# Purification and Characterization of Lipase Isolated from *Cyperus esculentus* (Tiger nut) Milk and Determination of Factors Inhibiting the Lipase Activity

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## ABSTRACT

Enzymatic catalysts hold significant importance across diverse industrial domains, including food processing, detergent manufacture, and pharmaceuticals. This study focuses on evaluating the effectiveness of lipase inhibitors in enhancing the shelf life of Cyperus esculentus (tiger nut) milk, a nutritious drink popular in northern Nigeria but plagued by rapid spoilage, leading to both consumer dissatisfaction and economic losses. The investigation involved the purification and characterization of lipase, using para-nitrophenyl palmitate as a substrate. The extraction of the lipase enzyme from tiger nut milk was successfully executed, followed by a purification process involving ammonium sulfate precipitation, sephadex G-100 gel filtration, and DEAE-Cellulose ion exchange chromatography. Thorough biochemical techniques were employed to characterize the purified enzyme. The lipase displayed a high specific activity of 78.0679 Units/mg protein, a purification fold of 3.8, and a yield of 12.1%. Optimal conditions for lipase activity were identified: a pH of 8.0, a temperature of 60°C, and a substrate concentration of 2.5mg/dl. Kinetic parameters were determined, revealing a Km value of 3.2877mg/ml and a Vmax of 0.5283 µmol/min. The study then assessed the impact of two commercial lipase inhibitors, (EDTA and Orlistat) on enzyme activity. Orlistat was found to significantly reduce lipase activity to 12.6 U/mL at a concentration of 10mM, outperforming EDTA, which decreased activity to 23.1 U/mL at 5mM and 56.4U/mL at 10mM. The study shows that lipase contributes greatly in the rapid spoilage of the milk by hydrolysing the abundant lipid in the milk to fatty acid, glycerol and other alchohols which then becomes rancid. its inhibition by agents such as Orlistat can substantially improve the milk's shelf life by inhibiting the enzyme's activity thereby reducing microbial growth and preserving the milk's nutritional content.

## 1. INTRODUCTION

Lipases, known as serine hydrolases, play a critical role in both the synthesis and breakdown of long-chain triacylglycerols, making them one of the most versatile and widely utilized enzymes in biotechnological sectors. Their ability to catalyze the hydrolysis of carboxylic ester bonds has garnered significant interest from researchers worldwide [1].

These enzymes are omnipresent across various life forms, including bacteria, fungi, plants, and animals.

These enzymes, present in numerous amount in animals, plants, and microbial species, are found to exhibit varying enzymological properties and substrate specificities [2], [3]. The production of lipases in microorganisms typically involves cultivating in a nutrient-rich medium that includes sources of carbon, nitrogen, and phosphorus [4]. Compounds like triglycerides, glycerol, and bile salts serve as inducers for lipase production [5]. Physicochemical properties of lipases, such as stability, specificity, and optimal pH and temperature, enhances their utility as biocatalysts [6]. Their robust stability across

wide range pH and temperature makes the enzyme to withstand various condition both in plants and animals [7], [8].

Lipases have application in a variety of fields, including textile and dairy sectors, detergent industry, biodiesel, oil processing, and the production of surfactants, all thanks to their microbial origins [9], [10]. They play a crucial role in the processing of polyunsaturated fatty acids, the production of food colorants like gamma-linolenic acid, and the synthesis of methyl ketones, which are key flavor components in blue cheese [9], [10]. Lipases has significant biotechnological applications and it is attributed to their ability to catalyze enantioselective reactions across a broad spectrum of substrates and their stability under a wide range of temperatures and pH levels [11], [12].

Besides animals and plants Lipases can be extracted from microorganisms, including bacteria, and fungi [11], [13]. Plant lipases are drawn from various plant parts, such as fruits, latex, and seeds. Notably, seeds, particularly oil-rich ones. In seeds, these enzymes play a pivotal role in supporting embryo growth by supplying energy, in tiger nut seeds prominence becomes usually noticeable during germination stages. However, lipase activity was notable even after the germination. [3], [14].

