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BIOSYNTHESIS OF METHYL ESTERS FROM USED COOKING OIL (UCO) USING LIPASE ENZYME FROM *ASPERGILLUS ORYZAE* ON MOLDY COPRA

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ABSTRACT

The successful production of methyl esters from used cooking oil (UCO) using lipase enzymes from *Aspergillus oryzae* on moldy copra has been achieved. This method offers an eco-friendly substitute for crude palm oil (CPO) in generating methyl esters or biodiesel, contributing to waste reduction, economic benefits, and lowered greenhouse gas emissions for sustainable development. This study aimed to synthesize methyl esters from UCO using the *Aspergillus oryzae* lipase enzyme sourced from moldy copra. The enzyme was purified through ammonium sulfate fractionation and gel filtration column chromatography. Electrophoresis validated its purity, and activity was assessed through the Erdmann and Lowry method. Methyl ester synthesis involved transesterification with a UCO (1 mol): methanol (9 mol): lipase enzyme (15% v/v) ratio. The enzyme displayed notable characteristics, including 43.76 units/mg protein activity, a 41.7 kDa molecular weight, optimum pH of 8.2, temperature preference of 35°C, K_m of 0.046, and a 1.926 µmol/minute V_{max}. This enzyme efficiently catalyzed UCO (triolein) into methyl ester (methyl oleate), yielding 75.65%. Characterization using Fourier Transform Infrared (FTIR) revealed specific functional groups like –OH carboxylic acid, C=C alkenes, C=O esters, methyl (CH3-), and methylene (-CH2-). Gas Chromatography-Mass Spectrometry (GC-MS) analysis identified prominent compounds: methyl palmitate (12.53%), methyl vacsenate (16.44%), and, notably, methyl oleate (41.08%). This underscores the potential of *Aspergillus oryzae* lipase as an effective biocatalyst for UCO transesterification, yielding valuable methyl esters or biodiesel.

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INTRODUCTION

The growing demand for biodiesel has spurred scientists to generate biodiesel using diverse origins. Biodiesel can be synthesized from mineral, synthetic, and vegetable oils. However, biodiesel obtained from mineral and synthetic oils presents limitations, with just a 20-40% degradation rate achievable by

soil microorganisms [1]. In contrast, vegetable-derived biodiesel exhibits greater eco-friendliness than other variants, given its remarkable potential for up to 98% degradation.

The process of transesterification can transform vegetable oil into methyl esters. This reaction involves triglycerides reacting with alcohol to yield fatty acid methyl ester (FAME) and glycerol. Fatty acids are organic acids characterized by linear hydrocarbon chains featuring a carboxyl group (-COOH) at one end and a methyl group ($-CH_3$) at the other. When the resulting methyl ester has a shorter chain, it holds potential as biodiesel. Conversely, if the produced methyl ester has a longer chain, it can be a lubricant[2].

Generally, the transesterification reaction can be carried out using acid, base, and enzyme catalysts. Using an acid catalyst requires a longer reaction time, a higher molar ratio of alcohol and oil, and more corrosion so that it can damage equipment. An alkaline catalyst can form soap if vegetable oil's free fatty acid (FFA) content exceeds 0.5%. The disadvantages of using acid and base catalysts can be overcome using enzyme catalysts. The enzyme that can be used is the lipase enzyme. The advantages of using lipase enzymes are that they can react at low temperatures (30-50^oC), are easily regenerated and separated from products, are environmentally friendly, and do not form soap [3].

The interaction mechanism between the lipase enzyme and triacylglycerol (substrate) and the substrate hydrolysis process encompasses two sequential reaction phases: acylation and deacylation. The acylation phase signifies the initial formation of the enzyme-substrate (ES) complex [4-5]. Commencing the acylation step involves the activation of the serine nucleophile, facilitated by proton transfer to histidine. This leads to the creation of an oxyanion during catalysis. Subsequently, this oxyanion attacks the carbonyl carbon within the triacylglycerol ester linkage, generating a serine-substrate (SS) tetrahedral configuration called the ES intermediate. The stability of this ES intermediate is jeopardized due to the positively charged oxygen atom within the serine structure. To counter this instability, histidine protons (with acidic attributes) are transferred to substrate oxygen (with alkaline attributes). This transfer prompts the heterolytic cleavage of the serine-substrate bond, resulting in the formation of enzyme-acyl (EA) and diacylglycerol intermediates. This entire progression constitutes a sequence termed acylation[6].

Subsequently, the deacylation phase represents the hydrolysis stage of the EA complex, yielding methyl ester (biodiesel) and liberated lipase enzymes. The initiation of deacylation involves the transfer of methanol protons (with acidic attributes) to serine oxygen (with alkaline attributes), inducing the heterolytic rupture of the serine-acyl bond. This rupture generates free enzymes and methoxy ions (CH_3O^-). Consequently, the methoxy ion engages the unstable acyl group, forming methyl ester (biodiesel) [7].

Lipase enzymes are a group of enzymes that generally function in the hydrolysis of triacylglycerols (triglycerides) to

produce long-chain fatty acids and glycerol [4]. This enzyme also hydrolyzes triacylglycerols into diacylglycerols and free fatty acids. Diacylglycerol is a glycerol ester used as an emulsifier and stabilizer for food, cosmetics, and pharmaceutical products [8]. Microbial lipases have been used as catalysts in producing oleochemical-based products, including modified fats and oils such as low-calorie triglycerides [9], EPA, and DHA-rich oils [10].

Several types of mold are known to grow in habitats that contain oil and produce lipase enzymes, including Aspergillus, Mucor, Rhizopus, and Penicillium [11,12]. Coconut serves as a fundamental raw material in vegetable oil production, with copra being a byproduct of its processing. Traditionally, the copra production technique entails the removal of coconut husks, followed by sun-drying to achieve desiccation. This conventional method of coconut transformation often yields damaged copra, characterized by mold infestation. Research findings indicate that roughly 1-5% of coconuts subjected to copra processing eventually succumb to mold-induced spoilage, potentially culminating in waste. Remarkably, moldy copra holds the potential to serve as a rich reservoir of lipase-generating microorganisms. Notably, due to its elevated moisture content, protein, lipid (oil), carbohydrate, and ash content, as well as a favorable C/N ratio and C/P ratio, copra presents an optimal substrate for microbial proliferation, particularly molds. The mold strains that thrive on moldy copra have been identified as originating from the Aspergillus and Penicillium genera. [13].

This investigation aimed to fabricate methyl ester through the lipase enzyme obtained from *Aspergillus oryzae* on moldy copra as a biocatalyst, targeting the conversion of used cooking oil (UCO). Our findings substantiate the viability of employing the lipase enzyme sourced from *Aspergillus oryzae* on moldy copra as an effective biocatalyst for facilitating the transesterification process of UCO, resulting in the generation of methyl esters. Consequently, this study presents a promising foundation for potential industrial-scale biodiesel production, warranting further exploration and development in this direction.

METHODS

1. Instruments and Materials

The instruments used were analytical balance (Ohaus), oven (Memmert), autoclave (Astel), laminar airflow (Esco), water bath (Corning Hot Plate), magnetic stirrer, micropipette (Accumulate Pro), centrifugation (MPW Med Instruments), incubator (Ratek), ose needle, Bunsen, Shimadzu® FTIR Prestige-21 spectrometer (Shimadzu Corporation, Kyoto, Japan), and GC-MS spectrometer. The glass tools used are measuring flask (Pyrex), Erlenmeyer (Pyrex), beaker (Pyrex), petri dish (Pyrex), measuring cup (Pyrex), stir bar, spatula, test tube (Pyrex), separatory funnel (Pyrex), volume pipette (Pyrex), capillary tube, and chamber.

