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**Article** *in* International Journal of Biochemistry and Biophysics · August 2020

DOI: 10.13189/ijbb.2020.080202



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# Production, Partial Purification and Characterization of Lipase Enzyme Expressed by *Klebsiella pnemoniae* of Vegetable Oil Contaminated Soil

**Moses Bali Emmanuel[1,2,*], Egwim Chidi Evans[2], Abdulkadir Abubakar[2], Liman Mubarak Labaran[3], Alex Victoria Ali[1], Manya Zabe[1]**

[1]Department of General Studies, School of Agricultural Technology, Nuhu Bamalli Polytechnic, Nigeria
[2]Department of Biochemistry, School of Life Sciences, Federal University of Technology, Nigeria
[3]Department of Science Laboratory Technology, School of Applied Sciences, Nuhu Bamalli Polytechnic, Nigeria

*Received May 14, 2020; Revised August 13, 2020; Accepted August 25, 2020*

***Cite This Paper in the following Citation Styles***

***(a):*** *[1] Moses Bali Emmanuel, Egwim Chidi Evans, Abdulkadir Abubaka, Liman Mubarak Labaran, Alex Victoria Ali, Manya Zabe , "Production, Partial Purification and Characterization of Lipase Enzyme Expressed by Klebsiella pnemoniae of Vegetable Oil Contaminated Soil," International Journal of Biochemistry and Biophysics, Vol. 8, No. 2, pp. 30 - 39, 2020. DOI: 10.13189/ijbb.2020.080202.*

***(b):*** *Moses Bali Emmanuel, Egwim Chidi Evans, Abdulkadir Abubaka, Liman Mubarak Labaran, Alex Victoria Ali, Manya Zabe (2020). Production, Partial Purification and Characterization of Lipase Enzyme Expressed by Klebsiella pnemoniae of Vegetable Oil Contaminated Soil. International Journal of Biochemistry and Biophysics, 8(2), 30 - 39. DOI: 10.13189/ijbb.2020.080202.*



**Abstract** Lipases are a versatile class of enzymes owing to their ability to perform a specific range of biotransformations. Bacteria for lipase production were cultured on nutrient agar (NA) plates and identified using Gram Stain and biochemical tests. Screening for lipase producers was performed on NA media supplemented with 3% olive oil at 37 °C. Seven bacteria were isolated and identified as *Bacillus megaterium, Klebsiella pneumoniae, Escherichia coli, Bacillus subtilis, Bacillus licheniformis and Micrococcus luteus.* Lipase production was carried using standard methods and the best lipase producer was purified and characterized. The lipase enzyme from *K. pneumoniae* had a yield of 18.8% and was purified 26.3 fold. The optimum pH for the partially purified lipase was determined to be 8 with maximum activity at 30 °C. The lipase enzyme had affinity for substrates in the following order, olive oil > groundnut oil > palm oil and the activity of the enzyme was enhanced by metal ions in the following order, $MgCl_2$ > $CaCl_2$ > KCl whereas inhibitory effects were observed in the following order, $CoCl_2$ > $HgCl_2$ > $CuSO_4$ > $FeCl_3$. The lipase enzyme had $V_{max}$ of 0.0006 U/Sec, $K_m$ of 0.4960 mM and $K_{cat}$ of 0.0125 $S^{-1}$.

**Keywords** *Klebsiella pneumoniae*, Lipase, Production, Purification, Biochemical Properties

## 1. Introduction

Lipases, classified as triacylglycerol acylhydrolase (EC 3.1. 1.3) belong to the family of hydrolases which act on carboxylic ester bonds. Their physiological role is to hydrolyze triglycerides to diglycerides monoglycerides, fatty acids and glycerol [1]. They constitute one of the most important group of biocatalyst for biotechnological applications [2]. Lipase-catalyzed processes generally offer cost-effectiveness as against the traditional downstream processing having the problems of energy consumption and toxic by-products [1].

Microbial enzymes are considered more useful than enzymes of plant and animal derivatives due to their stability, selectivity, and broad substrate specificity [3,4], versatility of molecular structure, the great variety of catalytic activities they possess, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations, rapid growth of microorganisms

on an inexpensive media and low waste production [5]. They are also safer and have the possibility of use in continuous operation, ease of recovery and reuse, cost benefit and low required downstream processing [6]. Also, lipolytic enzymes secreted into culture medium by the various fungi and bacteria have attracted substantial attention as a result of their biotechnological potentials [7].