*Cyperus esculentus* milk has been noted for its rich mineral content, containing higher levels of iron, magnesium, and carbohydrates compared to conventional animal milks such as cow milk [15]–[18]. Additionally, it offers distinct advantages by containing minimal amounts of casein and lactose, making it a suitable option for individuals with lactose intolerance. Furthermore, absence of cholesterol in the milk makes it a favorable choice for those managing hypertension [19]–[21]. Given the expanding applications and importance of lipases, this study aims to thoroughly investigate the characteristics of lipase present in tiger nut milk and assess its potential role in hastening the spoilage of this milk variant.

## 2. MATERIALS AND METHOD

### 2.1 Materials

Dried, smaller-sized brown *Cyperus esculentus* (tiger nut) were procured from Kasuwar Kurmi Kano and transported to Bayero University Kano in a a sterile plastic bag. The botanical identification was conducted at the Herbarium Botany Department of Bayero University Kano by Dr. Yusuf Nuhu, resulting in accession number BUKHAN 367. The tiger nuts were meticulously cleaned by removing stones and impurities, followed by washing and soaking in distilled water for 24 hours.

### 2.2 Methods
### 2.2.1 Sample Isolation

The *Cyperus esculentus* (tiger nut) were soaked in distilled water for 2 hours, washed, and homogenized for 5 minutes with 80 ml of Tris-HCl buffer (0.1M, pH 7.5). The resulting Tris-HCl buffer filtrate was filtered through four layers of cheesecloth and centrifuged at 1000 rpm using a refrigerated centrifuge (4 °C) for 30 minutes. The obtained pellets and supernatant were separated, with the crude lipase enzyme extracted for subsequent laboratory analyses.

### 2.2.2 Lipolytic Activity Determination

Lipolytic activity was assessed through a spectrophotometric assay employing pNPP (Para-nitrophenyl palmitate) as the substrate, following the methodology adopted from Ali et al. [13]. The reaction mixture comprised 0.1 ml of enzyme extract, 0.8 ml of 0.05 M Tris buffer (pH 8), and 0.1 ml of 0.01M p-NPP dissolved in isopropanol. The mixture was incubated at 60 °C for 20 minutes in a water bath, followed by the addition of 0.25 ml of 0.1M $Na_2CO_3$ to terminate the reaction. After centrifugation at 10,000 rpm for 15 minutes, the Optical Density (O.D.) was determined at 410 nm. Lipase activity was quantified as the amount of enzyme that liberated 1 μmol of p-nitrophenol per minute from p-nitrophenyl palmitate, using the provided calculation or the formula: lipase Activity = O.D410 = 5.43020 × Conc. (mM) + 0.00303.

### 2.2.3 Protein Estimation Using Bradford Method

The Bradford protein assay, a colorimetric technique, was employed for protein concentration determination. The Bradford reagent (acidified Coomassie Brilliant blue G-250) was used, leading to a visible colour change upon binding to proteins, detectable in the absorbance spectrum from 470 nm to 595 nm. The protein concentration of alpha amylase and lipase were established using the procedure outlined by Bradford (1976), with bovine serum albumin (100 μg/ml) employed as the standard. A calibration curve ranging from 0 to 100 μg/ml standard was created through duplicate dilutions using water as a diluent to a final volume of 800 μL [22].

For analysis, test tubes labelled as: test, standard, and blank were used. Bradford reagent (200 μL) was added to all test the tubes, followed by dispensing 700 μL of distilled water into each. Subsequently, 100 μL of the sample, BSA, and distilled water were added to the respective tubes. The contents were mixed and incubated for 2 minutes at room temperature. The absorbance of the blue-colored solution was measured at 595 nm against the reagent blank [22].