The materials used are mold from *Aspergillus oryzae* isolated from moldy copra. The culture is maintained and propagated on agar media. The agar media used were: peptone 0.5%, 0.1% KH_2PO_4, 0.001%

$FeSO_4.7H_2O$, 1.5% bacto agar, and 1% olive oil. The composition of the production media (fermentation) consists of 0.5% peptone, 0.1% KH_2PO_4, 0.001% $FeSO_4.7H_2O$, and 1% olive oil. Ammonium sulfate, 30% acrylamide, bis-acrylamide, casting buffer [1.5 M tris-HCl pH 8.8 (for resolving gel preparation) and 0.5 M tris-HCl pH 6.8 (for stacking gel preparation)], HCl 6 N, Laemmli loading buffer (bromophenol blue 0.004%, 2-mercapto-ethanol 10%, glycerol 20%, SDS 4%, tris-HCl 0.125 M), walking buffer (tris-HCl 25 mM, glycine 200 mM, SDS 0.1%), LDK 10%, ammonium persulfate 10%, SDS 10%, APS 10%, TEMED, gel fix solution (methanol and glacial acetic acid), Coomassie solution (CBB R-250 0.1%, methanol 40%, 10% glacial acetic acid, destain solution (methanol and glacial acetic acid), gel storage solution (glacial acetic acid and water), Whatmann No.1 filter paper, Q sepharosa FF, sephadex G-75, boric buffer, p-nitrophenol, p-nitrophenylbutyrate, SDS-PAGE gel 10%, BSA, used cooking oil, methanol p.a (Merck), n-hexane p.a (Merck), ethanol p.a (Merck), phenolphthalein indicator p.a (Merck), and activated carbon from avocado seeds.

2. Production and Isolation of Lipase Enzymes [10]

Peptone (1% v/v) and olive oil (3% v/v) were added to the shaken flask as media for enzyme production. The mixture was inoculated with a suspension of activated pure *Aspergillus oryzae* culture while stirring at 150 rpm. The isolate was fermented at 37 ^oC for 8 days. Fermented *Aspergillus oryzae* cells were separated from the media by centrifugation at 4^oC at 3500 rpm for 30 minutes. The supernatant obtained was used as a crude enzyme.

3. Lipase Enzyme Purification [10]

The crude enzyme was fractionated with 60-80% ammonium sulfate and precipitated by centrifugation at 4^oC at 10,000 rpm for 20 minutes. The solution was put into a vixing cellophane bag, dialyzed with 0.05 M borate buffer, and stirred with a magnetic stirrer for 1 night at 5^oC. Every 3 hours, the buffer is replaced. The ammonium sulfate fraction was further purified by ion exchange column chromatography according to the method of Mingrui [4], which was modified using a Q sepharose FF matrix (column length 35 x 1 cm with a flow rate of 30 drops/minute), which had been saturated with the appropriate buffer. The ammonium sulfate fraction was further purified by gel filtration column chromatography with a Sephadex G-75 matrix (column length 35 x 1 cm with a flow rate of 6 drops/minute) saturated with the appropriate buffer.

4. Lipase Enzyme Purity Test [11,12]

The enzyme solution obtained at each purification stage was tested for its purity electrophoretically with 10% SDS-PAGE gel. The enzyme purity test using the SDS-PAGE method was carried out in three stages: gel preparation, sample preparation, and gel staining.

Table 1. Solution Gel Preparation.

Gel (%)	Water (mL)	30% Acrylamide (mL)	1.5 M Tris-HCl, pH 8.8 (mL)	10% SDS (µL)	10% APS (µL)	TEMED* (µL)
10%	3.8	3.4	2.6	100	100	10

*TEMED must be the last ingredient added

a. Gel Preparation.

The glass plates and spacers of the gel casting unit were cleaned with deionized water and ethanol. Glass plates with spacers were assembled on a stable and level surface. The solution gel solution was prepared using volume (10 mL) for 10% gel, as in Table 1.

The gel solution was poured into glass plates arranged with spacers. The surface is coated with water or isopropanol to maintain the finishing gel's even and horizontal surface. The gel is allowed to harden for about 20-30 minutes at room temperature. The stacking gel solution was prepared using the volume (10 mL) of 5% gel in Table 2.

Table 2. Stacking Gel Solution

Gel (%)	Water (mL)	30% Acrylamide (mL)	1.5 M Tris-HCl, pH 8.8 (mL)	10% SDS (µL)	10% APS (µL)	TEMED* (µL)
5%	5.86	1.34	2.6	100	100	10

*TEMED must be the last ingredient added

The water or isopropanol coated on the resolving gel was discarded. The 5% gel solution was added sequentially until it overflowed. The comb is inserted immediately to ensure no air bubbles are trapped in the gel or near the wells. The gel is allowed to harden for about 20-30 minutes at room temperature.

b. Sample Preparation.

The enzyme sample (lysate cells or tissue) is added to the buffer with the same loading volume. This mixture was boiled at 95 ^{o}C for 5 minutes. The mixture was centrifuged at 16000 rpm for 5 minutes. This sample is stored at -20 ^{o}C or can be used for gel electrophoresis.

c. Gel Staining.

Protein or enzyme gel staining was performed using the Coomassie Brilliant Blue R-250 procedure. After electrophoresis, the gel was placed in a plastic tray containing the gel-fix solution. The tray is placed on a rocking table, and the proteins are strained for 2 hours. The fix gel solution was removed, and Coomassie's solution was added. The gel is placed on a rocking table and stained for 2-4 hours. After the staining stage, the gel was washed several times with distilled water to remove residual stains. The destain solution is added to the gel. The gel was placed on a shaking table and allowed to stand for 4 hours until a clear blue band with a clear background was visible. After destaining, the gel was stored in a gel storage solution and photographed as needed.

d. Purity Criteria.

Column chromatography Q sepharose FF and Sephadex G-75 determined the purity level of the lipase enzyme. The more bands that appear on the sepharose FF and Sephadex G-75 chromatograms, the lower the purity level of the lipase enzyme. Conversely, the fewer bands that appear on the sepharose FF and Sephadex G-75 chromatograms, the higher the purity of the lipase ezyme.

5. Lipase Enzyme Activity Test [13]

Lipase enzyme activity was determined using the method of Erdmann. Lipase enzyme solution or blank (0.1 mL) was added to a buffer (0.89 mL) containing 0.05 M Tris-HCl pH 7.0. Then 0.01 mL of 0.1 M *p*-nitrophenylbutyrate

substrate (dimethyl sulfoxide solvent) was added, shaken, and then incubated for 10 minutes at 37 ^{o}C. The absorption of the reaction mixture was measured using a UV-Vis spectrophotometer at a wavelength of 410 nm. Lipase enzyme activity was calculated based on the *p*-nitrophenol formed from the hydrolysis of the lipase enzyme on the *p*-nitrophenylbutyrate substrate.

6. Determination of Lipase Enzyme Protein Levels [14,15]

Lipase enzyme protein levels were determined using the Lowry method with the following steps.

a. Preparation of protein standard solutions for calibration curves.

A 10 mL volumetric flasks were labeled (70, 140, 210, 280, 350, 420, 490, and 560 µg/mL). Pipette standard protein (BSA) 1000 µg/mL (0.7, 1.4; 2.1; 2.8; 3.5; 4.2; 4.9; and 5.6 mL) into a volumetric flask. The solution was diluted with distilled water up to the mark and then homogenized.

b. Sample Preparation.

The lipase enzyme solution (1 mL) was put into a 10 mL volumetric flask. The solution was diluted with distilled water up to the mark and then homogenized—the solution of dilute (1 mL) into a 10 mL volumetric flask. The solution was diluted with distilled water up to the mark and then homogenized.

c. Maximum Wavelength Determination.

A 70 µg/mL (1 mL) protein standard solution (BSA) was placed in a test tube. Reagent C (5 mL) was added to the standard solution, homogenized, and allowed to stand for 15 minutes at room temperature. The standard solution was added with reagent E (3 drops), homogenized, and allowed to stand for 30 minutes at room temperature. The standard solution was put into the cuvette; then, the absorbance was measured in triplo with a UV-VIS spectrophotometer at 450-610 nm wavelength. The absorbance data of the standard solutions obtained from each reading are recorded in a spreadsheet.

d. Measurement of Standard Solutions, Samples, and Blanks.

The standard solution, sample, and blank (1 mL) were each put into a test tube. Reagent C (5 mL) was added to this solution, homogenized, and allowed to stand for 15 minutes at room temperature. Reagent E (3 drops) was added to this solution, homogenized, and allowed to stand for 30 minutes at room temperature. The standard solution, sample, and blank were each put into a cuvette; then, the absorbance was measured triplo with a UV-VIS spectrophotometer at 450-610 nm wavelength. The absorbance data of standard solutions, samples, and blanks obtained from each reading were recorded in a spreadsheet.

e. Creation of Calibration Curves.

