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Isolation and Optimization of Lipase Producing Microorganism from Degrading Palm (*Elaeis guineensis*) Kernel Nut Oil

Ariyo Dokun Olanrewaju[1*], Ayodele Bosede Adetutu[1] and Fasusi Oluwaseun Adeyinka[1]

[1]Department of Microbiology, Federal University of Technology Akure, Ondo State, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author ADO designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors ABA and FOA managed the analyses of the study. Author FOA managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: Most commercially useful lipases are of microbial origin. The increasing tendency of its market shows the importance to search new microbial resources to produce these enzymes.
Study Design: The study is designed at isolating lipase producing microorganism and to optimize the cultural conditions for the biosynthesis of extracellular lipase.
Place and Duration of Study: The study was conducted between April and September, 2015 at the Microbiology Laboratory of the Federal University of Technology, Akure, Ondo State, Nigeria.
Methodology: Palm kernel seeds were purchased from Oja Oba, Akure, Ondo State Nigeria. The oil was extracted using n-hexane by Soxhlet extractor. The oil samples were stored at room temperature (25°C±1) for three months. Microorganisms were isolated from the oil and identified every two weeks of storage. The isolated microorganisms were screened for lipolytic activity using qualitative plate assay method. The effects of pH, incubation period, substrate concentration and temperature were observed on the lipolytic activities of the microorganism that shows maximum

**Corresponding author: E-mail: lanredariyo@yahoo.com;*

activity. The isolate showing maximum activity was identified by following Berger's manual.
Results: All the isolates were tested for lipase activity and only the fungi *Aspergillus flavus, A. saprophyticus, Penicllium notatum and Articulosporium inflate* showed sign of lipolytic activities. *Aspergillus saprophyticus* showed the maximum activity. The optimum activity was produced for *Aspergillus saprophyticus* at 35°C, incubation period of 5 days, pH of 7 and substrate concentration of 1.5 ml.
Conclusion: This study showed palm (*Elaeis guineensis*) kernel oil as source of four major lipase producing microorganism namely *Articosporium inflate, Aspergillus flavus. A. saprophyticus and Penicillium notatum*. Optimization studies on the cultural condition for maximum lipase activity were done on the isolated *Apergillus saprophyticus.* The optimization improved the lipase production but further studies are needed to enhance lipase production of the organism.

Keywords: Apergillus saprophyticus; lipolytic activity; palm (Elaeis guineensis) kernel oil.

1. INTRODUCTION

Lipases (*Triacylglycerol acylhydrolases* EC 3.1.1.3) are a class of hydrolases which catalyze the hydrolysis of triglycerides to glycerol and free fatty acids on an oil-water interface [1]. In addition they are carboxylesterases that catalyze the hydrolysis and transesterification of esters. The syntheses of esters can exhibit enantioselective properties [2]. Several enzymatic activities have been described for lipases, such as hydrolysis, transesterification and esterification. However, the hydrolytic activity of lipases has been studied in detail in contrast to the transesterification activity. It is hypothesized that the transesterification activity of lipases is similar to the serine-protease catalytic activity, because both enzymes share the same catalytic triad Ser–His–Asp/Glu [3].

Lipases occur widely in nature, but only microbial lipases are commercially significant [4,5]. It is well known that lipases are the most widely used enzymes in organic synthesis and more than 20% biotransformations are performed with lipases [6]. In addition to their role in synthetic organic chemistry, these also find extensive applications in chemical, pharmaceutical, food and leather industries [7,8].

Palm kernel oil is a common cooking ingredient; its increasing use in the commercial food industry throughout the world is buoyed by its lower cost, the high oxidative stability (saturation) of the refined product when used for frying, and its lack of cholesterol and trans fatty acids, both viewed as being heart-healthy attributes [9]. The approximate concentration of fatty acids (FAs) in palm kernel oil is as follows: Lauric saturated C12 48.2%, Myristic saturated C14 16.2%, Palmitic saturated C16 8.4%, Capric saturated C10 3.4%, Caprylic saturated C8 3.3%, Stearic saturated C18 2.5%, Oleic monounsaturated C18:1 15.3%, Linoleic polyunsaturated C18:2 2.3% and others/unknown 0.4% [10].