Calculation: Protein concentration = (absorbance at 595 nm - intercept (C)) / slope (M)

### 2.2.4 Ammonium Sulphate Precipitation for Lipase Purification

In the purification process of lipase, the initial step involves ammonium sulphate precipitation. This process aims to isolate the lipase from the crude extract by optimizing the concentration of ammonium sulphate added. The saturation concentrations ranging from 10% to 90% are tested [13]. Ammonium sulphate is slowly added to the crude extract while maintaining it on ice with gentle stirring. This gradual addition facilitates the precipitation of proteins. After reaching the desired saturation, the mixture is centrifuged at 10,000 rpm for 15 minutes at 4 °C to separate the precipitated protein. Subsequently, the protein pellet obtained is re-suspended in 1.0M Tris-HCl buffer at pH 7.5 and then dialyzed overnight against the same buffer to remove any excess salt and impurities. This process was repeated for each test tube to determine the most efficient salt concentration for protein precipitation.

### 2.2.5 Gel Filtration using Sephadex G-100 for Lipase Purification

Following the initial purification step, the lipase enzyme is subjected to gel filtration chromatography using Sephadex G-100 to further purify it. In this step, 3 g of Sephadex G-100 is soaked overnight in 150 mL of Tris-HCl buffer (0.05M, pH 7.5). A glass column (2.5 x 30 cm) was prepared by rinsing it with distilled water and packing it with sterile cotton wool at

the bottom. The Sephadex G-100 slurry is then poured into the column, and the same buffer is used as the elution buffer. Thirty fractions of 3 ml each are collected at a flow rate of 0.4 mL per minute. The protein concentration of each fraction was determined using a spectrophotometer at 280 nm, and the enzyme activity of each fraction is also assessed. Fractions exhibiting the highest specific enzyme activity were pooled together for the subsequent purification step [13], [23].

### 2.2.6 Ion Exchange Chromatography on DEAE Cellulose for Lipase Purification

For the next purification step, ion exchange chromatography on DEAE Cellulose was employed. Initially, DEAE Cellulose is prepared by dissolving 2g of the powder in 100 mL of 0.1M phosphate buffer at pH 7.8 overnight. The resulting slurry was then poured into a 2.5 x 30 cm column, which is rinsed with distilled water followed by the same phosphate buffer used for dissolving the anion-exchanger. The column is packed with clean and sterile cotton wool. Fractions collected from this chromatography step were also assessed for protein concentration to monitor purification progress [13].

### 2.2.7 Molecular Weight Determination of Purified Lipase using SDS-PAGE

Finally, the molecular weight of the purified lipase is determined using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Pooled fractions with the highest specific activity from the ion exchange chromatography were loaded onto a precast 14% gel along with protein standards. The gel was run at 40V for 40 minutes until the dye enters the resolving gel, and then the voltage was increaswd to 80V until the dye reaches the bottom of the gel, allowing for complete separation of protein bands. After electrophoresis, the gel was stained with Coomassie blue, and then destained using a mixture of acetic acid, menthol and distilled water for 24hours and the protein bands were visualized for molecular weight determination [2].

### 2.2.8 Characterization of Alpha-Amylase and Lipase
### 2.2.8.1 Effect of pH and Temperature on Lipase activity

The optimal pH range was investigated from 3 to 11 at 50 °C using 0.1M tris-acetate buffer solutions with pH values spanning from 3 to 11. The spectrophotometric assay method, as outlined earlier, utilized p-NPP as substrate.The Optimal temperature for activity was determined by the spectrophotometric assay using p-NPP as substrate at different temperatures (30-90ºC) [24].

### 2.2.8.2 Effect of metal ions concentration on lipase activity

The effect of metal ions ($Na^+$, $K^+$, $Ca^{2+}$, $Mn^{2+}$, $Fe^{3+}$ and $Zn^{2+}$) were ascertained on the activity of Alpha Amylase and lipase, their effects were determined in the presence of each of the ions at a concentration of 1mM following described in the assay protocol. The enzyme activity in the absence of the effectors was taken as 100% and relative activity was calculated [7].