The calibration curve is made by plotting the absorbance data of the standard solution on the y-axis and the concentration on the x-axis. The calibration curve should appear linear and have non-linear parts - this is the limit of linearity (LOL), a sign that the instrument's detection is close to saturation.

f. Data Adjustment with Linear Regression.

Data were adjusted by linear regression using statistical software. The output is the following equation (1):

$$y = mx + b \quad \text{.....(1)}$$

where

y = dependent variable (absorbance),

x = independent variable (concentration),

m = slope,

b = y-intercept.

Lipase enzyme protein levels can be obtained from the linear regression equation above by entering the absorbance data of the sample solution.

7. Characterization of Lipase Enzymes

Enzyme characterization includes: determining the optimum pH and temperature and determining the molecular weight, K_m, and V_{max} values of the fractions obtained from the purification results using Sephadex G-75 column chromatography. The optimum temperature is determined by testing the enzyme activity at 20-50 ^oC. The optimum pH was determined by testing the activity of enzymes in the pH 7.0-9.0 range using a borate buffer.

8. Purification of Used Cooking Oil (UCO) [16]

UCO (150 mL) and avocado seeds activated charcoal (10 g) are put into 500 mL beakers. The mixture was stirred with a magnetic stirrer for 2 hours at 70 ^oC. The mixture was allowed to stand for 12 hours and filtered with Whatman No. 42 filter paper. Pure oil is collected in a beaker, and the avocado seeds' activated charcoal residue is discarded.

9. Determination of FFA Content [16]

Pure oil (2 g), hexane (15 mL), and 95% (v/v) ethanol (20 mL) are put into the erlenmeyer flask. The mixture is stirred until homogeneous. To the mixture are added 3 drops of phenolphthalein indicator. The mixture was titrated with 0.1 M KOH until a pink color was formed. The FFA content of oil is calculated using equation (2).

$$A = \frac{\text{V KOH(mL) x M KOH}\left(\frac{\text{mol}}{\text{mL}}\right)\text{x Mr FFA}\left(\frac{\text{g}}{\text{mol}}\right)}{\text{Sample Mass(g) x 1000}} \text{ x } 100\% \quad \text{.....(2)}$$

If the FFA content of oil is < 2% (w/w), then methyl ester biosynthesis can be continued to the transesterification stage. Conversely, if the FFA content of oil is > 2% (w/w), then methyl ester biosynthesis starts from the esterification stage.

10. Biosynthesis of Methyl Esters [10,20,21]

Triolein substrate ($C_{57}H_{104}O_6$) (Mr = 885.4321 g/mol; ρ = 0.921 g/cm^3) (961 mL, 1 mol) [16], methanol (CH_3OH) (Mr = 32.04 g/mol; ρ = 0 .7918 g/cm^3) (364 mL, 9 mol) [17], and lipase enzyme (15% v/v) [18] were put into a 1500 mL Erlenmeyer flask. The mixture was put into the incubator shaker. The mixture was incubated at 50^{o}C for 24 hours. The mixture was centrifuged at 150 rpm for 60 minutes until two layers were formed. The top layer is methyl ester (main product), and the bottom is glycerol (side product) [19]. A separatory funnel separates the product. The percentage yield of methyl ester is calculated using equation (3.

$$\%\text{Yield} = 100\% \quad \text{.....(3)}$$

RESULTS AND DISCUSSION

1. Lipase Enzyme Activity Test at Each Purification Stage

Lipase enzyme activity was determined using the Erdmann method [12]. Lipase enzyme solution or blank (0.1 mL) was added to a buffer (0.89 mL) containing Tris-HCl 0.05 M pH 7.0. Then 0.01 mL of 0.1 M *p*-nitrophenylbutyrate substrate (dimethyl-sulfoxide solvent) was added, shaken, and

then incubated for 10 minutes at 37 ^{o}C. The absorption of the reaction mixture was measured using a UV-VIS spectrophotometer at a wavelength of 410 nm. The activity of the lipase enzyme was calculated based on the p-nitrophenol formed from the hydrolysis of the lipase enzyme on the *p*-nitrophenylbutyrate substrate. The unit of enzyme activity used is the unit/mg protein.

Enzymes stand as protein catalysts endowed with biochemical functionality. The operational efficacy of an enzyme is gauged by its activity, which, in turn, serves as a gauge of its degree of purity. A heightened specific activity of an enzyme correlates with an elevated level of purity. Through the process of enzyme purification, the step-by-step augmentation of specific activity signifies the successful elimination of impurities. As the purification procedure entails segregating the target protein from other proteins within the amalgamation, successive phases of enzyme purification can bolster the enzyme's proportion vis-à-vis the overall protein amalgam. This outcome consequently contributes to a relatively elevated specific activity for the particular enzyme within the mixture. Hence, quantifying the lipase enzyme's specific activity during each purification stage holds significance in assessing its purity level.

Table 3 shows the results of the lipase enzyme activity test at each stage of purification.

Table 3. Results of Lipase Enzyme Activity Test at Each Stage of Purification

Purification Stages	Volume (mL)	Total Proteins (mg)	Lipase Enzyme Activity		Recovery (%)	Purification Levels (x)
			Total (unit)	Specific (unit/mg proteins)		
Crude extract	1820	33.67	72.800	2.16	100	1
Fraction $(NH_4)_2SO_4$ 60-80%	10	15.00	43.753	7.87	60.10	3.64
Column Q Sepharosa FF	6	399.75	11.022	27.50	15.40	12.85
Gel Filtration Column Sephadex G-75	3	137.37	6012	43.76	8.25	20.25

Table 3 exhibits the lipase enzyme's performance (crude extract) under the conditions of 3% (v/v) olive oil concentration, 1% (v/v) peptone, and a stirring speed of 150 rpm, resulting in an activity of 2.16 units/mg protein. The evaluation of the purified lipase enzyme, following the introduction of 60-80% saturation ammonium sulfate, yielded a specific enzyme activity of 7.87 units/mg protein. This signifies a 3.64-fold enhancement in purity compared to the crude enzyme. Subsequent assessment of the lipase enzyme's activity post-purification through Q sepharose FF column chromatography showcased a specific activity of 27.50 units/mg protein. The outcome reflects a significant boost in purity, attaining a level 12.85 times higher than that of the crude enzyme. The lipase enzyme, subjected to purification via Sephadex G-75 gel filtration column chromatography, manifested a distinct specific enzyme activity of 43.76 units/mg protein. Emphasizes an outstanding surge in purity, reaching a level 20.25 times higher than the crude enzyme.

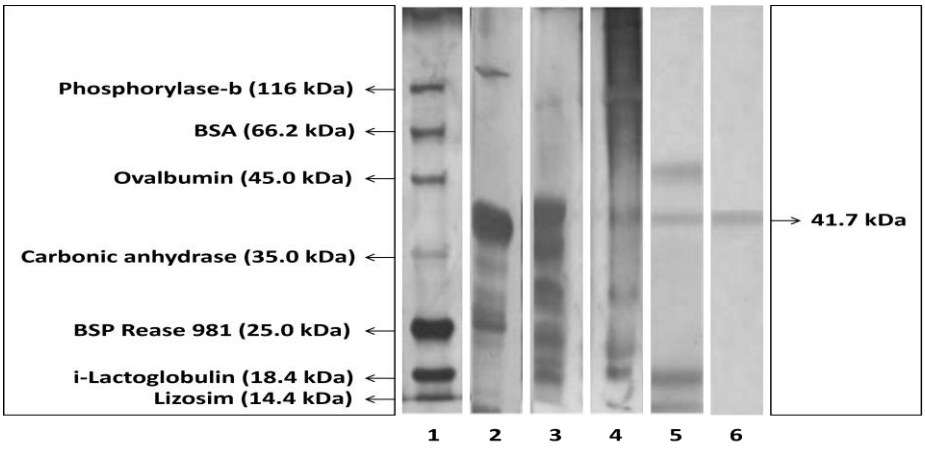
The lipase enzyme purification process at each stage is quite efficient. This can be seen from the efficiency level of the

purification process, which is increasing starting from the crude extract stage (1.00%), fraction $(NH4)_2SO_4$ 60-80% (27.47%), column Q sepharosa FF (28.33%), up to the Sephadex G-75 gel filtration column stage (63.46%). These results indicate that successive stages of enzyme purification can increase the lipase enzyme's efficiency.