Lipase-producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil, etc [11]. The aim of this study is to find and select lipolytic microorganisms showing transesterification activity and to optimize the cultural conditions for the biosynthesis of extracellular lipase.

2. METHODS

2.1 Collection of Samples

Fresh Palm kernel nut was collected from retailers at Oja oba Akure, Ondo State Nigeria and transported to the laboratory in a clean bag. They were stored in the laboratory in a clean environment at a room temperature of 28°C.

2.2 Extraction of Oil

Palm kernel oil samples were extracted from the crushed sample by Soxhlet extractor using n-hexane as solvent [12]. The extraction was carried out according to the procedure of AOAC [13]. After every 3 days, in each dessicator, sample of the cashew kernel from the different storage were blended, 25 g of the grinded kernel was packed in a filter paper and introduced into porous thimble. In this case, n-hexane was used as the extracting solvent and this was effected for 6-7 h. At the end of the extraction, the mixture was concentrated by distilling off the solvent. The oil was desolventized and concentrated.

2.3 Isolation of Microorganisms

2.3.1 Isolation bacterial

A stock solution of each of the oil samples was made by dissolving one millilitres (1 ml) of each sample in nine millilitres (9 ml) of sterile Tween 80. Five – fold serial dilution was made from each stock solution. One millitre aliquots of the last two dilutions of each sample were inoculated into Nutrient agar in triplicates using the pour plate method. All the plates were incubated at 37°C in an incubator for 24 hours. Colonies were counted using colony counter after 24 hours and results expressed as colony forming units per millilitre [14]. Colonies formed after incubation was sub-cultured on nutrient agar to produce distinct pure cultures of bacteria and these were further studied for identification. The cultures were preserved by inoculating them on agar slant prepared from double strength nutrient agar in McCartney bottles.

2.3.2 Yeast and moulds

A stock solution of each of the samples was made by dissolving one millilitre (1 ml) of each sample in nine millilitres (9 ml) of sterile Tween 80. Five – fold serial dilution was made from each stock solution. Aliquots of the last two dilutions of each sample was inoculated on Sabouroud Dextrose Agar (SDA) and incubated at room temperature in a canister for 7 days. Colonies were counted after 7 days and results expressed as colony forming units per milliliter [14]. Spores formed after incubation were sub-cultured on nutrient agar and Potato Dextrose agar to produce distinct pure cultures of bacteria and fungi respectively and these were further studied for identification. The cultures were preserved by inoculating them on agar slant prepared from double strength nutrient agar and Potato Dextrose agar in McCartney bottles.

2.4 Identification of Microbial Isolates

Bacterial isolates were identified by morphological and biochemical tests using standard procedures. The appearance of each colony on the agar media and characteristics such as shape, edge, colour, elevation and texture were observed as described by Olutiola et al. [15]. Relevant biochemical tests like Oxidase test, Catalase test, Methyl Red test, Voges-Proskaeur test, Urease test and Sugar Fermentation test were carried out as described by [16].

Isolated fungi were characterised by macroscopic (physical appearance on agar plates) and microscopic techniques (under light microscope) including colour of aerial and substrate mycelia comparing them with those of known taxa as contained in stardard fungal compendium [17].

2.5 Qualitative Plate Assay

2.5.1 Screening of the microbial strains for lipase production on solid agar

A plate detection method containing a chromogenic substrate (Congo red) was used to screen the strains for lipase-producing ability. The medium used for screening has the following composition in (g/l): peptone 10; NaCl 5; Calcium chloride; 0.1; castor oil, 1 ml agar, 50; Congo red, 0.5; and distilled water, 1,000 ml. The sterile medium was pour-plated and allowed to solidify. The agar plates were spot inoculated with the bacterial and fungal isolates and the plates were incubated at 30°C for 24-48 hours for bacterial isolates and 3-5 days for fungal isolates. Lipolysis was indicated by the appearance of clear zone of inhibition around the spot of inoculation. The diameters of the colonies and clearance zones were measured after 24 and 48 hours for bacteria isolates and 3 and 7 days for fungal isolates [18].