### 2.2.8.3 Effect of Substrate concentration on Lipase activity

Effect of different substrate concentrations of lipase Enzyme activity was carried out using pNPP as a substrate using varying substrate concentrations (0.05-0.4mg/dL) at (50ºC and pH 7.5). The absorbance was measured at 410 nm. $K_m$ and $V_{max}$ values were calculated at pH 7.5. Specific activity of the enzyme was tested with increasing concentrations of substrate.

### 2.2.9 Evaluation of inhibitory effects of Different Inhibitors on Lipase

The inhibitory effect was carried out using standard operating protocol (SOP) as described by Patel and Shah, [25]. The effects of inhibitors were ascertained by determining the enzyme's activities in the presence of 0.5, 1.0, 1.5 and 2.0mM concentration of EDTA and Orlistat using para-nitrophenol palmitate (pNpp) as substrate at pH 6.0 and temperature of 50 ºC. The inhibitor with the highest inhibitory effect was selected for further analysis.

### 2.10 Statistical Analysis

Data was analysed using IBM SPSS advanced statistics version 21.0 (SPSS Inc., Chicago, IL). Numerical data was expressed as mean and standard deviation. Tukey's multiple comparison test was applied under one-way ANOVA.

## 3. RESULTS

### 3.1 Lipase Purification Table

Table 1 presents a comprehensive summary of the purification process and outcomes for Lipase isolated from Cyperus esculentus (Tiger Nut) milk. Initially, at the crude level, the enzyme exhibited a total protein content of 1.9620 mg/mL and a specific activity of 20.1560 units/mg protein. Upon further purification using (NH4)2SO4 saturation at 70%, the total protein content decreased to 1.6756 mg/mL, while the specific activity increased to 27.4688 units/mg protein. Throughout the purification steps, both the total protein content and yield decreased. However, there was a consistent increase in specific protein activity and purification fold as the purification process progressed from one step to the next.

**Table 1**. Summary steps for Lipase Extraction from *Cyperus esculentus* (Tiger Nut) Milk

| Purification steps | Total protein (mg/ml) | Total activity (Units) | Specific activity (Units/mg protein) | % Yield | Purification Fold |
|---|---|---|---|---|---|
| Crude Lipase Enzyme | 1.9620 | 0.0974 | 20.1560 | 100 | 1 |
| (NH4)2SO4 (70%) Precipitation | 1.6756 | 0.0610 | 27.4688 | 62.6 | 1.36 |
| Gel filtration on Sephadex G-100 | 1.3523 | 0.0307 | 45.7299 | 31.5 | 1.66 |
| Ion Exchange on DEAE Cellulose | 0.9243 | 0.0118 | 78.0679 | 12.1 | 1.71 |

### 3.2 Molecular weight of Lipase from Cyperus esculentus (Tiger Nut) Milk

Fractions 15 and 19, exhibiting the highest specific activities of 76.6935 units/mg protein and 79.4424 units/mg protein respectively, were combined and subjected to Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The apparent molecular weight of the partially purified lipase was deduced from the obtained results, indicating an approximate enzyme molecular weight of 35 kDa, as depicted in Figure 1.
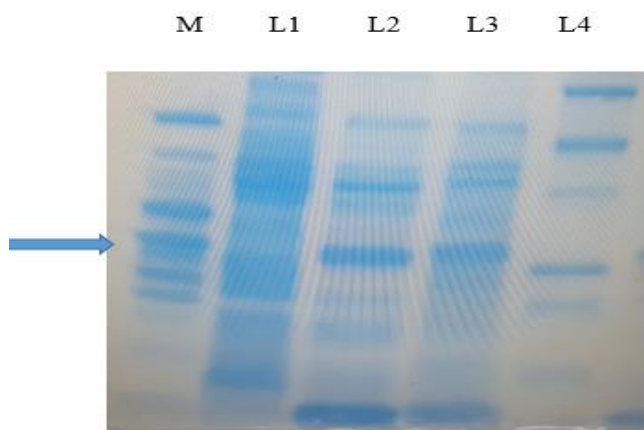
**Fig. 1.** SDS-PAGE of Purified Lipase

**Key**: lane M= molecular weight marker, lane 1 crude Lipase, lane 2= Lipase from ammonium sulphate, lane 3= purified Lipase from sephadex G-100, lane 4= purified Lipase from sephadex DEAE-cellulose column.

