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Properties and Application of *Luffa aegyptiaca* and *Citrullus lanatus* Seed Lipases in the Degradation of Palm Oil Effluent (POE)

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ABSTRACT

Enzyme pretreatment of palm oil effluent (POE), a significant environmental pollutant, offers an eco-friendly and cost-effective alternative over chemical pretreatment methods. Hence, this research explored the properties of lipases from *Luffa aegyptiaca* and *Citrullus lanatus* as well as their potential in the pretreatment of POE. Lipases were obtained on the 4th and 6th day of *Luffa aegyptiaca and Citrullus lanatus* seeds germination, with specific activities (SAs) of 211.81 and 170.56 U/mg, respectively. After ion exchange and gel filtration chromatography, the SAs were 546.88 and 490.55 U/mg, respectively. The enzymes were stable at a temperature and pH range of 50-80 °C and 5.0- 9.0. The pH optima of the *Luffa aegyptiaca* and *Citrullus lanatus* lipases were 7.0 and 6.0, respectively. The optimal temperatures for the *Luffa aegytiaca* and *Citrullus lanatus* lipases were 60 and 50 °C, respectively. The Michaelis constant (K_m) and maximum velocity (V_{max}) of 0.32 mM and 200 µmol/min were obtained for *Luffa aegyptiaca* lipase, whereas 0.370 mM and 197.27 µmol/min were obtained for *Citrullus lanatus* lipase. Metal ions (Fe^{2+}, $Ca^{2+,}$ and Co^{2+}) significantly ($p<0.05$) enhanced the lipase activity more than Mn^{2+} in a concentration-dependent manner. Pretreatment of POE with lipases enhanced the reduction of total organic carbon, biochemical oxygen demand, and total organic matter. Also, the pH and dissolved oxygen content of the effluent were increased. The properties of *Luffa aegyptica* and *Citrullus lanatus* lipases and their ability to degrade palm oil effluent make them ideal for the sustainable and successful pretreatment of POE.

Keywords: Lipase, Characterization, *Citrullus lanatus, Luffa aegyptiaca*, Palm oil effluent, degradation.

Introduction

Palm oil production is associated with large amounts of waste product known as palm oil effluents (POE), which contain a considerable quantity of organic matter such as total suspended solids (TSS), volatile suspended solids (VSS), grease, and residual oil that increases chemical and biochemical oxygen demand (BOD). These organic matters constitute serious environmental problems, making POE hazardous to both fauna and flora in water bodies if left untreated.[1,2,3,4] Enzymatic pretreatment of POE can efficiently contribute to the reduction of these organic matters, creating a green and sustainable way of POE biotransformation.[5] The POE degradation is a multifaceted process that includes hydrolysis, acidogenesis, acetogenesis, and methanogenesis.[6] Lipase carries out the first step, which is the breakdown of long-chain triacylglycerol into glycerol and free fatty acids.[6] Lipases (EC 3.1.1.3) are triacylglycerol acyl hydrolases that hydrolyze triacylglycerol into glycerol, monoacylglycerol, diacylglycerol, and free fatty acids at an aqueous-lipid interface.[5,7,8] Lipases can catalyze different reactions, including trans-esterification, aminolysis, acidolysis, alcoholysis, esterification, and inter-esterification in non-aqueous and micro-aqueous environments.[8] Lipases can be sourced from different species of yeast, fungi, bacteria, animals, and plants.[5,9,10]

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Substrate specificity, catalytic efficiency, reaction rates, and production approaches are affected by the enzyme source.[11,12] Lipase production from plants involves simple methodologies, which do not require genetic modification processes. This makes lipase production from plants more cost-effective than that obtained through submerged fermentation using microorganisms. Also, plant-derived lipases are more suitable for use in pharmaceutical and food production.[13,14,15] Hence, using lipases from more available and underutilized plant sources such as *Luffa aegyptiaca* and *Citrullus lanatus* seeds that belong to the family Cucurbitaceae, can provide a better option for lipase production and application.[16] These plants are locally called Asisa or Ogbo and Ugu mmiri, respectively, in the Igbo language. In this study, the properties of lipases from the seeds of two underutilized varieties of melon (*Luffa aegyptiaca* and *Citrillus lanatus*) were evaluated, and their applications in the pretreatment of palm oil effluent were explored.