2.6 Assay for Lipolytic Activity

2.6.1 Growth medium

The mineral growth medium (MGM) contained (in g/L): NaH_2PO4 12, KH_2PO_4 2, $MgSO_4 \cdot 7\ H_2O$ 0.3 and $CaCl_2$ 0.25. Ammonium sulphate at 1% (w/v) and olive oil at 2% (v/v) were used as nitrogen and carbon sources, respectively. The initial pH was adjusted to 6 for yeast and fungi and to 7 for the bacterium [19].

2.6.2 Lipase production

The culture was grown in 100-mL Erlenmeyer flasks containing 20 mL of mineral medium. The contents were sterilized by autoclaving at 121°C for 15 min. After cooling, the sterilized medium was inoculated with spores (10^7/mL) from a 7-day-old culture. The flasks were incubated at 30°C in a rotating Shaker at 100 rpm for 8 days. The mycelium was harvested by filtration under vacuum and later centrifuged at 12 000 rpm for 5 min. The clarified supernatant was used as a source of extracellular enzyme [19].

2.7 Spectrophotometric Assay Method

The transesterification reaction was performed by using para-nitro phenyl palmitate (pNPP) as the substrate [20]. Ten millilitre of isopropanol containing 30 mg pNPP (Sigma) was mixed with 90 ml 0.05 M sodium phosphate buffer (pH 8) containing 207 mg sodium deoxycholate and 100 mg gum arabic. A total volume of 2.4 ml freshly prepared substrate solution was pre-warmed at 37°C and mixed with 0.1 ml enzyme solution. After 15 min incubation at 37°C, absorbance at 410 nm was measured against the blank. One enzyme unit was defined as amount of enzyme required to release 1 µmol of p-nitrophenol from the substrate in milliliters per minute.

2.8 Optimization of Media Parameters for Enzyme Activity

2.8.1 Effect of substrate concentration on lipase activity

The Effect of substrate concentration on lipase activity was determined by varying the concentrations of palm kernel oil in the medium, ranging from 0.5 to 2.5 ml, according to the method of Hosseinpour et al. [21]. The pH of the medium was adjusted to 7 and was incubated at 30°C in a rotating Shaker at 100 rpm for 8 days [19].

2.8.2 Effect of pH on lipase activity

The optimum pH for lipase production was determined by varying the pH of the broth from 5 to 9 whereas the other parameters were unaltered. The substrate concentration was 1ml, while the temperature and incubation period was 30°C and 7 days respectively.

2.8.3 Effect of incubation periods

The optimum incubation period for the production of lipase was carried out by varying the incubation days from 3 to 11 days. The pH of the medium was adjusted to 7 and was incubated at 30°C in a rotating Shaker at 100 rpm [19].

2.8.4 Effect of temperature on lipase activity

For selection of optimum temperature for the production of lipases the temperatures varying from 25 to 45°C were selected by keeping the remaining parameters same.

3. RESULTS

3.1 Microorganisms Isolated from Palm Kernel and Cashew Oil

Three bacterial *isolates Bacillus licheniformis, Staphylococcus aureus and Psuedomonas aeroginosa,* and fungal isolates *Articosporium inflate, Aspergillus flavus, A. saprophyticus and Penicillium notatum* were isolated from palm kernel and oil sample.

3.2 Qualitative Assay of Lipolytic Microorganism Isolated

The result for qualitative assay of lipolytic microorganism isolated from Palm oil is presented in Table 1. From the study, it was observed that of all the Isolates tested for Lipase activities only the fungi show Signs of lipolytic activities.

