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Research Article

# Development of a Novel Support Modification for Efficient Lipase Immobilization: Preparation, Characterization, and Application for Bio-flavor Production

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## Abstract

The low cost and excellent catalytic properties of lipase for industrial processes are highly desirable. A promising new approach involves the support modification of lipase and spacer arm, which enables the enhancement of lipase properties. This study investigates the immobilization of crude lipase from *Mucor miehei* onto a Polyurethane Foam (PUF) surface using various coating techniques. The PUF matrix was obtained through isocyanate and polyol reactions. Subsequently, the PUF was coated by adsorbing lipase and adding edible support material. The immobilized lipase was then utilized in the hydrolysis of coconut oil to produce fatty acids. Furthermore, the immobilized enzyme was employed in the esterification of fatty acids to produce bio-flavors. The results demonstrate that the attachment reaction using support material, namely lecithin, gelatin, $MgCl_2$, and Polyethylene glycol 6000 (PEG), all of which are simple and edible, was able to enhance the stability and reusability of lipase. This immobilization technique increased triglyceride hydrolysis into FFA by 422%. The successful edible support modification of immobilized lipase from *M. miehei* on PUF, coupled with significantly enhanced enzyme stability and catalytic activity, offers a promising, environmentally friendly solution for diverse applications in the food industry.



***Keywords***: Immobilization; Bioflavor; *Mucor miehei* lipase; Polyurethane foam; Support modification

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## 1. Introduction

Over the past decades, lipase enzymes (triacylglycerol hydrolases E.C.3.1.1.3) have garnered extensive attention due to their pivotal role in hydrolyzing lipids, prompting exhaustive research across various domains including medical, pharmaceuticals, food, and cosmetics [1–3]. Lipase also finds broad application across diverse sectors, such as wastewater treatment, textiles, agriculture, and the fuel industry [4,5]. However, the utilization of free enzymes is often hindered by drawbacks, such as low stability and poor reusability [6,7]. Recently, diverse methods and novel support materials have been developed, along with the utilization of different bioreactors, aiming to enhance the immobilization of lipase [8].

Currently, research on enzyme immobilization technology is primarily categorized based on the type of supports and methods of immobilization [9]. Several techniques have been proposed for lipase immobilization, including encapsulation, carrier-free cross-linking, adsorption, and covalent multipoint attachment [10]. Employing an appropriate

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immobilization method can enhance certain enzyme properties of the immobilized lipase, such as stability, activity, selectivity, and even purity [11,12].

The use of PUF as a support has been explored by previous researchers [13,14], leveraging its inert and rigid nature. However, comprehensive studies on the specific roles of each support material have not been conducted. Additionally, although simple support modification techniques have been previously reported, a comprehensive investigation of these methods is lacking. Moreover, there is a dearth of studies on bio-flavor synthesis via immobilized lipase in a solvent-free system. Furthermore, existing reports predominantly utilize a commercially available lipase, Novozym 435, rather than crude lipase [15]. Commercial lipases offer high purity, consistent activity, and tailored characteristics for specific applications [16], unlike crude lipase, which may contain impurities and exhibit variable performance [17]. Extensive literature supports the reliability and versatility of commercial lipases, serving as a benchmark for researchers [18]. Nevertheless, research on crude lipase offers cost-effective, diverse, and potentially sustainable enzyme sources, aiding in process optimization and fostering innovation by uncovering novel enzymes and applications [19].