Microbial lipases are regarded as biotechnological machineries and this has increased the search for novel lipases and variants from natural sources [8]. Bacterial lipases are present in the strains of *Pseudomonas aeruginosa, Serratia marescens* [9] and *Bacillus* species [10]. Other genera of lipase producers which have been studied include; *Acinetobacter* [11], *Staphylococcus, Streptococcus, Burkholderia, Achromobacter, Arthrobacter, Alcaligenes, Chromobacterium* and *Streptomyces* [12,13].

Exploration of biodiversity is the emerging trend in present day biotechnology [14] yet not so much has been done in exploring microbial lipases from *K. pneumoniae*. The present study therefore sought to isolate lipase producing organisms from vegetable oil contaminated soils, partially purify and characterize the best lipase producer.

# 2. Materials and Methods

### 2.1. Sample Collection

Soil samples were collected from vegetable oil contaminated soil of Falke Factory, Kaduna State and Grand Cereal Factory, Plateau State. Five random spots of about 1m apart to a depth of 4 – 6cm were fetched with the help of a sterile spatula, bulked together and placed in sterile plastic bags. All the soil samples from Falke Factory, Kaduna were labelled as F while samples from Grand Cereal Factory, Plateau State were labelled as G. The soil samples were placed immediately in an ice packed flask and brought to the laboratory within 24 hr. The samples were sealed in sterile containers and stored in the refrigerator at 4 $^{o}$C until screening.

### 2.2. Isolation and Identification of Lipolytic Bacterial Species

Isolation of lipolytic microorganisms was carried out according to the method described by [15] with slight modification. Soil samples were serially diluted up to $10^6$ times in sterile distilled water, after which 1 ml inoculums of $10^6$ dilutions were placed on nutrient agar (NA) by spread plate method to quantify the total microbial population. The bacterial inoculated plates were incubated at 37 °C for 24 hr and colonies which appeared were immediately brought in subculture. The bacterial species were identified using grams stain and biochemical test as described by [16]. A pure culture of each isolate was maintained on NA slant at 4 °C until enzyme production.

### 2.3. Lipolytic Efficiency of Bacterial Species from Different Oil Contaminated Soil

All the strains were separately inoculated on NA media supplemented separately with 1 % (v/v) olive oil. The pH of the media in all cases were maintained at 7.2 and incubation carried out at 37 °C for up to 48 hr. Any clear zone produced surrounding bacterial colony indicated production of extracellular lipase. The lipolytic efficiency (LE) of each isolate was calculated by using the following formula [17]:

$$LE\ (\%) = \frac{Z - A}{Z} \times 100$$

Where,
Z: Zone of hydrolysis including colony diameter
A: Diameter of colony growth.

### 2.4. Lipase Production from Selected Isolates of Different Vegetable Oil Contaminated Soil

Microbial isolates for lipase production were finally selected based on their lipolytic efficiency. The composition of the basal medium used in this study was as follows (g/L): $MgSO_4$. $7H_2O$ (0.1), $KH_2PO_4$ (1.0), $K_2HPO_4$ (3.0), $Na_2SO_4$ (2.0), yeast extract (5.0), peptone (5.0), glucose (2.0) and olive oil (3% v/v). The initial pH of the medium was adjusted to pH 7.0. Lipase productions were carried out in 50 mL production media with initial inoculation of 0.5 % (v/v) inside 250 mL Erlenmeyer flasks under shaking speed of 150 rpm at 37 °C for successively 4 days. Total protein as well as lipase activity were assayed at 12 hr interval.

### 2.5. Enzyme Assay

Lipolytic activity was determined according to the method of [18] with slight modification following the hydrolysis of Tween 80 at 25$^{o}$C and measured spectrophotometrically at 450 nm. 200 µL of the enzyme was added to 800 µL of the substrate solution containing 1% (v/v) Tween 80 in 20 mM Tris-HCl (pH 8.5) and 80 mM $CaCl_2$. The hydrolysis rate of Tween 80 was directly monitored by measuring the change in absorbance over 5 min. Lipase with the best lipolytic efficiency and least time of incubation was further selected for purification.

### 2.6. Total Protein Determination

This was determined by using the method of [19]. Briefly, to 5 mL of Bradford reagent, 1 mL of the sample was added after which the reaction mixture was allowed to stand for 5 min at room temperature and absorbance was read spectrophotometrically at 590 nm. The amount of protein was determined by extrapolating from the egg

albumin (EA) standard curve (it was constructed by using a solution containing 1 mg/mL EA).

