**Optimization of production parameters and partial purification of a *Klebsiella pneumoniae* lipase isolated from freshly produced Palm Oil**

**Abstract**

 Lipases are important hydrolytic enzymes with numerous industrial applications and economic impact. Lipase producing microorganisms were isolated from freshly produced palm oil produced in Akure, Nigeria. The microbes were assessed for lipase production potential using submerged fermentation. The microorganism with the best production potential was used for optimization studies. The microorganism was identified as *Klebsiella pneumoniae*. Optimal lipase production was obtained at 48 hours incubation period with fructose as best carbon source and yeast extract as best nitrogen source at pH 9. *Klebsiella pneumoniae* lipase production was optimal at 40 °C. High lipase activity in fresh palm oil increases the production of free fatty acids. Findings from this study indicates that the *Klebsiella pneumoniae* lipase could be of biotechnological significance in various industries.

**Keywords**: lipase, fermentation, *Klebsiella pneumonia*, palm oil, activity

**1.0 Introduction**

 “The Oil palm tree is one of the most important oil crops in the world together with soybean, with more than 30 million tons of oil produced in 2004” (Ngando et al., 2006). “It is projected that the oil palm share of the market will need to increase to meet the doubled demand for vegetable oil predicted by 2030” (Murphy, 2009). The oil produced can represent up to 80 % of the dry weight of the mesocarp. “Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are a class of enzymes that catalyze the hydrolysis of long chain triglycerides to intermediate and short chain di and mono glycerides, free fatty acids and glycerol” (Alhebshi et al., 2023). “Apart from hydrolysis, lipases are also involved in a wide range of conversion reactions that include esterification, interesterification, transesterification, alcoholysis, acidolysis and aminolysis in non-aqueous media” (Savitha et al., 2007). “This enzymatic activity of microorganisms, particularly lipase secretion, facilitates the breakdown of fat-containing compounds into manageable by-products, which serve as a substrate for microbial growth” (Sutar et al., 2024).

 “Lipases possess a characteristic folding pattern of α/β-hydrolase with mostly parallel β-sheets, flanked on both sides by α-helixes in the structure. The active sites are characterized by formation of a catalytic triad (serine, aspartic/glutamic acid, and histidine), which is highly conserved” (Verma et al., 2020). “Lipase mediated reactions are reversible, hence, the enzyme can catalyze the esterification of glycerol from mono, di and triglycerides. This versatility makes lipases the enzyme of choice for potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic and paper industries” (Demirkan et al., 2021; Houde et al., 2004). “Mesocarp lipase (EC 3.1.1.3) is one of the lipid-related enzymes of palm oil which has been the most studied because of its strong negative impact on oil quality” (Ngando et al., 2006; Sambanthamurthi et al., 2000).

 “The non-solubility of the assay substrate and the strong dependence of the activity on the physico-chemical state of the substrate, makes lipase assay difficult, especially in palm oil which is complicated by the non-stability of the enzyme” (Beisson et al., 2000; Bharathi & Rajalakshmi, 2019).

 “In the food industry, lipases play a vital role during the fermentative steps of sausage manufacturing and to determine changes in long-chain fatty acids liberated during ripening. Lipases remain enzymatically active in organic solvents (Klibanov, 2001; Verma et al., 2008) which enhances their potential and flexibility as biocatalysts against a wide range of unnatural substrates” (Ghori et al., 2010).

 “The microflora of processed palm oil product is influenced greatly by the environment from where the product was originally obtained, the microbial quality of the product in its raw or unprocessed state and the sanitary conditions under which the product was handled and processed” (Sundram et al., 2003).

 Palm oil quality is usually assessed on the strength of its acidity (indicator of FFA content) and impurities (De Almeida et al., 2013; Tagoe et al., 2012). However, acidity values as a result of lipase activity are a reflection of oil quality impairment with its attendant effect in the taste and odour of the palm oil. Without refining, such oil may be unsuitable for human consumption (Constant et al., 2017).