Materials and Methods

Plant collection and identification

The fresh and mature seeds of *Luffa cylindrica* (voucher no: UNN/13110) and *Citrullus lanatus* (voucher no: UNN/13111) were respectively obtained from Umuezebi village, Nsukka, and Ogige Market, Nsukka, Enugu, Nigeria (Latitude: 6° 51'28.19" N, Longitude: 7° 23'44.77" E), on the 5th of May 2019. The plants were identified by Felix I. Nwafor, and the voucher specimens were deposited at the University of Nigeria Herbarium (UNN), domiciled in the Department of Plant Science and Biotechnology, Faculty of Biological Sciences. The UNN is indexed in Index Herbariorum- A global directory of the world's herbaria (https://sweetgum.nybg.org/science/ih/herbarium-details/?irn=126760). The details of this collection(s) are verifiable at https://unnvirtualherbarium.com.ng/.

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Isolation of lipase

Lipases were extracted as described by Eze *et al*.[17] The mature *Luffa aegyptiaca* seeds were thoroughly washed and soaked in distilled water containing 0.5% hypochlorite to prevent microbial growth for 48 h. The seeds were spread on a dampened jute bag in a dark room to germinate. A quantity (10 g) of the seeds was homogenized with a pestle and mortar using 0.1 M acetate buffer at pH 7.0. The mixture was filtered using a muslin cloth, centrifuged for 30 minutes at 10,000 x g using a centrifuge (Gallenhemp, Germany), and the supernatant was tested for lipase activity. Also, lipase was isolated from *Citrullus lanatus* seeds following the procedures described above.

Protein determination and assay for lipase activity

The concentration of the protein in each seed was quantified according to Lowry *et al*.[18] using bovine serum albumin (2 mg/mL) as the standard. Lipase activity was assayed as explained by Lokrakul and Dharmsthiti.[19] Known volumes of lipase solution (0.5 mL), 2 mM p-nitrophenyl palmitate (0.5 mL), and acetate buffer (1 mL, 0.1 M, pH 5.0) were mixed in test tubes and incubated for 30 min at 50 °C in a water bath. The reaction was terminated with 4 mL of NaOH (0.1 M). The absorbance was measured at 410 nm using a spectrophotometer (Spectrumlab 23A, England). One unit of lipase activity (U/mL) was defined as the amount of lipase required to produce 1 µmol of p-nitrophenol (p-NP) per minute under assay conditions.

Purification of lipase

The crude enzymes (lipases from *Luffa aegyptiaca and Citrullus lanatus*) were brought to 70% and 80% saturation using ammonium sulfate salt.[17] The precipitates were dialyzed against 100 mM phosphate (pH 6.0) buffer and 100 mM Tris-HCl buffer of pH 7.5, respectively, for 12 h. The dialysate (10 mL) was introduced into a column (1 x 30 cm) of pre-swollen diethylaminoethyl cellulose (DEAE-cellulose) gel treated with 10 mM phosphate buffer of pH 7.5. The column was washed with the respective buffers to remove unbound protein molecules. Enzyme fractions (5 mL each) were collected in a stepwise manner with sodium phosphate buffer (0.01 M, pH 7.5) containing different concentrations (0.1 to 1 M) of NaCl. The active fraction (10 ml) was added into a Sephadex G-100 gel column (80 x 2.0 cm), pre-equilibrated, and eluted with phosphate buffer (10 mM, pH 6.0). The enzyme fractions were obtained at a flow rate of 0.33 mL/min using 10 mM phosphate buffer of pH 6.0. The concentration of protein and lipase activity were assayed, and the active fractions were combined for further studies.

Effects of pH and temperature on Luffa aegyptiaca and Citrullus lanatus lipases

The effects of pH on the activities o f *Luffa aegyptiaca and Citrullus lanatus lipases* were determined using 2 mM p-nitrophenyl palmitate prepared in different buffer systems, which included 0.1 M acetate, 0.1 M phosphate, and 0.1 M Tris-HCl with buffering capacities at pH ranges of 3.5-5.5, 6.0-7.0 and 7.5-9.0, respectively, at intervals of 0.5 units as described by Liu *et al*.[20] A known volume (0.5 mL) of lipase solution was mixed with 2 mM p-NPP (0.5 mL), 1 mL of the respective buffer and incubated at 50°C for 30 min in a water bath and terminated with 4 mL of 0.1 M NaOH. The absorbance was read at 410 nm using a spectrophotometer (Spectrumlab 23A, England). The pH stability studies of the lipases were carried out by incubating 20 mL of the enzymes at pH 5, 6, 7, 8, and 9, respectively, at 60 °C for 1 h. An aliquot (1 mL) of lipase was withdrawn at intervals of 10 minutes and tested for lipase activity. The lipase residual activity (in percent) was determined and plotted against the incubation time. The effect of temperature on *Luffa aegyptiaca and Citrullus lanatus lipases* activity was assayed by incubating the enzymes in (0.1 M) phosphate buffer solution (pH 6.5) containing 2 mM p-NPP at various temperatures ranging from 30-70 °C for 30 minutes in a water bath (HH-2, China). The enzyme activity was assayed and plotted against pH. Thermal stability studies were carried out by incubating 20 mL of the enzymes at 50, 60, 70, and 80 °C for 1 h. Lipase solution (1 mL) was pipetted at intervals of 10 min and tested for lipase activity. The percentage residual activity was calculated and plotted against the incubation time.