### 3.3 Effect of pH on partially purified Lipase activity

The activity of Lipase isolated from C. esculentus in this study shows lower relative activity at lower pH from pH 3 to 6 where there was a steady increase in the activity of the enzyme from 19.6 U/mL to 26.2 U/mL , while from pH 6 to 7 there was a rapid increase in the activity of the enzyme from 26.2 U/mL to 43.1 U/mL. the highest relative activity of 58.6 U/mL was found at an optimum pH 8. From the optimum pH the relative activity of the enzyme rapidly decreased as the pH is increasing, at the highest pH 11, the relative activity was found to be 28.9 U/mL. This indicates that lipase from the study was found to function optimally in a slightly alkaline medium as shown in Figure 2.
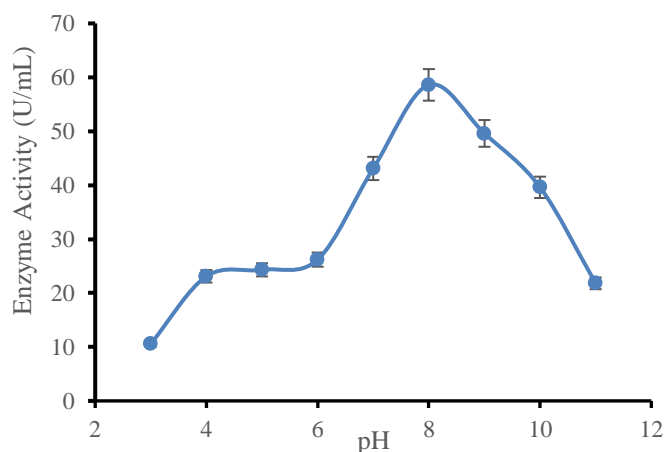


**Fig. 2.** Effect of pH on purified lipase Activity

### 3.3 Effect of Temperature on Lipase Activity

The effect of temperature on lipase activity is presented in Figure 3. The partially purified lipase from Tiger nut milk showed varying activity over a wide range of temperatures from 30 °C to 90 °C. The optimum activity for lipase was observed at 60 °C. However, the lipase activity showed a tendency to decrease with further increases in temperature beyond its optimum.
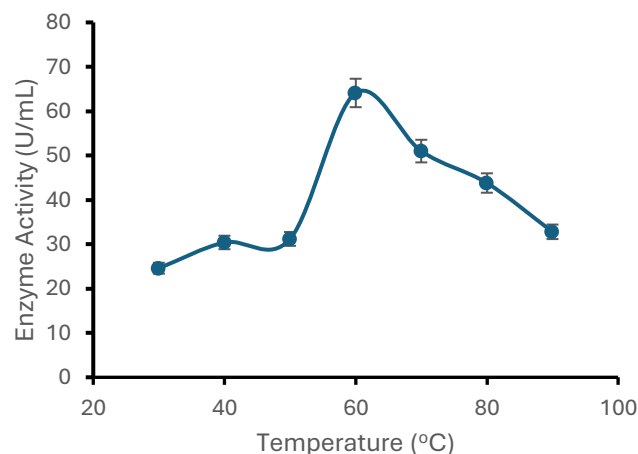