2. Lipase Enzyme Purity Test at Each Purification Stage

The evaluation of lipase enzyme purity following each stage of purification through SDS-PAGE gel electrophoresis, a method employed for protein separation based on size. This approach gauges the lipase enzyme's purity throughout the purification process. SDS-PAGE operates on the principle of forming a polyacrylamide gel matrix via the reaction of acrylamide and bis-acrylamide (N,N'-methylene bisacrylamide), resulting in a highly cross-linked gel structure. This gel matrix functions as a molecular sieve, facilitating protein movement under the influence of an electric field. Proteins inherently possess positive or negative charges, enabling their migration towards the isoelectric point where their net charge is neutral. By denaturing proteins and imparting them with a uniform negative charge, the separation based on size is achievable as the proteins travel toward the positive electrode. This technique proves invaluable for assessing the lipase enzyme's purification efficiency in a scientific context.

The results of the lipase enzyme purity test at each stage of purification are shown in Figure 1.



Column: 1. Protein standard; 2. Crude extract of lipase enzyme; 3. Ammonium sulfate fraction (60-80%); 4. Dialysis results; 5. Purification results with Q sepharose FF matrix; 6. Purification results with Sephadex G-75 matrix.

Figure 1. The results of SDS-PAGE Gel Electrophoresis (10%) from Lipase Enzymes Produced from *Aspergillus oryzae* cells in Moldy Copra.

Figure 1 The analysis demonstrates that the degree of lipase enzyme purity is relatively modest in the crude enzyme fraction (2) and the ammonium sulfate fraction (3), a characteristic evidenced by the number of discernible bands. Within the context of column chromatography fraction Q sepharose FF (5), three bands are noticeable, while in the Sephadex column chromatography fraction G-75 (6), only one band is observable. These findings underscore that the purification process involving Sephadex G-75 column chromatography (6) yields lipase enzymes with considerably enhanced purity levels compared to the preceding phases. By aligning the marker (standard protein) (1) and the calculated molecular weight outcomes, the molecular weight of the isolated lipase enzyme is determined to be 41.7 kDa. This result closely resembles the molecular weight of lipase enzymes originating from various microbial sources, such as *Mucor sp* 42 kDa [22], *Bacillus cereus* C71 42 kDa [23], *Yarrowia lipolytica* 38 kDa [4], and *Aspergillus oryzae* 40.7 kDa [10].

Table 3 shows a positive correlation between the results of the purity test and the lipase enzyme activity test at each stage of purification. The higher the specific activity of the lipase enzyme, the higher the purity of the enzyme. The higher the purity level of the lipase enzyme, the higher the quality of the enzyme.

3. Characterization of Lipase Enzyme

Many factors, including pH and temperature, influence the activity of an enzyme. Figures 2 and 3, respectively, show the effect of pH and temperature on lipase enzyme activity (45%). Purified results were tested on *p*-nitrophenylbutyrate (0.2 M) and 35°C. The results showed that the lipase enzyme was active at pH 7.0, and the optimum pH was reached at pH 8.2 (Figure 2). Lipase enzyme activity began to decrease after the optimum pH was reached, and at pH 8.8, lipase activity began to stop.

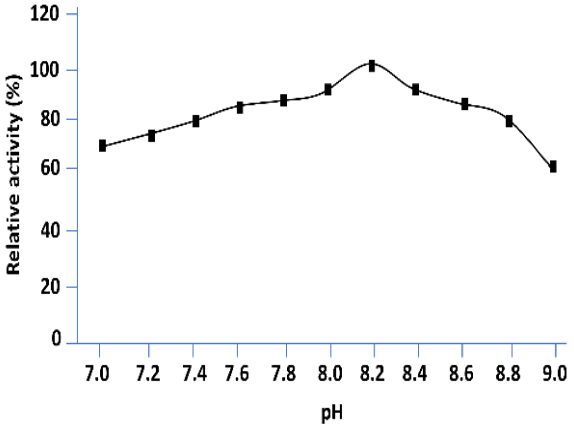


Figure 2. Effect of pH on The Lipase Enzyme Activity Produced from *Aspergillus oryzae* Cells in Moldy Copra.

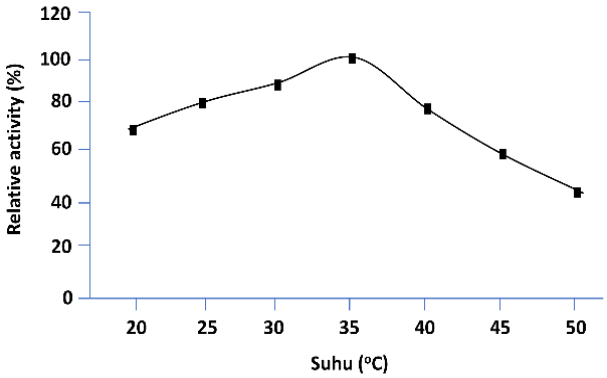


Figure 3. Effect of Temperature on The Activity Of The Lipase Enzyme Produced from *Aspergillus oryzae* Cells in Moldy Copra.

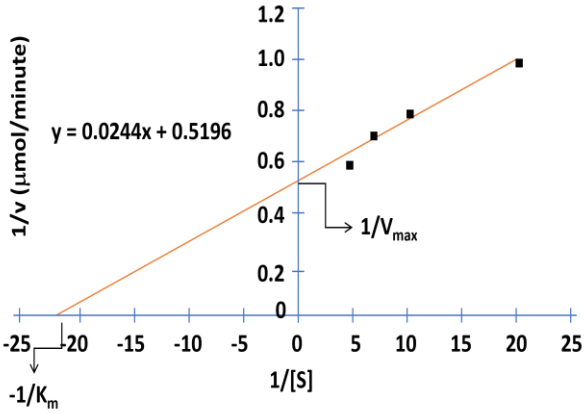


Figure 4. Lineweaver-Burk Curve of Lipase Enzyme Produced from *Aspergillus oryzae* Cells in Moldy Copra.

The findings illustrated in Figure 2 further indicate a reduction in the reaction rate of the lipase enzyme beyond the optimal pH threshold (pH = 8.2). This trend can be attributed to four underlying factors[24]. Firstly, the protein structure of the lipase enzyme may undergo denaturation due to pH extremes, whether excessively high or low. Secondly, the effectiveness of protein lipase enzymes hinges on the presence of ionized amino acid groups in their side chains, which could be functionally active solely in a specific ionization state. Thirdly, the substrate may alter proton content, thereby activating the lipase enzyme in just one charge form. Lastly, as pH decreases or [H+] concentrations rise, the negatively charged protein groups of the lipase enzyme can become protonated to neutralize their negative charge. Conversely, with an elevation in pH or an increase in [OH-] concentrations, the positively charged protein groups of the lipase enzyme undergo dissociation, resulting in their neutralization

The data in Figure 3 illustrates a direct correlation between the temperature and the activity of the lipase enzyme up to a point of 35°C. With the temperature elevation, the kinetic energy of each lipase enzyme molecule intensifies, consequently augmenting the interactions between these enzyme molecules. However, when the temperature surpasses 35°C, the lipase enzyme activity diminishes, primarily due to the initiation of enzyme protein denaturation caused by excessive heat. This establishes 35°C as the optimal temperature for the lipase enzyme derived from *Aspergillus oryzae* cells within moldy copra.

The main characteristics determined in studying the kinetic properties of enzymes are the maximum catalytic speed (V_{max}) and the substrate concentration when the catalytic speed reaches half the maximum (K_m). Lipase activity test using *p*-nitrophenylbutyrate substrate at intervals (0-0.05 M) and *p*-nitrophenol as a standard. The V_{max} and K_m constants were determined by the Lineweaver-Burk method.