The effect of various concentrations of p-NPP on lipase activity

The effect of various concentrations of substrate was conducted as described by Abdella *et al*.[21] with some modifications. The mixtures contained various concentrations of p-nitrophenyl palmitate (0.1 to 0.7 mM in 0.1 M phosphate buffer solution (pH 6.5)) and the enzyme (0.5 mL). The enzyme activity was assayed as described above. The Km and V_{max} were calculated from the Lineweaver-Burk plot of the initial velocity data at various concentrations of p-NPP.

Effects of metal ions on Luffa aegyptiaca and Citrullus lanatus lipase activities

Studies on the effects of some metal ions encompassing calcium, manganese, iron, and cobalt ions (Ca^{2+}, Mn^{2+}, Fe^{2+}, and Co^{2+}) on lipase activity were done by incubating the enzyme in different concentrations (30-50 mM) of metal ions for 30 min. *Luffa aegyptiaca and Citrullus lanatus lipase* activities were assayed and compared with the activity obtained from the reaction devoid of metal ions.[20]

Analysis of total organic carbon content (TOC), total organic matter content (TOM), biochemical oxygen demand (BOD), and pH of POE

Palm oil effluent (POE) was analyzed following the procedure as contained in Shafwah *et al*.[6] with the following modifications: POE (5 mL) was stirred vigorously in a conical flask and incubated for 20 min. The pH was monitored with a pH meter. The total organic carbon content (TOC) was determined by adding 10 mL of POE solution into a solution containing 10 mL of 0.5M $K_2Cr_2O_7$ and 20 mL of 18 M sulfuric acid. The mixture was vortexed for 30 min and diluted with 20 mL of deionized water. A volume of 10 mL of 14.7 M phosphoric acid and four (4) drops of ferroin indicator were mixed with the solution and titrated against 50 mL of ferrous ammonium sulfate (FAS). The total organic carbon content (TOC) was calculated using:

$$\text{TOC (mg)} = \frac{\text{Vb}-\text{Vs x16000}}{\text{Volume of sample}}$$

Where Vb=titer value of the blank solution, Vs=titer value of the test solution.

The total organic matter (TOM) was calculated using

$$\text{TOM} = 1.23 \text{ x TOC}$$

The dissolved oxygen concentration of the POE was determined using a dissolved oxygen concentration meter by dipping the calibrated dissolved oxygen (DO) electrode into the effluent solution. The sample was incubated for five days, and the BOD was deduced using the equation below:

$$BOD_5 = DO_0 - DO_5$$

The 5 in BOD represents the period of pretreatment under standard temperature.

Lipase-assisted degradation of Palm Oil Mill Effluent (POE)

The enzymatic pre-treatment of POE was carried out in a 250 mL Erlenmeyer flask. A volume (5 mL) of the enzyme was introduced into 150 mL of POE, and the flask was covered to prevent evaporation during the process. The flask was placed into a rotary shaker set at 180 rpm and allowed to stand for 72 h. The performance of the enzyme was assessed by evaluating the levels of TOM, BOD, TOC, and pH.[6]

Results and Discussion

Two lipases were isolated using acetate buffer (0.1 M) of pH 7.0 on the 4th and 6th day of *Luffa aegyptiaca and Citrullus lanatus* seeds germination, with specific activities (SAs) of 211.81 and 170.56 U/mg proteins, respectively. The high specific activities suggest that both lipases can be suitable for POE pretreatment. Al-Haidari *et al*.[22] isolated lipase from sunflower seeds using 0.1 M Tris-HCl buffer, pH

