**Optimization of production parameters of a *Klebsiella Pneumoniae* lipase isolated from fresh Palm Oil produced In Akure, Nigeria.**

**Abstract**

 Lipases are important hydrolytic enzymes with numerous industrial applications and economic impact. Lipase producing microorganisms were isolated from fresh palm oil produced in Akure, Nigeria. The microbes were assayed 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 was obtained at 48 hours incubation period with fructose and nitrogen as best carbon and 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.

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

**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 Ebongue *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 (Prazeres, Cruz and Pastore, 2006). 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 (Davranov, 1994; Savitha *et al.*, 2007).

 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 (Houde, Kademi and Leblanc, 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 (Sambanthamurthi, Sundram and Tan, 2000; Ngando Ebongue *et al.*, 2006).

 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).

 In the food industry, lipases play a vital role during the fermentative steps of sausage manufacture and to determine changes in long-chain fatty acids liberated during ripening. Lipases remain enzymatically active in organic solvents (Klibanov, 2001; Verma, M.L., Azmi, W. and Kanwar, 2008) which enhances their potential and flexibility as biocatalysts against a wide range of unnatural substrates (Ghori, M.I. Iqbal, M.J. and Hameed, 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, Sambanthamurthi and Tan, 2003).

 Palm oil quality is usually assessed on the strength of its acidity (indicator of FFA content) and impurities (Tagoe, Dickinson and Apetorgbor, 2012; De Almeida *et al.*, 2013). However, high 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.

**Materials and methods**

**Sample collection**

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

**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 (Veerapagu *et al.*, 2013; Furini *et al.*, 2018).

**Screening for lipolytic microorganisms**

 Screening was carried out using tributyrin agar (Oxoid); a differential medium. 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., Salihu, 2014).

**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

**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). 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.

**Optimization of lipase activity**

**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 (Wakil and Osesusi, 2017).

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

 Glucose present as carbon source in the production medium was replaced with different carbon sources like sucrose, 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.

**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 at a final concentration of 1% (w/v) by keeping the other parameters constant. Enzyme assay was carried out using standard assay procedure.

**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.

**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 described.

**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 described above.

**Partial purification of lipase enzyme**

 Cell-free medium was obtained by centrifugation at 5000 rpm for 30 min. The supernatant was collected and enzyme was concentrated using addition of 10–100% ammonium sulphate. Fractionated enzyme samples were then subjected to dialysis process for partial purification with the help of dialysis membrane.

**2.7. Estimation of protein content by Lowry’s method**

 Quantitative estimation of the protein content was done by (Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, 1951) method*.*

**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 ascension 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 and Aderiye, 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, Martinko and Parker, 2015) also highlighted the importance of carbon source in the facilitation of protein transport. (Alabras, R.; Kamal, A. and Ayman, 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 pneumonia* (Figure 5). This was followed by a gradual decrease in lipase activity. This is in agreement with reports by (Dahiya and Purkayastha, 2011; Dhiman and Chapadgaonkar, 2013) who reported optimal pH for *Bacillus* spp at pH 9. This is contrary to findings by (Olanrewaju and Adetutu, 2016) who reported decreasing lipase activity at pH 7 - 9. (Dheeman, Henehan and Frías, 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.

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 and 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 (Lawrence, R. C., Fryer, T. F. and Reiter, 1967; 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 and Purkayastha, 2011; Nwachukwu *et al.*, 2017) who reported optimal lipase production at 40 ℃ during optimization studies. (Guzmán, M.N., Vargas, V.A., Antezana, H. and Svoboda, 2008; Senthilkumar, P.K., Uma, C. and Saranraj, 2012) also reported optimum temperature for lipase activity between 35-45 ⁰C. The reports also differs to the results obtained by (Sirisha, Rajasekar and Narasu, 2010; Nagar, Mittal and Gupta, 2012) who reported maximum lipase activity at 35⁰C. (Salihu and 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. Partial purification of the enzyme was done using 40–60% ammonium sulphate precipitation method.

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