### 2.7. Enzyme Purification

The lipase enzyme culture from *Klebsiella pneumoniae* was centrifuged at 4000 rpm for 40 min at 10 °C to remove cells after which ammonium sulphate was added to 30 mL of the culture supernatant which was stirred constantly in order to bring saturation to 40 %. The mixture was left overnight at 4 ℃ and the precipitate removed by centrifugation at 4000 rpm for 1 hr. Five millilitre of the concentrated enzyme of ammonium sulphate fraction was loaded onto Sephadex G-75 column already equilibrated with 10 mM phosphate buffer (pH 7.0). The enzyme was eluted from the column at a flow rate of 0.5 mL/min using phosphate buffer (pH 7.0). Enzyme fractions (3 mL each) were collected and protein content measured as described in 2.6 above while lipase assay was performed as in 2.5 above.

### 2.8. Physicochemical and Biochemical Characterization of Purified Lipases from *Klebsiella pneumoniae* of Vegetable Oil Contaminated Soil

#### 2.8.1. Effect of pH

To determine the optimum pH of the enzyme, 200 µL of the enzyme was incubated with 1M Tris-HCl buffer at pH range 4 - 10 and assayed for lipase activity as in 2.5 above. A graph of lipase activity versus pH was plotted in order to determine the optimum pH.

#### 2.8.2. Effect of Temperature

To study the temperature response of the enzyme, Lipase assay was carried out as described in 2.5 above and incubated at different temperatures, from 10 to 80 ℃ for 30 minutes. A graph of lipase activity versus temperature was plotted in order to determine the optimum temperature.

#### 2.8.3. Effect of Different Substrates on Lipase Activity

The effect of different substrates was studied by substituting 1% Tween 80 with different oils (1%): olive oil, palm oil and groundnut oil and assayed as in 2.5 above.

#### 2.8.4. Effect of Different Metal Ions on Lipase Activity

Lipase activity was also determined in the presence of metal ions by incubating 200 µL of the enzyme separately in different test tubes with 0.2 µL of 1mM solution of each salt, i.e., $FeCl_3$, $CoCl_2$, $HgCl_2$, $MgCl_2$, $CuSO_4$, $CaCl_2$ and KCl at 25 °C for 1 hr and assayed for lipase activity as in 2.5 above. Comparative lipase activities were determined for each metal ion from the activity of the control sample which had no metal ion.

#### 2.8.5. Determination of Kinetic Parameters ($K_m$ and $V_{max}$)

The substrate concentration was varied over the range of 0.02 – 1.80 v/v of Tween 80. The activity of lipase for each was carried out in the standard enzyme assay in 2.5 above. The kinetic constants $K_m$ and $V_{max}$ for lipase were determined from the Lineweaver – Burk (double reciprocal) plot and $k_{cat}$ was calculated as well. The energy of activation (Ea) and half-life ($t_{1/2}$) of the purified enzyme were also determined by using the Arrhenius plot.

### 2.9. Statistical Analysis

Experiments were performed in triplicates and the means of results determined were calculated using Statistical Package for Social Science (SPSS) version 23.

## 3. Results and Discussion

In this study, *Bacillus megaterium, Escherichia coli, Bacillus subtilis, micrococcus luteus, Bacillus licheniformis and Klebsiella pneumoniae* were the bacterial species present in the different oil contaminated soil (Table 1). This however, shows the biodiversity of the different oil contaminated soil. Sharada and Ramakrishna [5] have reported microbial biodiversity from oil contaminated soil. The bacterial species isolated may be potential lipase producers with novel and specific properties along with lower processing and production cost.

**Table 1.** Lipolytic bacterial species isolated from different vegetable oil contaminated soil

| Bacteria Isolate | Gram Stain | Catalase Test | Coagulaase Test | Starch Hydrolysis Test | $H_2S$ Production Test | Citrate Utilization Test | Indole Production Test | Methyl Red Test | Voges Proskauer Test | Urease Production Test | Blood Haemolysis Test | Oxidase Production Test | Carbohydrate Utilization Tests | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | Lactose | Sucrose | Glucose |
| *Bacillus megaterium* | +R | + | - | + | - | - | - | - | - | - | - | - | - | + | + |
| *Escherichia coli* | -R | - | - | - | - | + | + | + | - | - | - | - | + | + | + |
| *Bacillus subtilis* | +R | + | - | + | - | + | - | - | + | - | α | - | - | + | + |
| *Micrococcus luteus* | +C | + | - | - | - | - | - | - | - | - | - | - | - | - | + |
| *Bacillus licheniformis* | +R | + | - | + | - | - | - | - | - | - | - | - | - | + | + |
| *Klebsiella pneumoniae* | -R | + | - | - | + | + | - | + | - | + | - | - | + | + | + |

Key: +R = Gram positive bacteria - R = Gram negative bacteria

+C = Gram positive cocci cells + = Positive (has reaction)