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7.5, and reported a maximum lipase activity of 0.669 U/ml after 3 days of seed germination. Salmanu *et al*.[23] isolated lipases from *Cyperus esculentus* with a high SA of 78.0679 U/mg. The variation in the levels of lipase activities could be due to the differences in the sources and extraction methods adopted by different researchers. The ammonium sulfate saturations of 70 and 80% were optimal for precipitating proteins with the highest lipase activities from *Luffa aegyptiaca* and *Citrullus lanatus* crude extracts. The ammonium sulfate saturations of 70 and 80% suggest that the two lipases were hydrophilic, requiring a high concentration of salt to precipitate. This is per the report of Al-Haidari *et al*. [22], who reported that 70% ammonium sulfate saturation was optimal to precipitate lipase from germinated sunflower with an SA of 2.576 U/mg protein and a yield of 69.21%.

Ion-exchange chromatography showed two major peaks for lipase obtained from *Luffa aegyptiaca*, whereas that for *Citrullus lanatus* lipase had one peak (Figures 1 and 2), indicating the presence of two isoforms in *Luffa aegyptiaca* and one isoform in *Citrullus lanatus* lipases. After gel filtration, a major peak was obtained for lipase from *Luffa aegytiaca* and three major peaks for lipase from *Citrullus lanatus* (Figures 3 and 4). Multiple peaks observed may indicate the presence of different isoforms. *Luffa aegyptiaca* seed lipase was purified up to 2.32-fold after ion exchange chromatography and gel filtration with a yield of 2%, and an increase in specific activity from 211.81 to 490.55 U/mg proteins (Table 1). *Citrullus lanatus* lipase was purified up to 3.2 fold with a specific activity of 546.88 U/mg protein after gel filtration (Table 2). The increase in specific activity observed here suggests that ion-exchange chromatography and gel filtration were suitable to purify lipases from *Luffa aegyptiaca* and *Citrullus lanatus*.

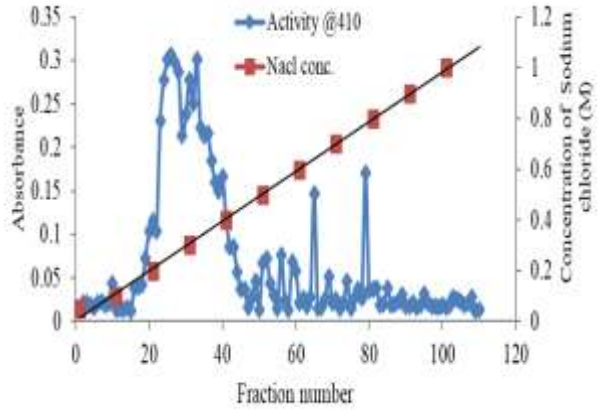

Figure 1: Ion-exchange chromatogram for *Luffa aegyptiaca* seeds lipase

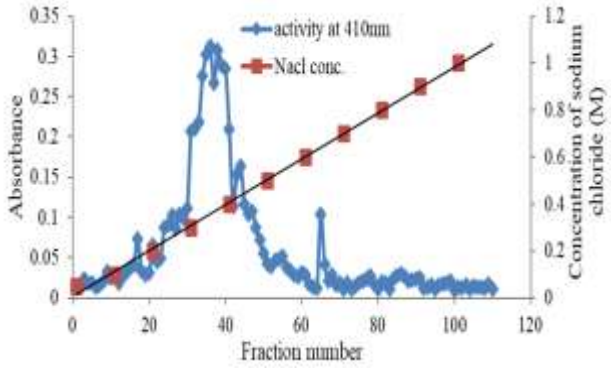

Figure 2: Ion-exchange chromatogram for *Citrullus lanatus* seeds lipase.