Figure 4. displays the plotted relationship between the reciprocals of the enzyme reaction rate (1/v) on the Y-axis and the inverse of the substrate concentration (1/[S]) on the X-axis. The resultant linear equation was derived as Y = 0.024X + 0.519 through linear regression analysis. Subsequently, by substituting the slope value from the linear regression into the Lineweaver-Burk equation, $1/V = (K_m/V_{max})(1/[S]) + (1/V_{max})$, the Michaelis-Menten constant (Km) was calculated to be 0.046 M and the maximum reaction rate (Vmax) was calculated to be 1.926 µmol/min^{-1}. This finding contrasts the research on triolein catalysis by lipase isolated from *Theobroma cacao*. L yielded a Km value of 0.011 M and a V_{max} value of 11.63 µmol/min^{-1} [23]. A higher K_m value indicates a diminished substrate affinity, whereas a smaller K_m value signifies a heightened substrate affinity, requiring less substrate concentration for achieving the maximal catalytic reaction speed (V_{max}).

4. Purification and Determination of UCO FFA Content

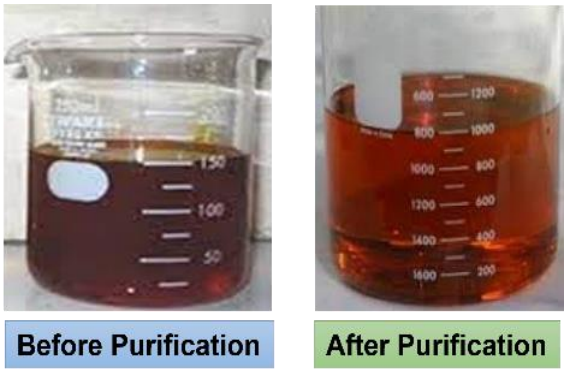


Figure 5. Changing the color of used cooking oil (UCO) before and after purification using avocado seeds activated charcoal.

Adsorption is a viable approach for mitigating the elevated levels of Free Fatty Acids (FFA) in Used Cooking Oil (UCO). In adsorption, the adsorbate (the substance subject to adsorption) adheres to the surface of the adsorbent (the substance promoting adsorption). This phenomenon's efficacy is exemplified in Figure 5, which depicts the observable color alteration of UCO before and post-purification. The initial hue of UCO was a dark brown shade, evolving into a reddish-brown tint following purification. This transformation underscores the activated charcoal adsorbent derived from avocado seeds, effectively capturing the particles responsible for UCO's opacity and dark hue. The efficacy of this adsorption stems from the avocado seed's activated charcoal adsorbent, boasting an expansive surface area measuring 19.62 m^2/g [25]. A substantial surface area augments the adsorbent's capability to accommodate adsorbate molecules, accentuating its adsorption capacity.

As stated by Langmuir-Hinshelwood, in instances where a solid surface facilitates a catalytic process involving a tri-molecular interaction between adsorbate molecules, a plausible mechanism encompasses the adsorption of the three adsorbate molecules—A, B, and C—onto an adjoining adsorbent surface site (S). Subsequently, the progression of the reaction unfolds through an intermediate activated complex, ultimately culminating in the generation of resultant reaction products—X, Y, and Z. (Figure 6) [26].

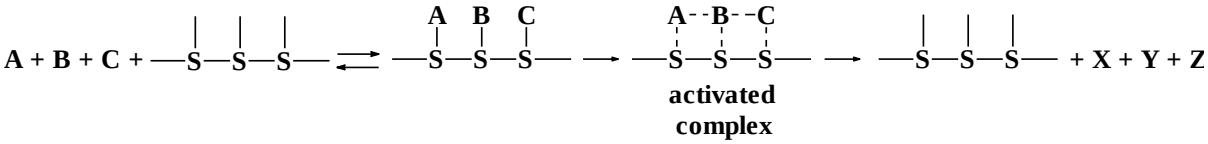


Figure 6. Mechanism of Absorption of Adsorbate Molecules on the Surface Site of The Adsorbent Through The Formation of Activated Complexes to Produce Products.

UCO FFA levels were determined based on ASTM D 664 procedures. The FFA content of oil is calculated using equation (1). If the FFA content of oil is < 2% (w/w), then methyl ester biosynthesis can be continued to the transesterification stage. Conversely, if the FFA content of oil is > 2% (w/w), then methyl ester biosynthesis starts from the esterification stage [15].

Table 4 shows the results of determining the FFA content of used cooking oil.

Table 4. The Results of Determining The FFA Content of Used Cooking Oil Before and After Refining Using Activated Charcoal From Avocado Seeds

Replication	KOH volume (mL)		FFA levels (%)		Decreased FFA Levels (%)
	Before Purification	After Purification	Before Purification	After Purification	
1	4.5	0.75	6.36	1.06	83.33
2	3.5	0.65	4.94	0.92	81.38
3	5.5	0.55	7.77	0.78	89.96
Average	4.5	0.65	6.36	0.92	84.89

Notes: KOH (0.1 M); UCO (2 g); Mr. FFA = 282.46 g/mol (as oleic acid, greatest relative abundance 41.08%)

Table 4 shows that activated charcoal from avocado seeds can reduce the FFA content of used cooking oil by an average of 84.89%. These results indicate that the average FFA content of

used cooking oil after refining is 0.92% < 2.00%, so the methyl ester biosynthesis process can proceed to the transesterification stage.

5. Methyl Ester Biosynthesis

Transesterification is the reaction of triglycerides with alcohols to produce methyl esters and glycerol as side products. The transesterification reaction conditions used in this study were UCO substrate (961 mL, 1 mol), methanol (364 mL, 9 mol), and lipase enzyme (15% v/v). The mixture was incubated at 50^{o}C for 24 hours and centrifuged at 150 rpm for 60 minutes. The result of UCO transesterification is a biphase solution (Figure 7). The lower phase is glycerol ($C_3H_8O_3$) (Mr = 92.09382 g/mol; ρ = 1.26 g/cm^3; td = 290 ^{o}C) [27] dark brownThee upper phase is biodiesel or methyl oleate ($C_{19}H_{36}O_2$) (Mr = 296.5 g/mol; ρ = 0.874 g/cm^3; td = 218 ^{o}C) [2,8] which is yellow and smells good.

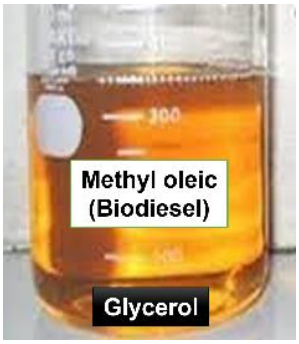


Figure 7. The Result of Biosynthesis of Methyl Esters from Used Cooking Oil uses The Lipase Enzyme As A Biocatalyst: Lower Phase (glycerol) and Upper Phase (methyl ester).

Table 5. The Percentage Yield of Methyl Ester from UCO Transesterification using Lipase as a Biocatalyst

Substrate Volume (mL)	Methanol Volume (mL)	Lipase Enzyme Level (% v/v)	Methyl Esters Volume (mL)	Percent Yield (% v/v)
961	364	15	727	75.65

Table 5 Illustrates a substantial methyl ester yield derived from UCO transesterification utilizing lipase (15% v/v) at an impressively elevated rate of 75.65% v/v. This underscores the effectiveness and efficiency of employing the lipase enzyme as a biocatalyst to convert triolein (triglycerides) into methyl oleate (methyl ester). This effectiveness is also substantiated by the employed mole ratio (triolein: methanol) of 1 9 in the biosynthesis of methyl esters. The reversible nature of the transesterification reaction is noteworthy. The introduction of an excessive amount of methanol moles into the equilibrium system elevates methanol concentration. This prompts the reaction between triolein and the surplus methanol, prompting an equilibrium shift from reactants (left) to products (right) (Figure 8). The phenomenon aligns with Le Chatelier's equilibrium principle, which posits that an equilibrium system adjusts to attain a balanced state in response to external pressure [29].