- = Negative (no reaction) α = Alpha blood haemolysis

**Table 2.** Lipolytic efficiency (LE) of bacterial species from different vegetable oil contaminated soil

| S/No | Bacterial Isolate | Source | LE (%) |
|---|---|---|---|
| 1 | *Bacillus megaterium* | G | 67.2 ± 0.64 |
| 2 | *Bacillus megaterium* | F | 25 ± 0.29 |
| 3 | *Bacillus subtilis* | F | 21.8 ± 0.12 |
| 4 | *Micrococcus luteus* | G | 18.9 ± 0.29 |
| 5 | *Bacillus lichenifomis* | F | 23.8 ± 0.98 |
| 6 | *Kblesiella pneumoniae* | F | 91.7 ± 5.02 |
| 7 | *Eschericia coli* | F | 0 |

Reference to Table 2, out of the six (6) bacteria species identified as lipase producers, *K. pneumoniae* from Falke Vegetable Oil Factory, Kaduna had the highest lipolytic efficiency (91.7 %) followed by *Bacillus megaterium* from Grand Cereals Vegetable Oil Factory, Plateau State (G) with lipolytic efficiency of 67.2 %. *Bacillus megaterium* from Falke Vegetable Oil Factory, Kaduna (F) had lipolytic efficiency of 25 %, *Bacillus licheniformis* from Falke Vegetable Oil Factory, Kaduna (F), 23.8 %, *Bacillus subtilis* from Falke Vegetable Oil Factory, Kaduna (F), 21.8 % and *Micrococcus luteus* having lipolytic efficiency of 18.9 % from Grand Cereals Vegetable Oil Factory, Plateau State (G). *Escherichia coli* from Falke Vegetable Oil Factory, Kaduna (F) showed no zone of hydrolysis. This shows that the above organisms (with the exception of *E. coli*) from the different oil contaminated soil have the ability to produce extracellular lipases in different quantities hence their purification and characterization for large scale production. Ghori [20] and Ramesh [21] reported lipase production from *Bacillus sp.* When considering source(s) of quantitative lipase production for different industrial and biotechnological applications, *K. pneumoniae* from Falke Vegetable Oil Factory, Kaduna (F) and *Bacillus megaterium* from Grand Cereals Vegetable Oil Factory, Plateau State (G) may be considered because of their ability to produce lipases in higher amounts.

Many studies have been carried out in order to determine the optimal culture and nutritional requirements for lipase production by submerged culture. Lipid as a carbon source is no doubt generally essential for obtaining a high lipase yield [22]. In the present study, olive oil was used as the substrate for lipase production. A number of other studies [22,21] verified olive oil as a sole specific substrate for enhanced lipase production when compared with other oil-inducers. *K. pneumoniae* was used for quantitative lipase production in liquid medium based on its earlier lypolytic efficiency. In the present study, lipase production from *K. pneumoniae* was observed to commence after 12 hr of incubation and the activity declined with increase in time of incubation. However, lipase production was observed to be highest at 36 hr as depicted in Figure 1. For this reason, the lipase from the present study may be employed for different industrial applications.

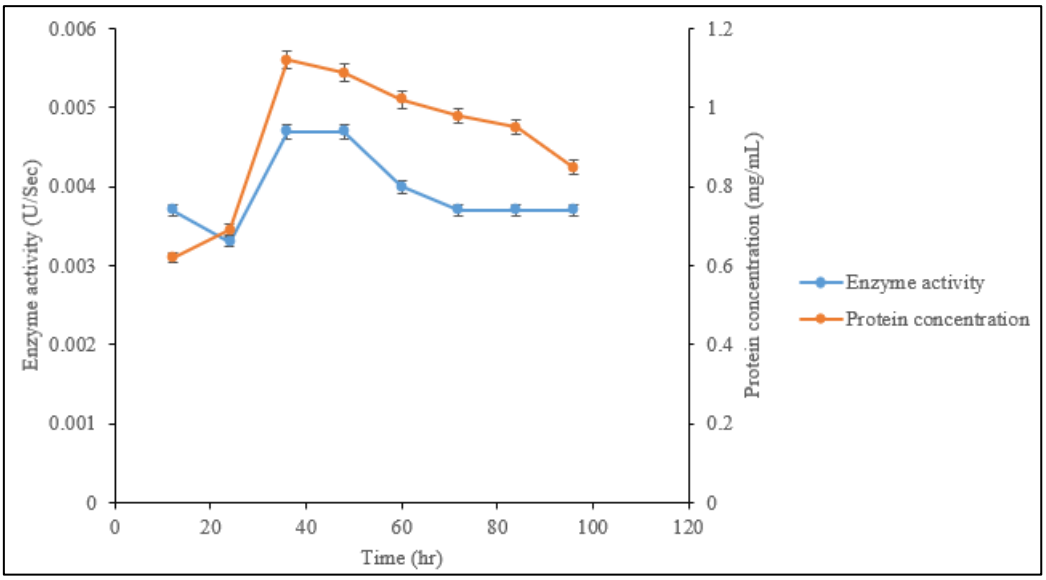


**Figure 1**. Time course for lipase production from *Klebsiella pneumoniae* isolated from a vegetable oil contaminated soil