Table 1. Qualitative assay of lipolytic microorganisms isolated from palm kernel oil

Isolates	Diameter of the clearance zone (cm)
Bacillus licheniformis	0.00±0.000
Staphylococcus aureus	0.00±0.000
Pseudomonas aeruginosa	0.00±0.000
Aspergillus flavus	0.50±0.000
Aspergillus saprophyticus	0.60±0.005
Penicillium notatum	0.40±0.000
Articulosporium inflate	0.10±0.003

Values are mean ± SD (n=3)

3.3 Optimization of Cultural Conditions for Enzymes Activity and Protein Concentration by *Aspergillus saprophyticus* Isolated From Palm Kernel Oil

Results of the optimization of cultural conditions (temperature, incubation periods, substrate concentration and pH) for lipase activity and protein concentration of *Aspergillus saprophyticus* are shown in Figs. 1, 2, 3 and 4 respectively.

Fig. 1 shows the effect of change in temperature on lipolytic activity and protein concentration by *Aspergillus saprophyticus* isolated from palm kernel oil. There was an exponential phase in lipase activity from 25°C to 35°C, after which was a decrease. The increase was rapid between 25°C to 35°C and later decreases rapidly

between 35°C to 45°C. The lipase activity was optimum at a temperature of 35°C while the protein concentration was optimum at 35°C.

The effect of change in incubation days on lipolytic activity and protein concentration of *Aspergillus saprophyticus* isolated from palm kernel oil is shown in Fig. 2. There was a gradual increase in the lipase activity and protein concentration between incubation day 3 to 5, after which was a gradual decline between day 5 to 7 and a sharp decrease between day 7 to 9 in the lipase activity while the protein concentration showed a sharp decrease in day 7 but later increases gradually between day 7 to 11. The lipolytic activity and protein concentration is shown to be optimum at an incubation period of 5 days.

The effect of change in substrate concentration on lipolytic activity and protein concentration of *Aspergillus saprophyticus* isolated from palm kernel oil is shown in Fig. 3. There was a sharp increase in the lipase activity and protein concentration at a substrate concentration of 1ml to 1.5 ml, after which a decline was observed between 1.5 ml to 2 ml and a gradual increase between 2 to 2.5 ml. The lipase activity was highest at a substrate concentration of 1.5 ml and lowest at 0.5 ml.

Fig. 4 shows the effect of pH on lipolytic activity and protein concentration of *Aspergillus saprophyticus* isolated from palm kernel oil. There was a gradual increase in the lipase activities between pH 5 to 7, after which was a rapid decrease between pH 8 to 9. The protein concentration shows a gradual increase between pH 5 to 7, after which was a gradual decrease between pH 7 to 9. The lipase activity and protein concentration was highest at a pH value of 5 and lowest at a value of 9.

4. DISCUSSION

The bacteria isolated from the oil sample were *Bacillus licheniformis, Staphylococcus aureus and Pseudomonas aerouginosa,* and fungal isolates are *Articosporium inflate, Aspergillus flavus, A. saprophyticus and Penicillium notatum.* The production of lipase by the test microorganisms showed appreciable differences in their lipolytic activity. The isolated bacteria did not show sign of lipolytic activity which is not in agreement with previous study of Escobar-Niño et al. [22]. It could be assumed that the fungal strain was able to efficiently use the palm kernel nut oil to enhance or promote the synthesis of the enzyme, while the test bacteria could not. Another possibility could involve the isolated bacterial being able to express intracellular lipase instead of extracellular way [19]. Among the strains tested, *Aspergillus saprophyticus* strain showed more lipase activity than any other followed by *Aspergillus flavus, Penicillium notatum* and *Articulosporium inflate*. Based on this, the optimization of cultural conditions for the production of lipase by *Aspergillus saprophyticus* strain was further investigated.