 “One of the main criteria for assessing the quality of palm oil is the level of free fatty acids. The free fatty acids present in palm oil have been attributed to the enzymatic hydrolysis of the oil under the influence of an endogenous lipase in the fruits” (Abigor et al., 1985). High lipase activity increases the free fatty acid content of fresh palm oil necessitating post-harvest inactivation by heat treatment of fruit bunches. Hence the need to study the lipase activity of microorganisms isolated from palm oil in an attempt to understand the hydrolysis of palm oil by lipase.

 This study aims to assess the lipase activity of microorganisms isolated from palm oil produced in Akure, Ondo State.

**2.0 Materials and methods**

**2.1 Sample collection**

 Fresh palm oil samples were collected in sterile sample bottles from oil palm processing mills in Akure, Ondo state, Nigeria.

**2.2 Isolation**

 Isolation of microorganisms present in the palm oil samples were carried out by serial dilution. Bacteria were isolated on nutrient agar plates, and identified based on biochemical characteristics and molecular method by 16S rDNA gene sequencing as described by (Furini et al., 2018a; Veerapagu et al., 2013).

**2.3 Screening for lipolytic microorganisms**

 Screening was carried out using tributyrin agar (Oxoid); a differential medium. . Tributyrin agar was prepared by melting the content of the sealed bottle in a boiling water-bath at 100°C (the caps were partially unscrewed) until completely dissolved. It was then allowed to cool to 45-50 °C, and mixed well to avoid the formation of bubbles. It was then aseptically dispensed into Petri dishes and allowed to solidify. The plates were then inoculated by streaking the isolates onto the surface of the medium and incubated at 30+/-1°C for three days. Tributyrin oil forms an opaque suspension in the agar. Production of clear halo zones around the organism is evidence of lipolytic activity (Asih et al., 2014).

**2.4 Production of lipase by the isolate**

 Culture medium containing the basal medium Na2HPO4 (0.9 g/L), NaCl (0.2 g/L), MgSO4.7H2O (0.2 g/L), NaNO3 (1.0 g/L), CaCl2 (0.002 g/L), MnSO4 (0.002 g/L), FeSO4.7H2O (0.002 g/L) and Olive oil (1% w/v) in a 1000 mL conical flask was prepared. The medium was autoclaved at 121oC, 1 atm for 15 minutes. The culture was inoculated with 6.0 McFarland and incubated on a shaker incubator at 28 oC for 48 hours at 150 g. The samples collected were centrifuged at 10,000 g at 4 oC for 15 minutes. Cell growth was determined by measuring the absorbance at 600 nm, uninoculated media was used as blank. The supernatant collected after centrifugation was preserved at 4 oC for lipase assay

**2.5 Lipase assay**

Lipolytic activity was determined spectrophotometrically based on the cleavage of *p*-nitrophenyl palmitate (*p*-NPP) at pH 8.0 (Denise et al., 2015). The reaction mixture contained 180 µL of solution A (0:062 g of *p*-NPP in 10 mL of 2-isopropanol, and sonicated for 6 min before use), 1620 µL of solution B (0.4% triton X-100 and 0.1% gum arabic in 50 mM Tris-HCl, pH 8.0) and 200 µL of properly diluted enzyme sample. The product was detected at 410 nm wavelength against a blank (without enzyme) after incubation for 15 minutes at 37 °C. Under this condition, the molar extinction coefficient of *p*-nitrophenol (*p* -NPP) released from *p*-NPP was 15000 M*−*1 (Furini et al., 2018b). The standard graph was prepared by using para-nitrophenol (0.4 to 4 μmoles). A unit of lipase activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol (*p*NPP) per millilitre of crude enzyme per minute. The best lipase producer was used for further processing.