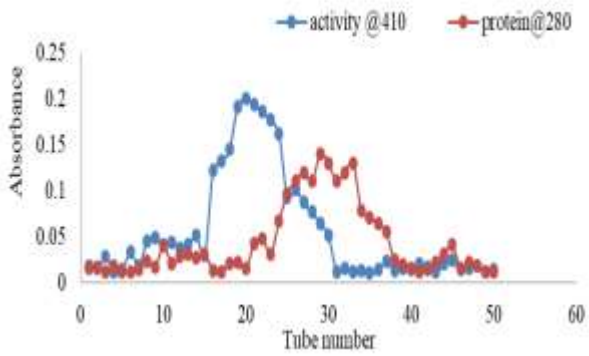

Figure 3: Gel filtration chromatogram for lipase from *Luffa aegytiaca* seeds

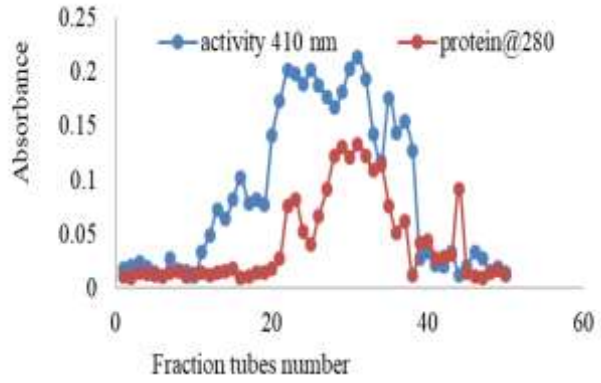

Figure 4: gel filtration chromatogram for lipase from *Citrullus lanatus* seeds

Optimal pH and temperature of enzymes are important factors to be considered in industry, since they give information on the suitable conditions for industrial application of enzymes. The optimal pHs of *Luffa aegyptiaca* and *Citrullus lanatus* lipases were 7.0 and 6.0, respectively (Figure 5). Eze and Ezema reported optimal pH of 5.9 and 7.5 for isoforms of lipase isolated from the seeds of white melon (*Cucumeropsis manni*).[24]

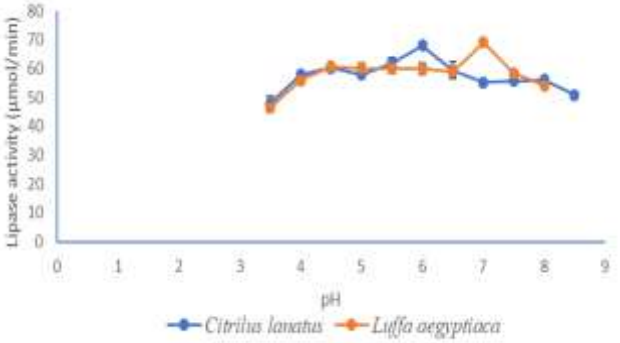

Figure 5: Effect of pH on lipase obtained from the seeds of *Citrillus lonatus* and *Luffa aegyptiaca*

The optimum temperature for the *Luffa aegyptiaca* lipase was 60 °C, whereas that for *Citrullus lanatus* lipase was 50 °C (Figure 6). Buratai *et al*.[26] reported optimal pH and temperature of 6.0 and 37 °C for lipase isolated from *Hibiscus sabdariffa*. Also, optimal pH and temperature of 8.0 and 60 °C were reported for lipase isolated from *Cyperus esculentus*.[23] However, Romo-Silva *et al*.[25] reported that *Hyphopichia wangnamkhiaoensis* and *Yarrowia deformans* lipases had optimal activities on p-NPP at a pH and temperature of 8.0 and 40 ◦C, respectively. The results of the effect of pH and temperature on *Luffa*

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aegyptiaca and *Citrullus lanatus* lipase activities suggest that the enzymes can be applied in POE degradation.

The K_m and V_{max}, which are kinetic parameters used to determine enzyme and substrate specificity and affinity, obtained from the Lineweaver-Burk plots (Figures 7 and 8), were 0.32 mM and 200 µmol/min for *Luffa aegyptiaca* lipase, and 0.370 mM and 197.27µmol/min for *Citrullus lanatus* lipase. These results indicate that lipases exhibited high affinity and catalytic efficiency towards the substrate and could be suitable for POE pretreatment. Buratai *et al*.[26] reported a Vmax of 3.92 µmol/ml and Km of 1.70 mg/mL, respectively, for lipase isolated from *Hibiscus sabdariffa* using 100 mg/mL of egg yolk as substrate. More so, Salmanu *et al*.[23] reported a Km and Vmax of 3.2877 mg/mL and 0.5283 µmol/min for lipase obtained from *Cyperus esculentus*. Acid and alkaline lipases with Km and Vmax of 15.67 g/L; 166.7 U and 13.28 g/L; 142.8 U were reported by Eze and Ezema.[24]