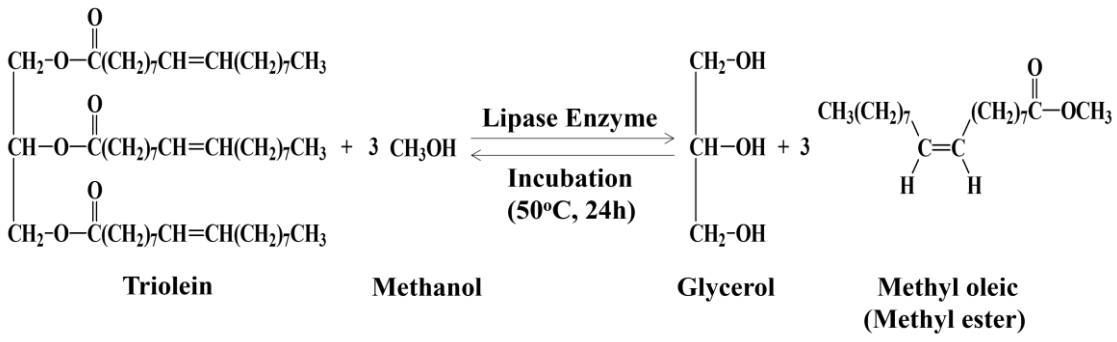


Figure 8. Triolein Transesterification Reaction forms Methyl Oleate using Lipase Enzyme Biocatalyst.

6. Characterization of Methyl Ester by FTIR

Samples of methyl ester solution were characterized using an FTIR spectrometer at wave numbers 4000-1400 cm^{-1} with a resolution of 1.2 Hz. The results of the interpretation of the FTIR spectrum of methyl esters (sample) and methyl oleic (standard) are shown in Table 6.

Table 6. The Results of Interpreting The FTIR Spectrum of Methyl Esters (Sample) and Methyl Oleic (Standard).

No.	Frequency (cm^{-1}) and Intensities		Frequency Ranges (cm^{-1})*	Group or Class	Type of Vibrations
	Methyl esters (sample)	Methyl oleic (standard)			
1	3448.72 (vs)	3448.72 (vs)	3500 - 3200	Carboxylic Acid, RCOOH	OH stretch
2	2924.09 (w)	2924.09 (w)	2940 - 2920 (w)	Aliphatic, RH	C-H stretches in $-CH_2-$
3	2852.72 (w)	2852.72 (w)	2970 - 2850 (w)		C-H stretch in $-CH_3$
4	1435.47 (m)	1465.90 (m)	1470 - 1435 (m)		CH_3 deformation
5	1635.64 (w)	1635.64 (w)	1665 - 1635 (w)	Ethenyl, -CH=CH-	C=C stretch (cis isomer)
6	1751.36 (vs)	1749.44 (vs)	1765 - 1720 (vs)	Esters, RCOOR'	C=O stretch
7	1263.37 (m)	1244.09 (m)	1290 - 1110 (m)		C-O-C antizyme stretch
8	671.23 (m)	580.30 (m)	675 - 575 (m)		O-C-O bend

◦Notes: vbr = very broad; vs = very strong; v = variable; s = strong; m = medium; w = weak.
*Sources: [15,27]

The FTIR spectrum data in Table 6 shows that both methyl ester (sample) and methyl oleate (standard) have functional group absorption types that are typical of unsaturated fatty acid methyl ester compounds [30]. Absorption bands from the ester group (RCOOR') appear at three frequencies, namely 1751.36 cm^{-1} originating from a very strong absorption band from C=O strain, 1263.37 cm^{-1} originating from a moderate absorption band from antisymmetric strain C-O-C, and 671.23 cm^{-1} comes from the moderate absorption band of the O-C-O bend [31]. The appearance of the C=O ester group at 1751.36 cm^{-1} is following the results of previous studies, namely 1740 cm^{-1} [32], 1743.65 cm^{-1} [33], 1749 and 1751 cm^{-1} [34], and 1749.44 cm^{-1} (methyl oleate standard). The strong absorption band at 3448.72 cm^{-1} comes from stretching the carboxylic acid OH group (RCOOH). This data follows the results of previous studies, namely 3471.87 cm^{-1} [32], 3448 cm^{-1} [33], and 3448.72 cm^{-1} (methyl oleate standard). Three aliphatic C-H stretching absorption bands appeared at 2924.09 cm^{-1} from the methylene group ($-CH_2-$), 2852.72 cm^{-1} from the methyl group ($-CH_3$), and 1435.47 cm^{-1} from the deformed CH_3 group. This data is by the results of previous studies, namely 2916 cm^{-1} ($-CH_2-$) and 2848 cm^{-1} ($-CH_3$) [31], 2924 cm^{-1} ($-CH_2-$), and 2854 cm^{-1} ($-CH_3$) [35], 2924.09 cm^{-1} ($-CH_2-$) and 2852.72 cm^{-1} ($-CH_3$) [32], 2926.01 cm^{-1} ($-CH_2-$), 2854.65 cm^{-1} ($-CH_3$), and 1423.47 cm^{-1} of the deformed CH_3 group [33], and 2924.09 cm^{-1} ($-CH_2-$), 2852.72 cm^{-1} ($-CH_3$), and 1465.90 cm^{-1} of the deformed CH_3 group (standard methyl oleate). One moderate absorption band that appears at 1635.64 cm^{-1} indicates the presence of an ethenyl group (-CH=CH-) from the alkene. This data is by the results of previous studies, namely 1627.92 cm^{-1} (-CH=CH-) [32], 1643 cm^{-1} (-CH=CH-) [34], 1635 cm^{-1} (-CH=CH-) [33], and 1635.64 cm^{-1} (-CH=CH-) (methyl oleate standard).

Based on the data in Table 6 and the description above, we can conclude that the transesterification reaction of UCO and methanol using a lipase enzyme catalyst can produce methyl esters with the functional groups C=O, C-O-C, O-C-O, -OH, $-CH_2-$, $-CH_3$, and -CH=CH- [35].

The FTIR analysis outcomes in Table 4 indicate a notable degree of precision and accuracy. The precision of the FTIR measurement setup is evident through the consistency or resemblance of the measured frequency and intensity values of the methyl ester (sample) to the genuine values of methyl oleate (standard). Meanwhile, the repeatability or precision of the FTIR measurement system is apparent from the findings of frequency and intensity measurements for the methyl ester (sample), which remain unchanged and closely mirror the outcomes of other research efforts. Consequently, it can be deduced that the margin of error associated with the measurement of frequency and intensity of methyl ester (samples) through FTIR analysis is exceedingly negligible.

7. Characterization of Methyl Ester by GC-MS

Methyl ester solution samples were characterized by the GC-MS analysis method. The GC-MS specifications used are He carrier gas with a pressure of 10-50 psi and a flow rate of 25-150 mL/min, a capillary column with a length of 5-100 m and a diameter of 0.2-0.7 mm, and the temperature is set to rise regularly from 30-180 ^{o}C for 35 minutes,

Table 7 shows that three methyl ester compounds are contained in the sample resulting from the UCO transesterification reaction using the lipase enzyme as a biocatalyst. The three compounds were methyl palmitate, with an abundance of 12.53% and a retention time of 25.008 minutes; methyl vaccinate, with an abundance of 16.44% and a retention time of 30.680 minutes; and methyl oleate, with an abundance of 41.08% (the highest) and a retention time of 47.443 minutes. These three compounds have also been reported by [36–45] as the main components of methyl esters in transesterification products from various vegetable oils.

Table 7. Results of Methyl Ester Analysis with GC-MS

No	Compound Name	Molecular Formula and Structure	Molecular Mass (g/mol)	Retention Time (minute)	Relative Abundance (%)
1	Palmitic acid methyl ester Hexadecanoic acid	$C_{16}H_{32}O_2$ $CH_3(CH_2)_{14}COOH$	256.42	25.008	12.53
2	Vaxenic acid methyl ester (11*E*)-Octadec-11-enoic acid	$C_{18}H_{34}O_2$ $CH_3(CH_2)_5$**CH=CH**$(CH_2)_9COOH$	282.46	30.680	16.44
3	Oleic acid methyl ester (9*Z*)-Octadec-9-enoic acid	$C_{18}H_{34}O_2$ $CH_3(CH_2)_7$**CH=CH**$(CH_2)_7COOH$	282.46	47.443	41.08

The data presented in Table 7 further demonstrates that short-chain esters elute from the column in advance of their long-chain counterparts, aligning with the principle of "like dissolves like." This principle signifies that polar compounds, such as short-chain esters, tend to migrate out of the column alongside the mobile phase, which is also polar. Conversely, longer-chain esters, characterized by their nonpolar nature, tend to be retained within the column.