**Fig. 3.** Effect of temperature on partially purified Alpha-amylase and lipase from tiger-?

### 3.4 Effect of Substrate concentration on Lipase activity

The substrate concentration ranged from 0.05 to 0.4 mg/mL of P-npp. The results revealed that lipase exhibited its highest enzyme activity, reaching 0.308 units, at a substrate concentration of 0.25 mg/mL of P-npp. Conversely, the lowest enzyme activity, measuring 0.064 units, was observed at the lowest substrate concentration of 0.05 mg/dL of P-npp, as depicted in Figure 4. This suggests that the activity of the lipase enzyme is dependent on the concentration of the substrate, with an optimal activity observed at an intermediate substrate concentration.
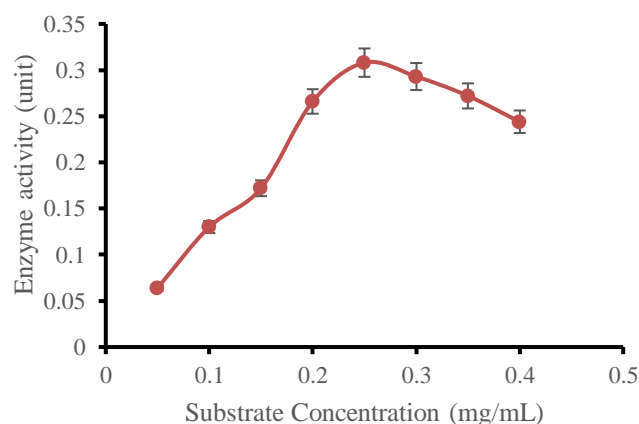


**Fig. 4.** Effect of substrate (p-nitrophenyl palmitate) oncentration lipase activity

### 3.5 Effect of metal ions concentration on lipase

The effect of metal ions concentration on lipase activity is presented in Figure 5. It was observed that $Fe^{3+}$ and $Zn^{2+}$ exhibit a modest inhibitory impact on the enzyme's activity, with $Fe^{3+}$ demonstrating the most pronounced inhibitory effect in comparison to the control group. Conversely, $Ca^{2+}$ was found to exert a stimulating influence on lipase activity. Moreover, $Na^+$ did not elicit a significant effect when compared to the control condition. On the other hand, $K^+$ and $Mn^{2+}$ were noted to exert a slight inhibitory effect on the activity of the lipase enzyme. These findings suggest a nuanced interplay between various metal ions and the enzymatic activity of lipase, with some ions acting as inhibitors while others exhibit stimulatory or negligible effects.
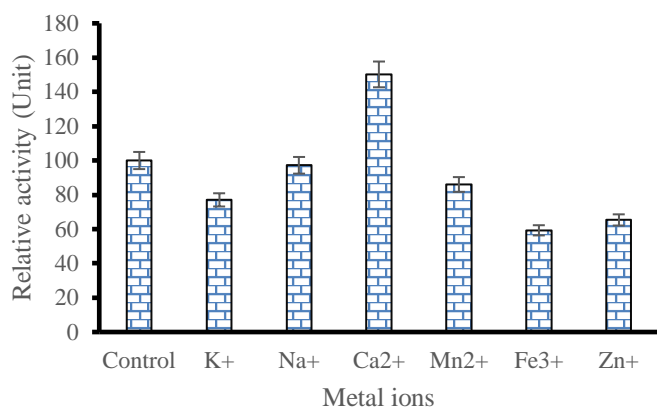
**Fig. 5.** Effect of metallic ions on the activity of lipase

### 3.6 Enzyme kinetics Study of purified

The enzyme kinetics investigation of the purified lipase from *Cyperus esculentus* (tiger nut) milk is illustrated in Figure 6. Steady-state kinetics analysis conducted through initial velocity studies employing P-nitrophenyl palmitate (P-npp) as a substrate, with concentrations ranging from 0.05 to 0.4 mg/dL, unveiled a Km value of 0.5283 µmol/min. Additionally, employing the formula slope=Km/Vmax, the Vmax was determined to be 0.917 mg/mL. Consequently, it can be inferred that the Lipase isolated from *C. esculentus* in this study exhibits a Km of 0.917 mg/mL and a Vmax of 0.5283 µmol/min.
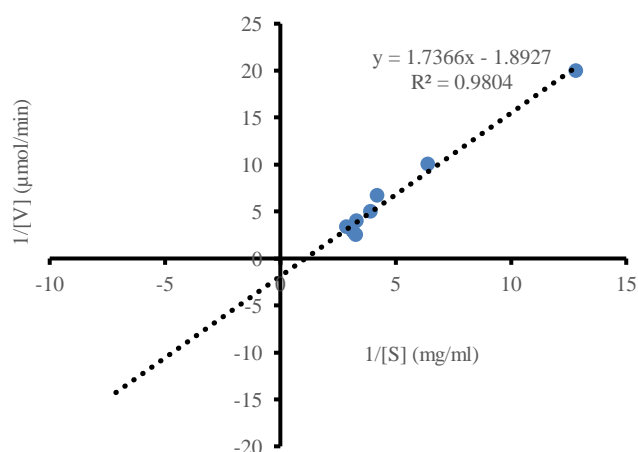