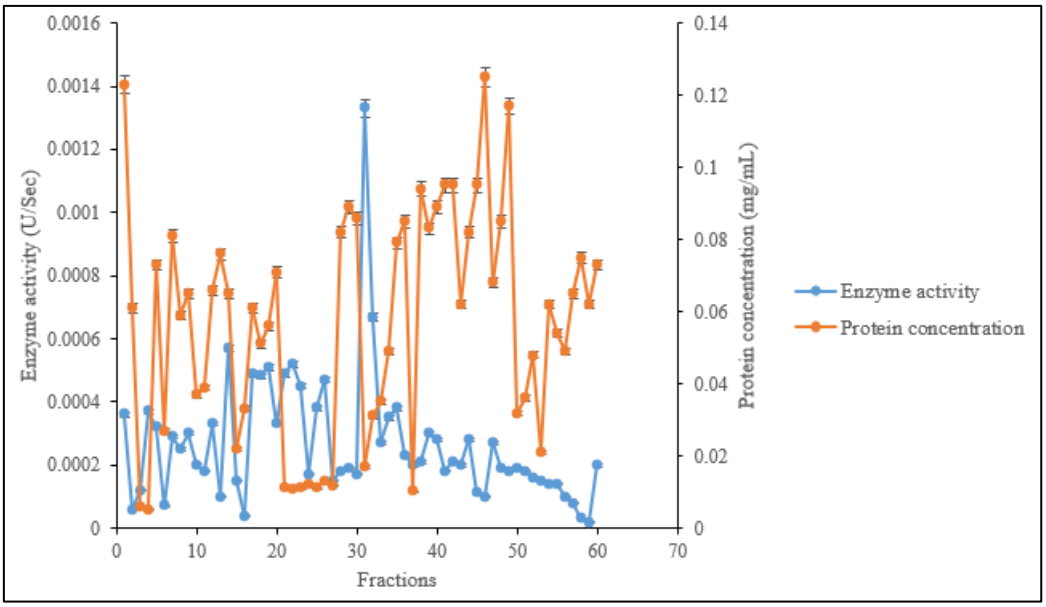


**Figure 2.** Elution profile of lipase from *Klebsiella pneumoniae* isolated from a vegetable oil contaminated soil

**Table 3.** Purification Table for Lipase from *Klebsiella pneumoniae* of a vegetable oil contaminated soil

| Fraction | Fraction volume (mL) | Enzyme activity (U/Sec) | Total activity (Units) | Protein concentration (mg/mL) | Total protein (mg) | Specific activity (U/mg/Sec) | Purification fold | Yield (%) |
|---|---|---|---|---|---|---|---|---|
| Crude | 500 | 0.0007 | 0.35 | 1.12 | 560 | 0.0006 | 1 | 100 |
| $(NH_4)_2SO_4$ | 30 | 0.0071 | 0.213 | 0.94 | 28.2 | 0.0076 | 12.7 | 60.9 |
| Sephadex G-75 | 5 | 0.008 | 0.04 | 0.04 | 0.2 | 0.02 | 26.3 | 18.8 |

Enzyme purification generally involves a series of fractionations by which an enzyme protein is separated from other proteins. Crude lipase enzyme from *Klebsiella pneumoniae* was therefore subjected to purification after which the enzyme showed an increase in enzyme activity from 0.0007 to 0.0071 U/Sec with a purification fold of 12.7 and 60.9 % recovery following ammonium sulphate precipitation at 40 % saturation (Table 3). Five millilitre of the ammonium sulphate fraction was loaded unto sephadex G-75 gel filtration column and one activity peak was observed (fraction 31) as depicted in Figure 2. The enzyme activity and protein concentration of the purified enzyme were determined to be 0.008 U/Sec and 0.04 mg/mL respectively (Table 3). The purification process resulted in 26.3-fold purification factor and a final recovery (yield) of 18.8 % of the enzyme with specific activity of 0.2 U/mg/Sec. Generally, it was observed that increase in the purity of the enzyme resulted to an increase in activity. This is in in accordance with expected theory which has it that, enzyme purification generally results to an increase in enzyme activity [2].