Table 1: Purification table of lipase isolated from *Luffa aegytiaca* seeds

Steps	Total Protein (mg/ml)	Total Activity (U)	Specific Activity (U/mg)	Purification fold	%Yield
Crude Extract	520.00	110140	211.81	1	100
$(NH_4)_2SO_4$	79.60	17298	217.31	1.03	15.71
Dialysis	18.64	6397	343.20	1.62	5.80
Ion-exchange chromatography	8.10	3345	412.96	2.00	3.04
Gel filtration	4.56	2098	490.55	2.32	2.00

Table 2: Purification table of lipase isolated from *Citrullus lanatus* seeds

Steps	Total Protein (mg/ml)	Total Activity (U)	Specific Activity (U/mg)	Purification fold	%Yield
Crude Extract	640	109160	170.56	1	100
$(NH_4)_2SO_4$	100.25	20373	230.22	1.19	18.66
Dialysis	19.67	5387	273.88	1.61	5.00
DEAEC	9.46	3570	377.32	2.21	3.30
Gel filtration	4.68	2541	546.88	3.20	2.30

Studies on the effects of metal ions on *Luffa aegyptiaca and Citrullus lanatus* lipases showed that at 0.05 M concentration, Ca^{2+}, Co^{2+}, and Fe^{2+} significantly ($p<0.05$) enhanced *Luffa aegyptiaca* lipase activity, whereas Mn^{2+} did not affect the enzyme activity (Figure 9).

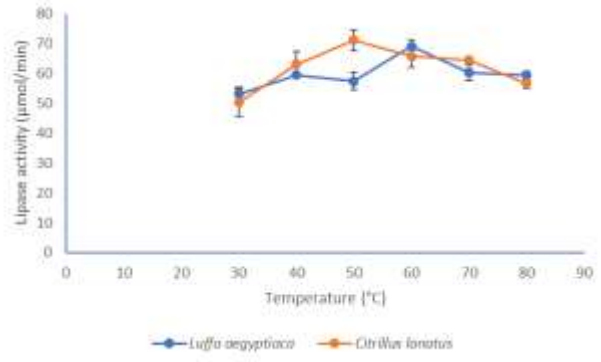

Figure 6: Effect of temperature on *Citrullus lonatus* and *Luffa aegyptiaca* seed lipase

At a lower concentration (0.03 M), the metal ions significantly ($p<0.05$) inhibited the *Luffa aegyptiaca* lipase compared to the control. *Citrullus lanatus* lipase activity was enhanced by a 0.05 M solution of the metals. Ca^{2+} and Co^{2+} enhanced the enzyme activity at 0.03 and 0.05 M when compared with the control (Figure 10). Metal ions Fe^{2+}, $Ca^{2+,}$ and Co^{2+} significantly ($p<0.05$) enhanced the lipase activity than Mn^{2+} in a concentration-dependent manner when compared with the control. This agrees with the reports of Eze and Ezema, who reported Ca^{2+} as a good activator of lipase.[24] Romo-Silva *et al*.[25] reported that *Hyphopichia wangnamkhiaoensis* and *Yarrowia deformans* lipases were activated by Ca^{2+} and Mg^{2+}. This suggests that *Luffa aegyptiaca and Citrullus lanatus* lipases are metalloenzymes, and their applications in the industry will require Ca^{2+}, Co^{2+}, and Fe^{2+} as cofactors.

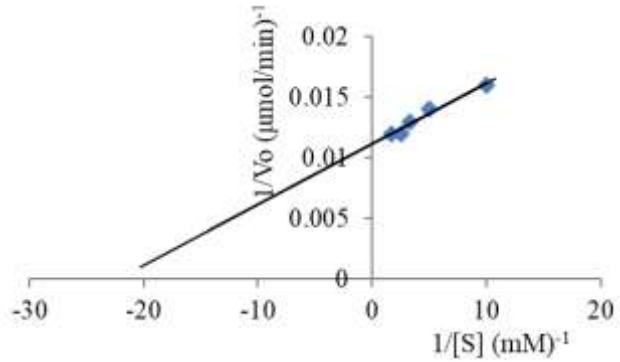

Figure 7: Linear-weaver-Burk plot of initial velocity data for lipase obtained from *Luffa aegyptiaca* seeds

Studies on the pH stability of *Citrullus lanatus* lipase showed that the enzyme maintained up to 50% of the initial activity after 60 min of incubation at all the pH tested (Figure 11). *Luffa aegyptiaca* lipase retained up to 50% of its initial activity after 45 min of incubation. Although at pH 6.0 and 9.0, *Luffa aegyptiaca* lipase retained up to 50% of its original activity. The enzyme lost more than 50% of its initial activity after 60 minutes of incubation (Figure 12).