**Fig. 6.** Lineweaver-burke plot relating initial velocity data of purified lipase with substrate (P-npp) concentration

### 3.7 Effect of Inhibitors (Orlistat and EDTA) on Lipase activities

Th effect of Orlistat and EDTA on Lipase activities is presented in Figure 7. EDTA shows the highest inhibitory effect of 16.7 U/mL at 2.0 mM of concentration and the lowest inhibitory effect of 56.4 U/mL at 1.0 mM of EDTA concentration. While the effect of Orlistat on the activity of Lipase shows the highest inhibitory effect of 12.6 U/mL relative activity at 1.0 mM Orlistat concentration and lowest inhibitory effect of 34.1 U/mL relative activity at 2.0mM of Orlistat concentration, this implies at the highest concentration of the inhibitors (2.0 Mm) the activity of the enzyme does not seem to be reduce, and thus, Orlistat has more inhibitory effects on the activity of lipase isolated from *Cyperus esculentus* than EDTA as shown in Figure 7.
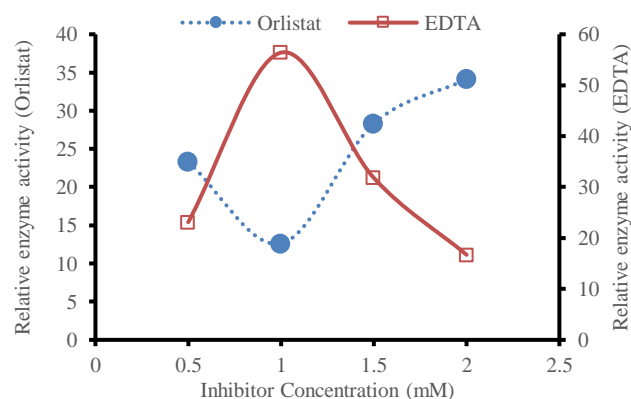


**Fig. 7.** Effect of Orlistat and EDTA on the activity of purified lipase

## 4. DISCUSSIONS

In the study, purification and characterization of lipase enzyme from *C. esculentus* (tiger nut) were conducted, this is in order to study its characteristics and assess the effect of inhibiting the activity of the enzyme in reducing the rapid spoilage of the milk. The purification process involved sequential steps, starting with crude extraction obtained by centrifugation and sieving. The specific activity of the crude lipase was found to be 20.1560 U/mg protein. Subsequent purification steps included precipitation with solid ammonium sulphate at various saturation levels, with the highest specific activity of 27.46888 U/mg protein achieved at 70% saturation. Further purification was carried out using desalting and Sephadex G-100 column chromatography, resulting in a specific activity of 45.7299 U/mg protein. The final purification step involved anion-exchange chromatography (DEAE Cellulose), yielding a lipase with the highest average specific activity of 78.0678 U/mg protein, with a 3.8-fold purification and 12.1% yield .

The molecular weight estimation of the purified lipase was performed using SDS-PAGE, revealing an estimated molecular weight of 35 kDa. This molecular weight finding aligns with previous studies on lipase enzymes from various sources, such as *Micrococcus sp*. L69 which reported a molecular mass of 40 kDa [27].