The optimum pH for lipase activity was observed at 8.0 from *K. pneumoniae* suggesting the lipase enzyme to be slightly alkaline in nature (Figure 3). The pH value obtained in this study is in tandem with the highest lipase activity at pH 8.0 from a *Bacillus* strain as reported by Saraswat [23] although, a pH range of 8 – 9 has been reported by Nawani [24] from some *Bacillus* species. Priji [25] reported the lipase from *Pseudomonas aeruginosa* strain with an alkaline pH to be suitable for detergent manufacturing. The lipase from *K. pneumoniae* may be used in detergent formulations because of its alkaline property. As seen in Figure 4, lipase from *K. pneumoniae* was optimally active at 30 ℃ suggesting it to be mesophilic. Litthauer [26] reported that bacterial lipases generally have temperature optima in the range of 30 – 60 °C. Conversely, reports also exist on lipases of bacterial origin with optima in both lower and higher ranges [27]. Saraswat [23] reported lipase activity from *B. subtilis* at a temperature range of 30 and 50 ℃. Salgado [28] have reported the use of mesophilic lipases in bioremediation and waste water treatment. The lipase from this study may as well be used for the same purpose.

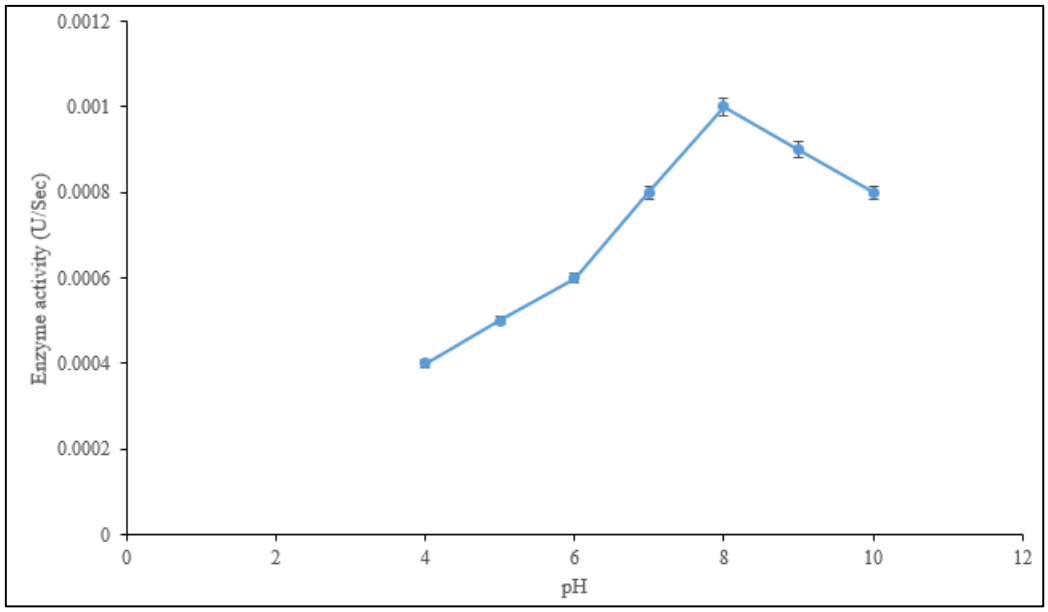


**Figure 3.** pH activity profile for purified lipase from *Klebsiella pneumoniae* using Tris-HCl buffer

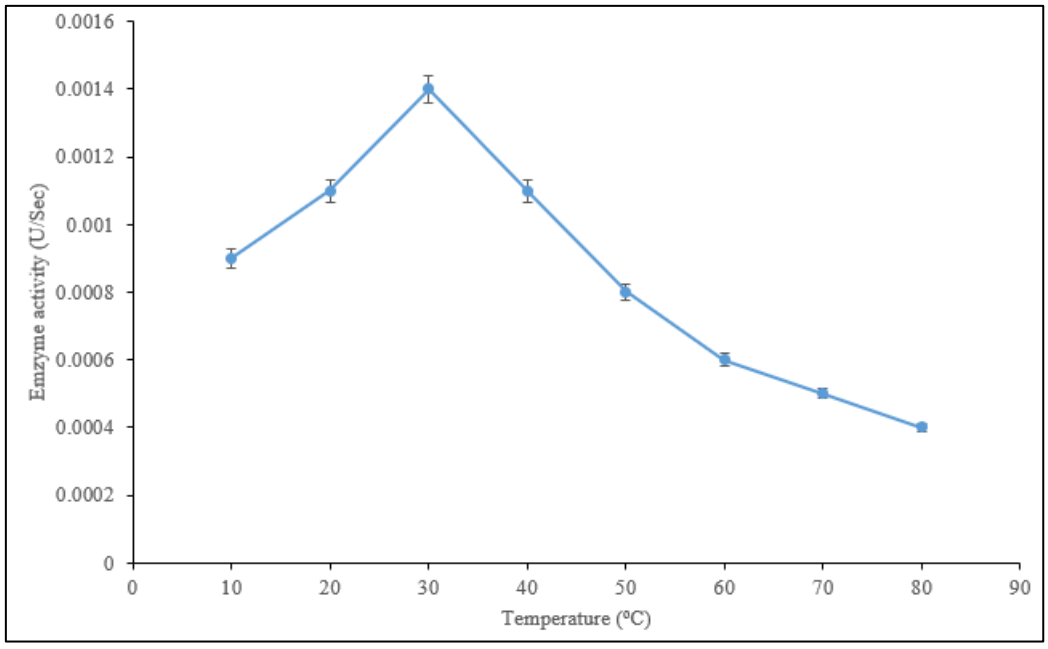


**Figure 4.** Optimum temperature of activity of purified lipase from *Klebsiella pneumoniae* isolated from a vegetable oil contaminated soil

**Table 4.** Effect of different substrates on the activity of purified lipases from *Klebsiella pneumoniae* from a vegetable oil contaminated soil

| Lipase activity (U/Sec × $10^{-4}$) | | |
|---|---|---|
| Palm oil | Groundnut oil | Olive oil |
| 11 ± 0.6 | 14 ± 1.7 | 15 ± 1.2 |

Lipase activity was determined to be highest when olive oil was used as the substrate than in groundnut oil and palm oil (Table 4). In a similar investigation by Ezema [29], the highest enzyme activity was obtained with olive oil. This suggests that, olive oil could be a potent substrate for lipases from isolates of the different oil contaminated soil. From the results obtained, olive oil may be the potent substrate among the three substrates studied.