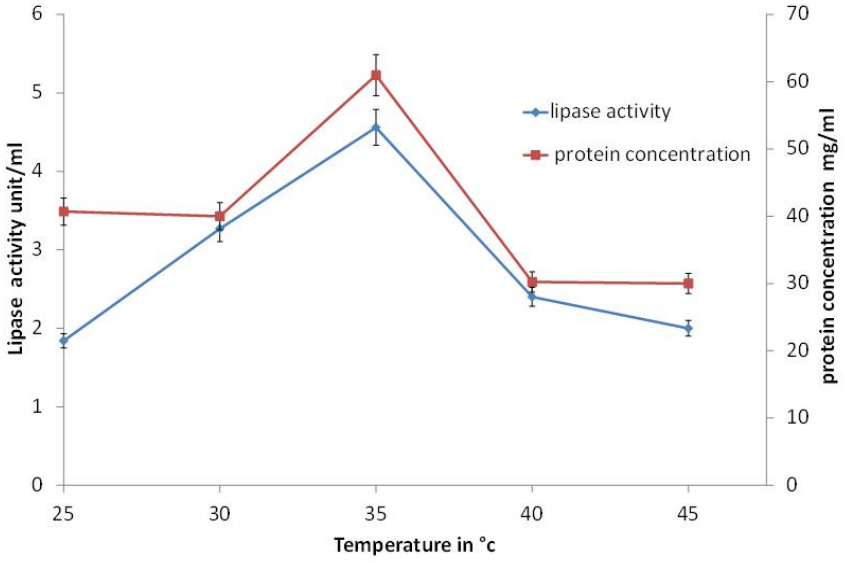

Fig. 1. Effect of temperature on lipolytic activity and protein concentration of *Aspergillus saprophyticus i*solated from palm kernel oil

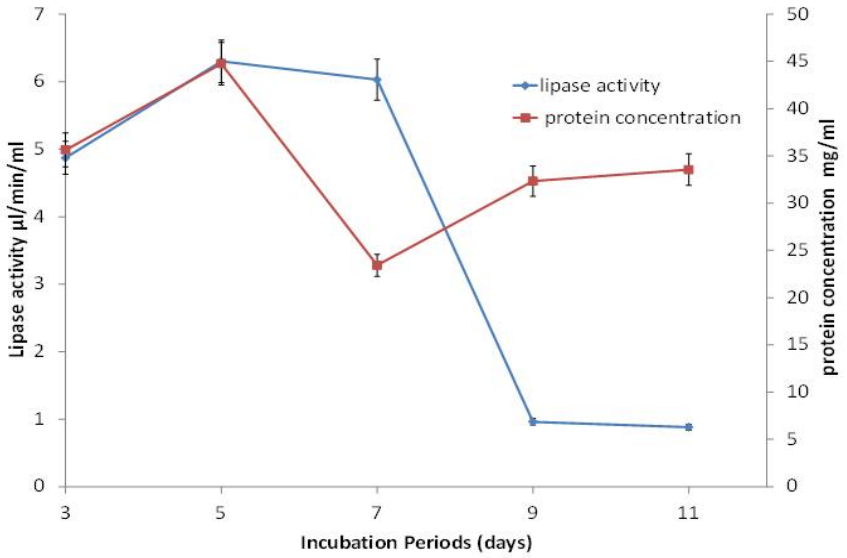

Fig. 2. Effect of incubation period on lipolytic activity and protein concentration of *Aspergillus saprophyticus* isolated from palm kernel oil