**2.6 Preparation of the Standard Curve**

 Standard graph for 4-nitrophenol was prepared for lipase assay. A series of samples containing 10 to 50 nanomole/ml of 4-nitrophenol were prepared in 50 mM Tris-HCl pH 7.2. Absorbance was measured at 400 nm. The standard graph was plotted between absorbance and the amount of 4-nitrophenol. The lipase activity against 4–nitrophenyl palmitate was determined by measuring the amount of 4-nitrophenol released by lipase – catalysed hydrolysis. The production of 4-nitrophenol was measured at 400 nm in a spectrophotometer and the released 4-nitrophenol concentration was calculated from the standard.

**2.7 Optimization of lipase activity**

Optimization of the various cultural parameters on enzyme production was carried out using the one-factor-at-a-time (OFAT) approach. In the OFAT approach, one variable is being optimized while all other variables are kept constant. Thereafter, the optimal conditions were pooled together for further production.

**2.8 Effect of incubation period on lipase activity**

 Isolates were inoculated into production medium broth containing Na2HPO4 (0.9 g/L), NaCl (0.2 g/L), MgSO4.7H2O (0.2 g/L), NaNO3 (1.0 g/L), CaCl2 (0.002 g/L), MnSO4 (0.002 g/L), FeSO4.7H2O (0.002 g/L) and Olive oil (1% w/v) in a 1000 mL Erlenmeyer flask. 2g Glucose and 10 ml olive oil at 37 °C in an orbital shaker at agitation speed of 150 rpm. The culture broth was harvested on a 24 hourly basis for 120 hours. Enzyme assay was carried out using standard assay procedure as described above (Ilesanmi et al., 2020; Wakil & Osesusi, 2017).

**2.9 Effect of different carbon sources on lipase activity**

 Glucose present as carbon source in the production medium described above was replaced with different carbon sources like sucrose, fructose, starch, maltose and lactose at 1% (w/v) final concentration by keeping the other parameters constant. Enzyme assay was carried out using standard assay procedure.

**2.10 Effect of different nitrogen sources on lipase activity**

 Different nitrogen sources (peptone, yeast extract, KNO3, NaNO3 and NH4Cl) were added to the production medium broth described earlier at a final concentration of 1% (w/v) by keeping the other parameters constant. Enzyme assay was carried out using standard assay procedure.

**2.11 Effect of pH on lipase activity**

 The optimum pH for the production of lipase was determined by varying the pH of the production medium broth, ranging from pH 4 to pH 8 while the other parameters are unaltered. Enzyme assay was carried out using standard assay procedure.

**2.12 Effect of agitation on lipase activity**

 In the determination of the optimal agitation speeds for peak enzyme activity, the isolates were cultured in an orbital shaker at varying agitation speed from 120 to 250rpm. Enzyme assay was carried out using standard assay procedure as earlier described.

**2.13 Effect of incubation temperatures on lipase activity**

 The optimum temperature for the production of lipase was determined by incubating the culture production medium broth at varying temperatures (30 ⁰C to 50 ⁰C) while keeping the other parameters constant. Enzyme assay was carried out using standard assay procedure as earlier described above.

**2.14 Partial purification of lipase enzyme**

 Cell-free medium was obtained by centrifugation at 5000 rpm for 30 min. The supernatant was collected and the lipase was concentrated using addition of 10–100% ammonium sulphate. Ammonium sulphate with various concentrations was gradually added, along with slowly stirring using magnetic stirrer for 30 minutes, until all the ammonium sulphate was dissolved, the mixtures were then kept at 4 ℃ for 18 hours overnight to allow for total precipitation of the enzymes. It was then centrifuged at 10,000 rpm for 10 minutes to remove the remaining salt from the protein. The precipitates obtained were dissolved in citrate-phosphate buffer. The precipitates were then pooled together for each enzyme by calculating enzyme activities and protein content.