It has been established that metal ions play a significant role in enzyme catalysis by binding to amino acid residues with negative charges at specific sites [30,31]. From this study (Figure 5), $MgCl_2$ and $CaCl_2$ enhanced the activity of the partially purified lipase from *K. pneumoniae* while KCl had no effect on the activity of the enzyme. $FeCl_3$, $HgCl_2$, $CuSO_4$ and $CoCl_2$ on the contrary showed inhibitory effects on the activity of the enzyme. Ghori [20] reported $Mg^{2+}$ to enhance the activity of lipase from *Bacillus* sp. This is analogous to the findings of this study and that of Saun [32] from the *Bacillus aerius* studied. Saraswat [23] and Wang [33] reported $K^+$ to speed up lipase activity. $K^+$ on the other

hand had no effect on the activity of lipases studied by Ghori [20]. This is in line with the findings of this study. Saraswat [23] have reported $Cu^{2+}$ and $Co^{2+}$ to inhibit lipase activity which corresponds to the findings of this study. Changes in the activities of lipases from different sources following the addition of various metal ions showed that different ions produced different level of conformational changes to the enzymes [34]. It may be concluded that $MgCl_2$, KCl and $CaCl_2$ produced a conformational change to a more active form of the enzyme studied.

The Lineweaver-Burk plot of the partially purified lipase from *Klebsiella pneumoniae* is depicted in Figure 6. The $V_{max}$, $K_m$ and $K_{cat}$ were found to be 0.0006 U/Sec, 0.4960 mM and 0.0125 $S^{-1}$ respectively. In a similar vein, the energy of activation and half-life of the enzyme were determined to be 26.689 KJ/mol and 0.16 hr correspondingly (Table 4).

**Table 5.** Energy of activation and half-life of purified lipase from *K. pneumoniae* of a vegetable oil contaminated soil

| Energy of activation (KJ/mol) | Half-life (hr) |
|---|---|
| $26.689 \pm 5.8 \times 10^{-4}$ | $0.16 \pm 5.8 \times 10^{-3}$ |