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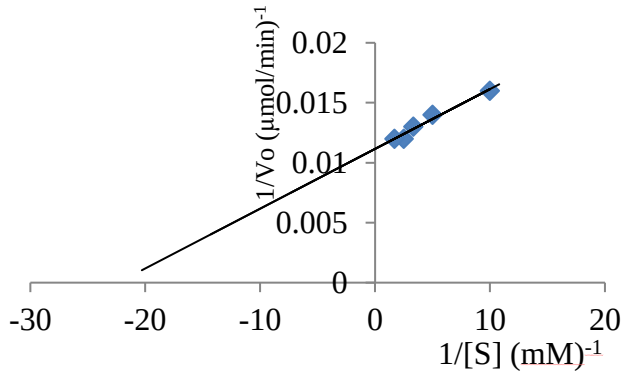

Figure 8: Lineweaver-Burk plot of initial velocity data for *Citrullus lanatus* lipase

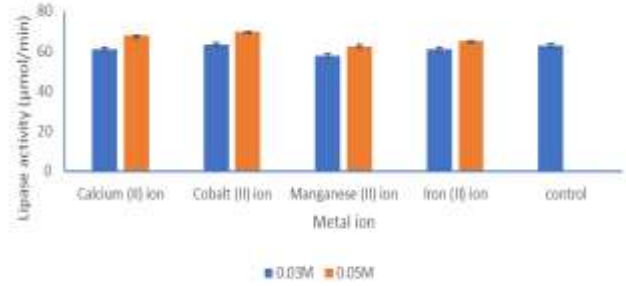

Figure 9: Effect of divalent metal ions on lipase from seeds of *Luffa aegyptica*

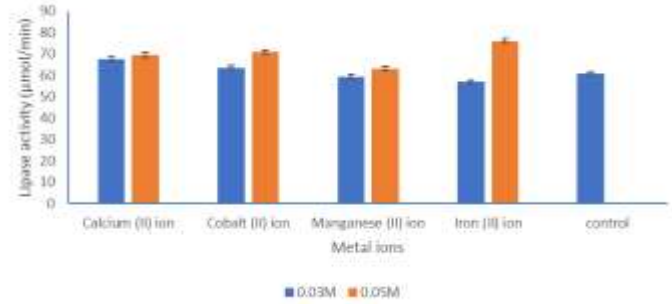

Figure 10: Effect of Divalent metal ions on lipase from seeds of *Citrullus lanatus*

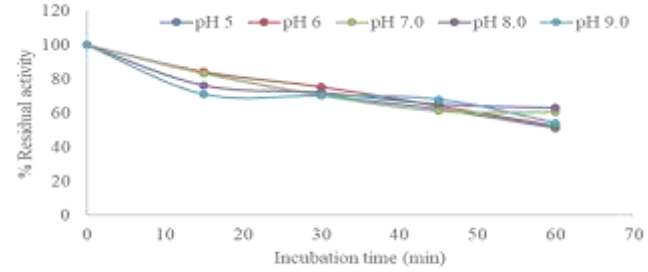

Figure 11: Plot of percentage residual activity against incubation time of lipase isolated from *Citrullus lanatus* seeds

More so, heat stability studies showed that *Luffa aegyptiaca* and *Citrullus lanatus* lipases retained up to 50% of their original activities after 60 min of incubation at all the temperatures tested, suggesting that the enzymes are stable in a wide pH range and may be suitable for POE pretreatment.

Enzyme stability, which is the enzyme's ability to withstand heat-induced unfolding in the absence of substrate, is a crucial factor that must be considered when applying enzymes in industry. Enzyme stability influences enzyme catalytic efficiency, productivity, and reaction timing.[27,28] Studies on the effect of temperature on enzyme stability showed that lipases obtained from the seeds of *Citrullus lanatus and Luffa aegyptiaca* retained more than 50% of the original activity after 60 min of incubation at 50, 60, 70, and 80 °C (Figures 13 and 14). However, Eze and Ezema reported that *Cucumeropsis manni* (White Melon) lipases were stable at 45 ◦C, beyond which the enzymes lost their activities.[24] This suggests that *Citrullus lanatus and Luffa aegyptiaca* lipases can withstand heat at 80 °C for 1 h, making them suitable for POE pretreatment.