The pooled protein precipitates from ammonium sulphate precipitation above were each dialyzed using citrate phosphate buffer solution (pH 7.5) to remove (NH4)2SO4 and other unwanted salts present (Wingfield, 2001).The precipitates were dispensed into dialysis tube (Dialysis tubing cellulose membrane, avg. flat width 33 mm). The dialysis tube was cut and tied into 20 to 25-cm sections, brought to boiling in distilled water, and cooled. They were well tightened at both ends to create turgor pressure, upon loading of the dialysate in the tube and submerged in citrate phosphate buffer pH 7.5 for 48 hours in refrigerated condition. The buffer in the medium was stirred and the buffer solution was changed at 2 hours interval (Ding et al., 2012).The buffer solutions were changed until clear solution was obtained.

**2.15 Estimation of protein content by Lowry’s method**

 The total protein content was determined using Lowry’s method (1951). The reagents used were prepared as solution A: 2 % Na2Co3 in 0.1 N NaOH; solution B: 1 % Sodium Potassium Tartarate in H2O; solution C: 0.5 % CuSO4.5H2O in H2O; solution D: Reagent I: 48 mL of A, 1 mL of B, 1 mL of C, solution E: Reagent II: 1part Folin-Phenol [2 N] and 1 part water and solution F: Bovine Serum Albumin (BSA) as standard (1 mg/mL). A 0.2 mL of bovine serum albumin (BSA) (standard) was pipetted in a series of test tubes and all the tubes were made up to 1 mL using distilled water. Blank containing 1 mL distilled water was also taken. A 0.5 mL of test solution in a test tube was taken and this was also made up to 1 mL with distilled water. 5 mL of alkaline copper reagent (Reagent I) was added to all the test tubes and kept for 10 minutes at room temperature. Then 0.5 mL of Folins Ciocalteau reagent (reagent II) was added to all the tubes, well mixed and incubated at 37 ℃ for 30 minutes. The blue colour developed was read at 660 nm. A standard graph was plotted with concentration of BSA on X-axis and optical activity on Y-axis.

**3.0 Results and Discussion**

 From the palm oil samples, five microorganisms were isolated based on the qualitative screening results on a selective agar. Quantitative lipase assay showed that isolate F had an activity of 12.10 U/ml, and was used for further studies. The isolate was characterized using morphological, biochemical and molecular characteristics as *Klebsiella pneumoniae* with accession number NC\_016845.1 and 99.3 % when submitted to the NCBI database upon blasting. Reports have shown *Klebsiella pneumoniae* as a notable lipase producer (Odeyemi et al., 2013). Emmanuel et al. (2020) also reported highest lipase activity from *K. pneumoniae* isolated in Falke Vegetable Oil Factory, Kaduna.

**Table 1. Biochemical characterization of bacterial isolated from palm oil**.

|  |  |
| --- | --- |
| ParametersMotility test | observation+ |
| Indole production test | + |
| MR test | + |
| VP test | + |
| Citrate utilization test | − |
| Carbohydrate fermentation test | + |
| TSI test | − |
| Oxidase test | + |
| Urease test | − |
| Catalase test | + |
| Nitrate reduction test | + |
| Hydrogen sulphide test | + |

Keys: MR = Methyl Red; VP = Voges-Proskauer; TSI = Triple Sugar Iron. +”sign = positive and - ”sign = negative

Optimization for incubation period showed the highest lipase activity for *Klebsiella pneumoniae* was observed at 48 hours (18.66 U/ml) (Figure 2). This slightly differs from studies by (Emmanuel et al., 2020) who reported highest lipase activity in *Klebsiella pneumoniae* at 36 hours.

The effect of various carbon sources on lipase production by *Klebsiella pneumoniae* is shown in Figure 3. It was observed that fructose had the highest lipase activity (18.83U/ml). (Madigan et al., 2015) also highlighted the importance of carbon source in the facilitation of protein transport. (Alabras et al., 2017) reported that the existence of oil in the bacterial medium stimulates the bacteria to produce lipase to utilize the oil as a nutrient source.