The effectiveness of the lipase enzyme in the methyl ester biosynthesis reaction from UCO is quite good. This can be seen from the percentage of methyl oleate produced in the study of 41.08%, which is quite high when compared to the results of previous studies, 38.84% [37], 34.86% [38], 31.29% [39], and 10.30% [46].

The precision and accuracy of the GC-MS analysis results for methyl esters in Table 5 exhibit considerable excellence. The accuracy of

the GC-MS measurement system is evidenced by the close resemblance between the measured retention time values and the relative abundance of methyl ester (sample) compared to the actual methyl oleate (standard) value. In parallel, the precision or replicability of the GC-MS measurement system is discernible from the consistent retention time values and relative abundance of methyl esters (samples), which remained constant and yielded congruent or akin outcomes as observed in other studies. As a result, we can affirm that the margin of error in the GC-MS analysis for the retention time values and relative abundance of methyl ester (sample) is exceedingly minute.

CONCLUSION

The lipase enzyme derived from the fermentation process of *Aspergillus oryzae* on moldy copra exhibits an activity of 43.76 units/mg protein, possessing a molecular weight of 41.7 kDa. Its optimal pH and temperature are 8.2 and 35°C, respectively, with a K_m of 0.046 and V_{max} of 1.926 µmol/minute. This lipase enzyme effectively catalyzes the transesterification reaction of UCO (triolein) into methyl ester (methyl oleate) with a substantial yield of 75.65%. The FTIR characterization of methyl esters reveals the presence of distinct functional groups such as –OH carboxylic acid, C=C alkenes, C=O esters, methyl (CH3-), and methylene (-CH2-) groups. GC-MS analysis further confirms the presence of three specific compounds: methyl palmitate (12.53% abundance, retention time 25.008 minutes), methyl vaccinate (16.44% abundance, retention time 30.680 minutes), and methyl oleate (highest at 41.08% abundance, retention time 47.443 minutes). These findings align with similar previous studies, underlining the applicability of the lipase enzyme derived from *Aspergillus oryzae* on moldy copra in catalyzing transesterification reactions. Moreover, these results propose the potential utilization of this lipase enzyme as a biocatalyst in the pharmaceutical and food ingredient industries.

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REFERENCES

[1] R. N. Sari, E. Hastarini, A. Henang, W. Widyajatmiko, and A. H. Tambunan, "Characteristics of Siamese Patin (*Pangasius hypophthalmus*) Oil Based Biolubricant," *JPB Kelautan dan Perikanan,* vol. 12, no. 2, pp. 158-167, 2021, doi: 10.15578/jpbkp.v15i2.675.

[2] D. Malhotra, J. Mukherjee, M. N. Gupta, and Munishwar, "Lipase Catalyzed Transesterification of Castor Oil by Straight Chain Higher Alcohols," *Journal of Bioscience and Bioengineering,* vol. 119, no. 3, pp. 280-283, 2015, doi: 10.1016/j.jbiosc.2014.08.005.

[3] L. Buchori, I. Istadi, and P. Purwanto, "Perkembangan Proses Produksi Biodiesel Sebagai Bahan Bakar Alternatif," *Prosiding Seminar Nasional Teknik Kima "Kejuang"*, pp.1-9, 2015.

[4] Y. Mingrui, Q. Shaowei, & T. Tanwei, "Purification and Characterization of the Extracellular Lipase Lip2 from *Yarrowia*

lipolytica," *Process Biochemistry*, vol. 42, pp. 384-391, 2007, doi: 10.1016/j.procbio.2006.09.019.

[5] C. Ling-Zhi, T. Chin-Ping, K. Long, M. S. A. Yusoff, N. Arifin, L. Seong-Koon, and L. Oi-Ming, "Production of a Diacylgliserol-Enriched Palm Olein Using Lipase-Catalyzed Partial Hydrolysis: Optimization Using Response Surface Methodology," *Food Chemistry*, vol. 105, pp. 1614- 1622, 2007, doi: 10.1016/j.foodchem.2007.03.070.

[6] G. P. McNeill, and P. E. Sonnet, "Low-calory Synthesis by Lipase-catalyzed Esterification of Monoglycerides," *JAOCS*, vol. 72, no. 11, pp. 1301-1307, 1995, doi: 10.1007/BF02546203.

[7] A. Halldorsson, B. Kristinsson, C. Glynn, and G. G. Haraldsson, "Separation of EPA and DHA in Fish Oil by Lipase-catalyzed Esterification with Glycerol," *JAOCS*, vol. 80, no. 9, pp. 915- 921, 2003, doi: 10.1007/s11746-003-0796-8.

[8] R. Sharma, Y. Chisti, and U. C. Banerjee, "Production, Purification, Characterization, and Application of Lipase," *Biotechnology Advances*, vol.19, pp. 627-662, 2001, doi: 10.1016/s0734-9750(01)00086-6.

[9] S. Dali, A. R. Patong, M. N. Jalaluddin and P. A. Parenrengi, "Exploration and Isolation of Lipase Enzyme from Indigenus Infective Fungi (genus *Aspergillus* and *Penicillium*)," *Lephas*, vol. 14, no. 1, pp. 2005, doi: 10.31258/jnat.14.1.26-31.

[10] S. Dali, A. R. Patong, M. N. Jalaluddin, and P. A. Parenrengi, "Pemurnian dan Karakterisasi Enzim Lipase dari *Aspergillus oryzae* pada Kopra Berjamur," *Jurnal Natur Indonesia,* vol. 14, no. 1, pp. 26-31, 2011, doi: 10.31258/jnat.14.1.26-31.

[11] J. Sambrook, T. Fritsch, and T. Maniatis, *Molecular Cloning: Laboratory Manual 1*, New York: Cold Spring Harbor Laboratory Press,1989, ISBN: 9780879695767.

[12] T. Vorderwulbecke, K. Kieslich, and H. Erdmann, "Comparison of Lipases by Different Assay. Enzyme," *Microb. Technol*., vol. 14, pp. 631-639,1992, doi: 10.1016/0141-0229(92)90038-P.

[13] S. P. Colowick, and N. O. Kaplan, *Methods in Enzymology*, New York: Academic Press. Inc. 1957.

[14] M. P. Deutscher, *Methods in Enzymology*: *Guide to Protein Purification*, vol. 128, New York: Academic Press Inc. 1990, ISBN: 9780121820831.

[15] N. D. Wela, A. D. Wela, S. Dali, A. Chairunnas, H. A. M. Amalia and S. A. A. Puspitasari, "Study of Reaction Conditions for the Synthesis of Methyl Oleic from Used Cooking Oil," *Akta Kimindo*, vol. 6, no. 1, pp. 41-57, 2021. doi: 10.12962/j25493736.v6i1.8106.

[16] J. Tong, M. Nakajima, H. Nabetani, Y. Kikuchi, "Characterization of surfactants used for monodispersed oil-in-water microspheres production by microchannel emulsification," *Studies in Surface Science and Catalysis*, vol. 132, pp. 1055-1059, 2001, doi: 10.1016/S0167-2991(01)82264-3.

[17] A. Basile and F. Dalena, *Methanol*, Elsevier, 2017, ISBN: 9780444639035.

[18] N. B. M. Zin, B. M. Yusof, S. N. Oslan et al., "Utilization of acid pre-treated coconut dregs as a substrate for production of detergent compatible lipase by *Bacillus stratosphericus*," AMB Express, vol. 7, no. 1, pp. 131, 2017, doi: 10.1186/s13568-017-0433-y.

[19] R. Handayani, and J. Sulistyo, "Lipase Application of lipase technology for transesterification of fatty acid ester,"

Biodiversitas, vol. 6, no. 3, pp. 164-167, 2005, doi: 10.13057/biodiv/d060304.

[20] N. Dali, and A. Dali, "Synthesis of Nitro Ethyl Oleic from Used Cooking Oil," *Al-Kimia*, vol. 7, no. 1, pp. 46-55, 2019. doi: 10.24252/al-kimia.v7i1.7642.