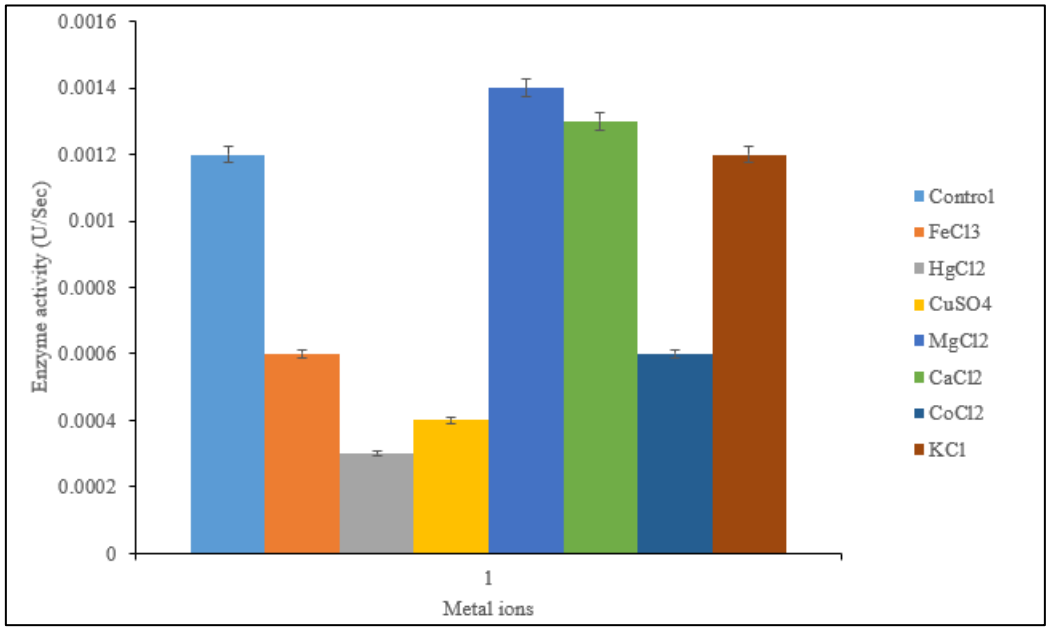


**Figure 5.** Effect of metal ions on the activity of purified lipase from *Klebsiella pneumoniae* isolated from a vegetable oil contaminated soil

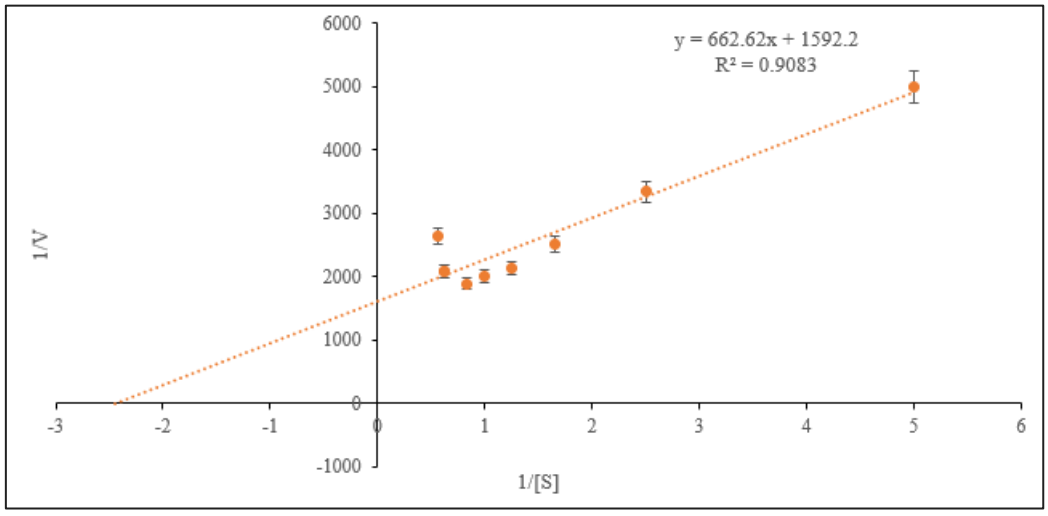


**Figure 6.** Lineweaver-Burk plot of purified lipase from *K. pneumoniae* isolated from a vegetable oil contaminated soil

## 4. Conclusions

An extracellular lipase from *K. pneumoniae* was partially purified by ammonium sulphate precipitation and gel chromatography with a yield of 18.8 % and purification fold of 26.3. The partially purified enzyme was maximally active at 30 ℃ and at pH 8.0. The enzyme had a wide affinity for different substrates with highest activity observed in olive oil substrate. $MgCl_2$ and $CaCl_2$ activated the enzyme to a more active form while KCl had no effect the activity of the enzyme. The findings of the present study suggest *K. pneumoniae* isolated from a vegetable oil contaminated soil as a novel lipase producer with the capacity to meet the numerous biotechnological applications such as bioremediation, detergent manufacturing and waste water treatment.

## Acknowledgements

Authors are very grateful to TETFUND Nigeria for funding this research.

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