In the optimization of various nitrogen sources for lipase production, yeast extract yielded the highest lipase growth (15.91U/ml) (Figure 4). This is similar to reports by (Jaiswal et al., 2017; Furini et al., 2018) who reported “yeast extract as the best nitrogen source for lipase production”. Costa *et al*. (2002) opined that “yeast extract provided essential amino acids, peptides, vitamins and carbohydrate which are necessary for rapid growth of microorganisms”. Also, Sharma et al. (2002) highlighted that “peptone and yeast extract are the best nitrogen sources for microbial lipases when compared to other sources such as meat extract, tryptone or wheat bran”.

The findings from this study showed that pH 9 yielded optimal lipase activity (21.76 U/ml) by *Klebsiella pneumoniae* (Figure 5). This was followed by a gradual decrease in lipase activity. This is in agreement with reports by (Dahiya & Purkayastha, 2011; Dhiman & Chapadgaonkar, 2013) who reported optimal pH for *Bacillus* spp at pH 9. This is contrary to findings by (Olanrewaju & Adetutu, 2016) who reported decreasing lipase activity at pH 7 - 9. (Dheeman et al., 2011) suggested that lipase activity was influenced by the pH of the medium, owing to molecular interactions and functions being directly related to pH of the fermentation medium, thus a slight shift in the pH of fermentation media influences biological activity (Demirkan et al., 2021).

The effect of agitation on lipase activity varied with the agitation speed of the shaker. However, results obtained showed that optimal lipase activity was observed at 120 rpm (Figure 6). This differs from reports by (Wakil & Osesusi, 2017) who reported optimal lipase activity for *Klebsiella* spp at a speed of 150 rpm. “The effect of agitation on lipase production could be attributed to varying oxygen transfer rate to the organism, increased surface area of contact with the media components and better dispersal of the oil substrate during fermentation under agitated condition” (Saxena et al., 2011).

Lipase production was optimal at a temperature of 40 °C (12.66U/ml) (Figure 7). This is similar to the reports by (Dahiya & Purkayastha, 2011; Nwachukwu et al., 2017) who reported optimal lipase production at 40 ℃ during optimization studies. (Guzmán et al., 2008; Senthilkumar et al., 2012) also reported optimum temperature for lipase activity between 35-45 ⁰C. The reports also differs to the results obtained by (Nagar et al., 2012; Sirisha et al., 2010) who reported maximum lipase activity at 35⁰C. (Salihu & Alam, 2014) attributed “the effect of temperature on lipase activity to non-covalent interactions resulting in the formation of salt-bridge which contributes to stabilization of the enzymes at elevated temperatures, by restricting the flexibility of the lipase enzyme active site”.

The cell-free supernatant was used as a crude enzyme for precipitation and dialysis process. The protein was precipitated using ammonium sulphate fractions from 20 to 80% (w/v). 40–60% (w/v) of ammonium sulphate fractions showed higher lipase precipitation activity than the rest of the fractions. No detectable activity was observed 80% (w/v) saturation and above. Precipitated enzyme was purified by dialysis method with the help of dialysis membrane. Protein content was 1.67 mg/ml.

**Conclusion**

From the study, it can be concluded that freshly produced palm oil can contain microorganisms of economic significance. The lipase enzyme production was optimized with different physiological conditions for high yield production, and the assay method was stable. Fructose and yeast extract could be a better source for maximum lipase production. The presence of microorganisms with this hydrolytic activity requires more attention to preserved the shelf life of artisanal produced palm oil. Hence, the lipase is of biotechnological significance, with potential for use in the formulation of detergents.

**Recommendation**

Further research is recommended on the methods to prevent enzymatic hydrolysis of palm oil by spontaneous microganisms, in an attempt to preserve the shelf life of the product. Also, this hydrolytic potential should be further assessed towards more biotechnological applications.

**Disclaimer (Artificial intelligence)**

We the author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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