Lipase from *C. esculentus* in this study is optimally active at pH 8.0. This value was similar to the optimum pH of lipase activities in the germinating seeds of *Cucumeropsis mannii* [28]. In general, there are no reports on microbial and plant lipases that are active at extremely acidic pH. Lipases from *Bacillus thermoleovorans* ID1 showed the optimal activity at pH 9 [8]. Lipases producing bacteria like *thermophilic Bacillus* which lie in the range of pH 7.2–8.5 have been reported in earlier species [29].

Additionally, Lipase isolated in this study has an optimum temperature of 60 ºC, the specific activity of lipase in the study was tested at temperatures ranging from 30 to 90 °C, using coconut oil as substrate. Similar results were found by different authors, Lipase from *Microbacterium* sp. showed activity in a range of 10–90 °C. The optimum temperature for *Microbacterium* sp. lipase was found to be 50 °C. Similar results were found for lipase a ctivity in *Bacillus sp.* J33 (60

°C). *Bacillus Bacillus gibsonii.* (60ºC) [30]) and *Bacillus thermoleovorans* ID-1 (60–65 °C) [8].

The optimum substrate concentration for lipase from C. esculentus was observed at 0.25 mg/mL, showing maximal enzyme activity.However, beyond this concentration, enzyme activity began to decline. This decrease could be attributed to various factors such as the enzyme-substrate concentration ratio, substrate inhibition, or alterations in physiochemical characteristics. Lineweaver bulk plot has revealed that Km and Vmax of the lipase isolated in the study to be 0.5283 mg/mL and 3.2871µmol/min respectively. Muhammed et al. [31] reported a similar observation, noting a saturation point where the Km and Vmax 7.345 mM 96.52 U/mg of lipases isolated from *Streptomyces sp.* SBLWN_MH2 [31].

The study also examined the effects of various metal ions, including $K^+$, $Na^+$, $Zn^+$, $Fe^{3+}$, $Ca^{2+}$, and $Mn^{2+}$, on the activity of lipase from *C. esculentus*. Results showed that $K^+$, $Na^+$, $Mn^{2+}$, and $Fe^{3+}$ inhibited the activity of the lipase by 22.14 U/mL, 2.88 U/mL, 13.17 U/mL, and 39.71 U/mL, respectively. Conversely, $Ca^{2+}$ increased lipase activity by 50%. Although all inhibitions were observed to varying extents, none were complete. Similar findings were reported for the esterolytic enzyme of *Anoxybacillus gonensis* A4 and many *Pseudomonas* lipases [30], [32]. These enzymes typically rely on the presence of $Ca^{2+}$ ions for maintaining their stable and active structures. $Ca^{2+}$ ions bind strongly to specific sites on the enzyme surfaces, which are primarily composed of negatively charged carboxylate side-chain groups of aspartyls and glutamyl residues, arranged through polypeptide chain folding [26].

Furthermore, the inhibitory effects of compounds on lipase activity were examined, revealing that Orlistat emerged as the most potent inhibitor, significantly reducing lipase activity to 12.6 U/mL at a concentration of 0.1 mM, surpassing the inhibitory effect of EDTA. This inhibitory effect of orlistat on lipase activity has been reported in previous studies on lipases from maize and castor seeds [14], [23].

## 5. CONCLUSION

The study purified and characterized a lipase enzyme from Cyperus esculentus (tiger nut) milk to understand its properties and inhibitory factors. Sequential purification steps led to increased specific activity, with SDS-PAGE indicating a molecular weight of 35 kDa. Optimal activity was observed at pH 8.0 and 60°C, with an optimal substrate concentration of 0.25 mg/mL. Metal ions such as $K^+$, $Na^+$, $Mn^{2+}$, and $Fe^{3+}$ showed inhibitory effects, while $Ca^{2+}$ enhanced lipase activity by 50%. Orlistat emerged as a potent inhibitor, significantly reducing lipase activity. These findings enhance understanding of the enzyme's biochemistry. However, Further research could explore structural and kinetic properties to optimize enzyme efficiency for suitability in industrial application.

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