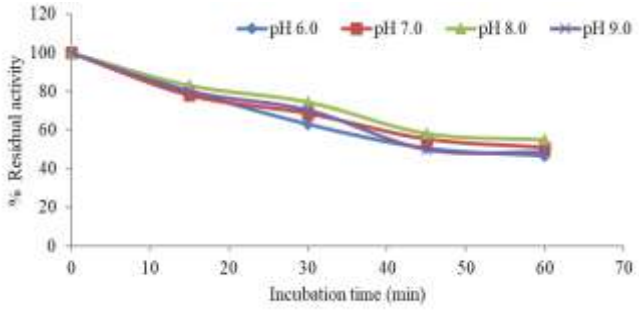

Figure 12: Plot of percentage residual activity against incubation time of lipase isolated from *Luffa aegyptiaca* seeds.

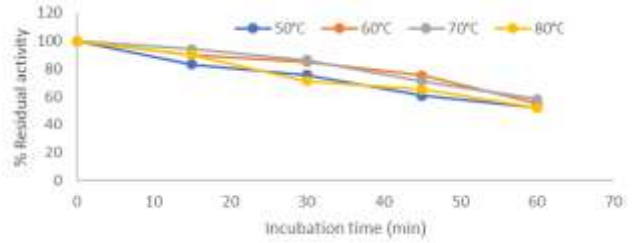

Figure 13: Plot of % residual activity obtained at 50, 60, 70, and 80°C against the time of incubation for lipase from *Citrillus lonatus* seeds

Hydrolysis of triacylglycerol, being the rate-limiting step in POE degradation, enhances the digestion efficiency of anaerobic digestion and methane production. Pre-treatment of POE with *Luffa aegyptiaca* and *Citrillus lanatus* lipases reduced the total organic carbon, BOD5, and total organic matter of the effluent, and increased the pH and dissolved oxygen content of the effluent (Table 3). The two lipases have shown the properties of good industrial enzymes for sustainable POE pre-treatment. Shafwah *et al.*[6] reported that POE pretreatment with lipase and xylanase was able to reduce total solids (TS) by 34.52%, total suspended solids (TSS) by 49.21%, and remove chemical oxygen demand (COD) by 49.7%.

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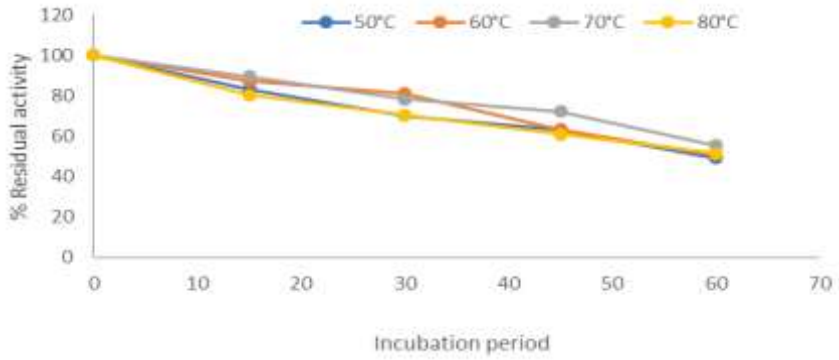

Figure 14: Plot of % residual activity obtained at 50, 60, 70, and 80°C against the time of incubation for lipase from *Luffa aegyptiaca* seeds

Table 3: Physicochemical parameters of POE before and after enzyme pre-treatment

Physiochemical parameters	**Before treatment**	**Pre-treatment With lipase from *Luffa aegyptiaca***	**Pre-treatment with lipase from *Citrillus lanatus***
pH	5.67	6.80	6.50
Dissolved oxygen content (mg/ml)	6.31	7.16	6.88
BOD5 (mg/ml)	4.87	2.76	2.89
Total organic matter content (TOM) (mg/ml)	100.70	70.54	68.15
Total organic carbon contents (TOC) (mg/ml)	81.87	61.58	56.20

Conclusion

This research presents the properties of lipases obtained from underutilized varieties of melon seeds (*Luffa aegyptiaca* and *Citrullus lanatus*). The high affinity of the enzymes for p-NPP and their stability at a wide range of pH and temperature in the presence of Fe^{2+}, Ca^{2+}, and Co^{2+} make them a good source for biotechnological applications. The enzymes obtained from *Luffa aegyptiaca* and *Citrullus lanatus* were able to enhance palm oil effluent degradation by reducing the total organic carbon, biochemical oxygen demand, and total organic matter of the effluent while increasing the pH and dissolved oxygen content of the effluent.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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