum concentration is the one with the larger inoculum size. In the final analysis, the driving force in fermentation is the inoculum concentration which is akin to the quorum of cells required to initiate many microbial biosynthetic processes including antibiotic and biosurfactant synthesis, bioluminescence, sporulation and synthesis of virulence factors required for microbial invasions during pathogenesis. The higher the inoculum concentration, the earlier a metabolite biosynthesis commences and the shorter the overall fermentation time. In this study, results showed that inoculum size significantly influenced lipase production by the bacterium. An inoculum size of 10^8 cells/mL was selected as best inoculum size for lipase production by *Enterobacter cloacae* strain POPE6 using palm kernel oil processing effluent as carbon source, NH_4NO_3 as nitrogen source and Na_2HPO_4/KH_2PO_4 (2:1) as phosphorus source and buffer. A similar inoculum size was observed by Ekpenyong *et al*. **[8]** in their biosurfactant study. The authors suggested that too little an initial cell population would delay biosurfactant production until such a time that its density reached the desired quorum. They also argued that too large an initial cell population might arrest production too early by reason of ineffective collisions between the bacterial cells (catalysts) and the substrates for want of space in a saturated system.

Two important environmental factors that influence enzyme activity include temperature and pH **[14]** and their effects were investigated in this study. Our results showed that duration of exposure was an important modulator at any particular temperature. Exposure of an enzyme to high temperatures usually results in the denaturation of the enzyme by breakage of peptide bonds and destabilization of intermolecular interactions that facilitated the formation of the protein. Increased denaturation and destabilization result if the exposure to such damaging temperature is prolonged. Selection of 35°C as optimal temperature for activity implies that at this temperature, *Enterobacter cloacae* strain POPE6 lipase attains maximal and prolonged catalytic activity on stearate (C_{18}) for 10 min. The preference of this enzyme for long chain fatty acids (C_8-C_{18}) confirms it as a true lipase **[20]**. **[19]** reported, in their research on lipolytic enzymes, that the effect of temperature on lipase activity is substrate-specific. The present study had earlier screened lipidic substrates for lipase activity and found stearate to be most suitable for test lipase action. Effect of temperature and pH on lipase activity was accordingly conducted solely on this substrate and the influence of exposure time on activity, as anticipated, became a major issue. Effect of pH on the activity of the test lipase was only conducted at the optimum temperature and time already established. It is possible that all four factors could interact to bring about an influence on the activity of the enzyme. A response surface methodology has therefore been set up in our laboratory to study the effects of these four variables and their possible interactions on lipase activity. The optimal pH for *Enterobacter cloacae* lipase in this study was 7.0 with an activity of 2.031 U/mL at 35°C for 10 min. This is in agreement with earlier reports on lipase activity studies as bacterial lipases frequently show high activities at neutral or alkaline pH **[1, 9]**

Conclusion

This research identified a rare lipolytic bacterium, *Enterobacter cloacae* strain POPE6 with the uncommon ability to use palm kernel oil processing effluent as substrate. The conversion of this waste into lipase is an important approach to solving waste pollution problems. This research also identifies lipase as one of the major virulence factors of the bacterium since it is generally known to be involved in nosocomial infections. However, the ability of the bacterium to use palm kernel oil processing effluent as carbon source for fermentative lipase production could be exploited as a waste management option.

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