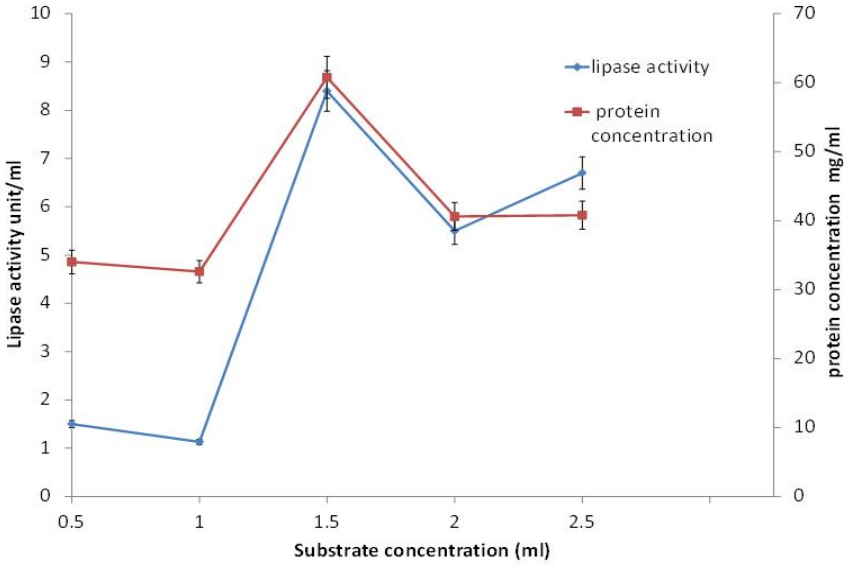

Fig. 3. Effect of substrate concentration on lipolytic activity and protein concentration of *Aspergillus saprophyticus* isolated from palm kernel oil

Considering temperature as a parameter for lipolytic activity, the highest lipolytic activity was observed at 35°C. This result is in conformity with the findings of Chander et al. [23] and Eitenmiller et al. [24] in *Penicillium roqueforti* and *Penicillium chrysogenum* lipases, respectively. Above this temperature, there was a decrease in lipolytic activity showing the enzymes to be very sensitive to high temperature, which could lead to denaturation.

Enzyme secretion by the isolates was·greatly influenced by varying pH of the environment. The maximum enzyme was produced at pH 7. This shows that the lipase activity is higher at neutral pH, similar results have been reported for other fungal lipases [25].

Incubation period had great influence on the production of extracellular lipase. The synthesis of the enzyme began around the third day of

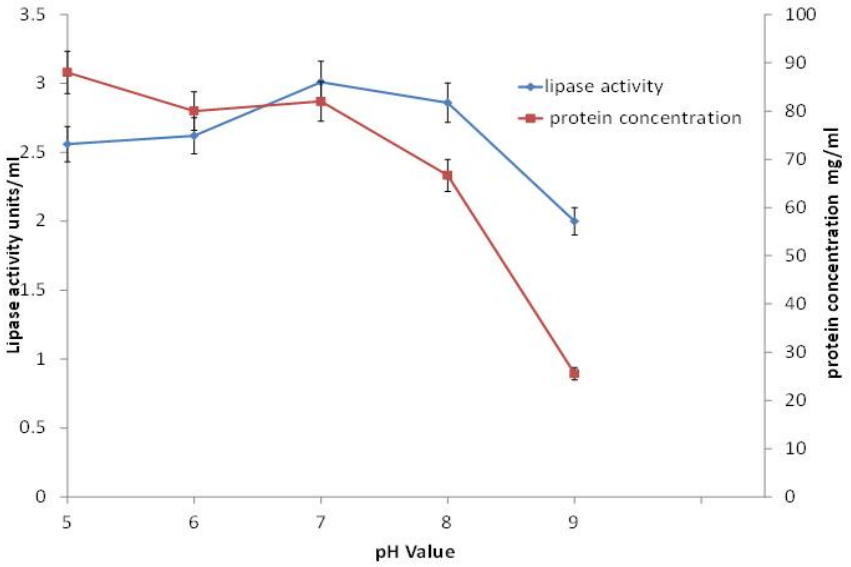

Fig. 4. Effect of pH on lipolytic activity and protein concentration of *Aspergillus saprophyticus* Isolated from palm kernel oil

fermentation and the maximal activity was recorded at the 5th day of incubation. The lipase production continued till the last day of investigation (11th day). This suggested that the production of lipase continued during the stationary phase.

5. CONCLUSION

This study showed four major lipase producing microorganism isolated from degrading palm kernel oil. Microorganism namely *Articosporium inflate, Aspergillus flavus. Aspergillus saprophyticus and Penicillium notatum*. *Aspergillus saprophyticus* showed the maximum activity and the cultural condition was optimized. The optimization of pH, temperature, substrate concentration and incubation period improved the lipase production. The optimum activity was produced at 35°C, incubation period of 5 days, pH of 7 and substrate concentration of 1.5 ml.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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