[21] H. Abbas, A. Hiol, V. Deyris, and L. Comeau, "Isolation and Characterization of an Extracellular Lipase from Mucor sp Strain Isolated from Palm Fruit," *Enzyme and Microbial Technology*, vol. 31, pp. 968-975, 2002, doi: 10.1016/S0141-0229(02)00190-4.

[22] C. Shaoxin, Q. Lili, and S. Bingzhao, "Purification and Properties of Enantioselective Lipase from a Newly Isolated *Bacillus cereus* C71," *Process Biochemistry*, vol. 42, pp. 988- 944, 2007, doi: 10.1016/j.procbio.2007.03.010.

[23] R. A. Samsumaharto, "Partial Characterization of Lipase from Cocoa Beans (*Theobromacacao* L.) of Clone PBC 159," *Indo. J. Chem*, vol. 3, pp. 448-453, 2008, doi: 10.22146/ijc.21604.

[24] D. S. Page, *Principles of Biological Chemistry,* New York: Willard Grant Press, 1981, doi: 9780871507136.

[25] Rahayu, "Pemanfaatan Limbah Lemak Ayam Broiler (*Gallus domesticus*) Sebagai Bahan Baku Pembuatan Biodiesel Melalui Proses Transesterifikasi," *J. Chemurgy,* vol. 2, no. 2, pp. 6-14, 2014, doi: 10.30872/cmg.v2i2.2232.

[26] M. S. A. Rahman, M. J. Jalil, M. Muain et al., "Epoxidation of waste cooking oil using catalytic in situ generated performic acid," *IOP Conference Series: Earth and Environmental Science*, vol. 476, 2020, doi: 10.1088/1755-1315/476/1/012143.

[27] M. Pagliaro, *Glycerol,* Elsevier, 2017, ISBN: 9780128122051.

[28] T.W. Abraham, R. Höfer, "Lipid-Based Polymer Building Blocks and Polymers," *Polymer Science: A Comprehensive Reference*, vol. 10, pp. 15-58, 2012, doi: 10.1016/B978-0-444-53349-4.00253-3.

[29] J. B. Lambert, S. Gronert, H. F. Shurvell, and D. A. Lightner, *Organic Structural Spectroscopy*, 2nd Ed., New Jersey: Pearson Prentice Hall, 2011, ISBN: 9781292054056.

[30] W. Kemp, *Organic Spectroscopy*, 3rd Ed., London: MacMillan Education Ltd., 1991. DOI: 10.1007/978-1-349-15203-2.

[31] H. Sastrohamidjojo, *Dasar-dasar Spektroskopi*, Yogyakarta: UGM Press, 2018, ISBN: 9789794208175.

[32] D. Suhendra, E. R. Gunawan, "Sintesis Asam-Asam Lemak Hidroksamik dari Minyak Kelapa Menggunakan Lipase sebagai Katalis," *Jurnal Natur Indonesia*, vol. 12, no. 2, pp.160-164, 2012, doi: 10.31258/jnat.14.1.160-164.

[33] J. H. Mandei, M. Edam, Y. F. Assah et al., "Metil Ester Minyak Kelapa Murni yang Telah Diekstrak Senyawa Fenolik dengan Variasi Waktu Transesterifikasi." *Jurnal Riset Teknologi Industri*, vol. 14, no. 2, pp. 309-319, 2020, doi: 10.26578/jrti.v14i2.6557.

[34] R. D. Kusumaningtyas, Normaliza, E. D. N. Anisa et al., "Synthesis of Biodiesel via Interesterification Reaction of *Calophyllum inophyllum* Seed Oil and Ethyl Acetate over Lipase Catalyst: Experimental and Surface Response Methodology Analysis," *Energies*, vol. 15, no. 20, pp. 7737, 2022, doi: 10.3390/en15207737.

[35] D. Malhotra, J. Mukherjee, M. N. Gupta, and Munishwar, "Lipase Catalyzed Transesterification of Castor Oil by Straight Chain Higher Alcohols," *Journal of Bioscience and Bioengineering,* vol. 119, no. 3, pp. 280-285, 2015, doi: 10.1016/j.jbiosc.2014.08.005.

[36] D. Susvira, R. Hartono and R. A. Fauzantoro, "Synthesis of Biodiesel from Waste Cooking Oil Using Heterogeneous Catalyst (CaO) Based on Duck Eggshell with Transesterification Reaction," *Jurnal Kartika Kimia,* vol. 5, no. 1, pp. 40-43, 2022,
doi: 10.26874/jkk.v5i1.122.

[37] Y. K. Salimi, N. I. Ischak, and Y. Ibrahim, "Karakterisasi Asam Lemak Hasil Hidrolisis pada Minyak Biji Kelor (*Moringa oleifera*) dengan Metode Kromatografi Gas-Spektroskopi Massa," *Jamb. J. Chem.*, vol. 01, no. 1, pp. 6-14, 2019.
doi: 10.34312/jambchem.v1i1.2101.

[38] M. T. Bintang, Aisyah, and A. Saleh, "Sintesis Biodiesel dari Minyak Biji Nyamplung (*Callophyllum innophylum L.*) dengan Metode Ultrasonokimia," *Chimica et Natura Acta*, vol. 3 no. 2, pp. 84-89, 2015,
doi: 10.24198/cna.v3.n2.9199.

[39] E. Setiawati, F. Edwar, "Teknologi Pengolahan Biodiesel dari Minyak Goreng Bekas dengan Teknik Mikrofiltrasi dan Transesterifikasi sebagai Alternatif Bahan Bakar Mesin Diesel," *Jurnal Riset Industri*, vol. VI, no. 2, pp. 117-127, 2012.

[40] E. G. Fransina, J. Latupeirissa, "Analysis of Fatty Acid Components in Blue Bubara Fish (*Caranx melampygus*)," *MjoCE*, vol. 2, no. 2, pp. 89-95, 2012.
doi: 10.30598/MJoCEvol2iss2pp89-95.

[41] R. Musta, L. Nurliana and M. M. Halulanga, "Synthesis of Methyl Ester Nitrate from Mahogany Seed Oil (*Swietenia mahagoni Linn*)," *Indonesian Journal of Chemical Research,* vol. 9, no. 1, pp. 63-68, 2021,
doi: 10.30598//ijcr.2021.9-rus.

[42] I. Aziz, M. N. Aristya, Hendrawati, and L. Adhani, "Peningkatan Kualitas Crude Glycerol dengan Proses Adsorpsi Menggunakan Sekam Padi," *Jurnal Kimia Valensi*, vol. 4, no. 1, pp. 34-41, 2018,
doi: 10.15408/jkv.v4i1.7498.

[43] D. Hashatan, J. Sunaryo, and L. N. Komariah, "Pengaruh Konsentrasi H_2SO_4 dan Waktu Reaksi terhadap Kuantitas Dan Kualitas Biodiesel dari Minyak Jarak Pagar," *Jurnal Teknik Kimia,* vol. 2, no. 18, pp. 26-36, 2012.

[44] F. C. Hidayati, Masturi, and I. Yulianti, "Pemurnian Minyak Goreng Bekas Pakai (Jelantah) dengan Menggunakan Arang Bonggol Jagung," *Jurnal Ilmu Pendidikan Fisika, v*ol. 1, no. 2, pp. 67-70, 2016,
doi: 10.26737/jipf.v1i2.67.

[45] Abdullah, Triyono, W. Trisunaryanti, and W. Haryadi, "Purification of Methyl Ricinoleate on Producing of Cetana Improver," *J. Phys. Conf. Series.,* vol. 82, no. 4, pp. 1-6, 2016,
doi: 10.1088/1742-6596/824/1/012018.

[46] J. H. Mandei, M. Edam, Y. Assah, A. Makalalag, and D. Silaban, "Metil Ester Minyak Kelapa Murni yang Telah Diekstrak Senyawa Fenolik dengan Variasi Waktu Transesterifikasi," *Jurnal Riset Teknologi Industri*, vol. 14, no. 2, pp. 307-316, 2020,
doi: 10.26578/jrti.v14i2.6557.