In this study, support materials including gelatin, lecithin, and $MgCl_2$ were utilized to improve the immobilization of crude lipase from *M. miehei* onto PUF. The selection of gelatin, lecithin, $MgCl_2$, and PEG for immobilizing crude lipase from *M. miehei* onto PUF was guided by several factors. Firstly, gelatin and lecithin were chosen for their adhesive properties, facilitating the binding of lipase molecules to the PUF matrix [20,21]. Moreover, these materials have been reported to enhance enzyme stability and activity when immobilized. Secondly, $MgCl_2$ was selected as a crosslinking agent to stabilize the immobilized lipase. Crosslinking agents like $MgCl_2$ help form stronger bonds between enzyme molecules and the support matrix, thereby improving the robustness of the immobilization process [22]. Lastly, PEG was included as a spacer molecule to create distance between enzyme molecules and the support matrix [23], reducing steric hindrance and enhancing enzyme accessibility to substrates [24]. This selection of materials aimed to improve the efficiency and performance of the immobilization process, ultimately enhancing the catalytic activity of the immobilized lipase on PUF. In this work, the effectiveness of the immobilization technique was assessed based on catalytic activity, thermal stability, and the PUF-immobilized ratio. Subsequently, the immobilized lipase was employed to hydrolyze coconut oil into fatty acids, followed by esterification of the fatty acids with citronellol to produce bio-flavors.

## 2. Materials and Methods

### 2.1 Materials

Virgin coconut oil (VCO), commercially known as Laitco, was obtained from Permata Agrindo Pendowoharjo (Sewon, Bantul, Indonesia). A stock culture of *M. miehei* was sourced from the Biochemical Technology Laboratory, Department of Chemical Engineering, Sepuluh Nopember Institute of Technology, Indonesia. Natural citronellol was obtained from Sigma-Aldrich, China. All other chemicals were procured from local markets. Fatty acid standards for GC-MS analysis were purchased from Sigma Aldrich.

### 2.2 Production of Lipase from *M. miehei*

*M. miehei* strain was maintained on Potato Dextrose Agar. Typically, 3.9 g of PDA was dissolved in 100 mL of water, poured into an erlenmeyer flask, and sterilized at 121 °C for 15 min. After cooling in an oblique position, it solidified to form the medium. The stock culture of *M. miehei was* aseptically inoculated and then incubated at 37 °C for 5 days. The resulting culture was suspended in 10 mL of water containing 0.1% (v/v) Tween-80. Subsequently, a 10% sample of this suspension was cultured in a solid-state fermentation medium composed of 5% peptone, 1.4% $KH_2PO_4$, 0.001% $FeSO_4.7H_2O$, 10% olive oil, 10% palm oil, 20% dried coconut grout, and 53.99% water. The initial pH of the culture medium was adjusted to 5. The suspension was then incubated at 37 °C in an orbital shaker operating at 120 rpm for 5 days. The enzyme was extracted using a phosphoric buffer solution (100 mM, pH = 7) under a solid-state fermentation medium to liquid ratio of 1:4 (w/w) at 37 °C for 30 min. The resulting culture was filtered using Whatman filter paper no. 2 and then centrifuged to obtain a supernatant of crude lipase. In this study, the inducer compounds (triacylglycerol) added to the media were coconut and olive oil. Tween 80 functions to reduce surface tension, facilitating the secretion of secondary metabolites [21,23].

The utilization of diverse buffer solutions for enzyme extraction and washing of the PUF-immobilized lipase constitutes a strategic approach in the experimental protocol. Initially, phosphoric buffer solution was opted for enzyme extraction due to its capacity to furnish an ideal pH milieu, thereby preserving enzyme stability and activity throughout the extraction process [25]. Renowned for its efficacy in solubilizing enzymes from source materials while upholding their functional integrity, phosphoric buffer is a

preferred choice in enzyme extraction procedures [26]. Conversely, for the washing of PUF-immobilized lipase, sodium phosphate buffer solution was selected to expunge any residual impurities or unbound enzyme molecules [27]. This selection was based on sodium phosphate buffer's compatibility with the immobilized enzyme, enabling efficient removal of contaminants while conserving enzyme activity. The utilization of distinct buffer solutions serves to optimize conditions for enzyme extraction and purification, thereby ensuring the effective recovery and stabilization of the enzyme for subsequent experimental analyses.

### 2.3 The Synthesis of PUF

The PUF matrix was made by polymerizing polyisocyanate (RCNO) and polyol (R-OH) in a 1:1 (v/v) ratio. In brief, 50 mL of polyisocyanate was placed in a plastic tube, followed by 50 mL of polyol, which was then evenly agitated for 3 min. The substance swelled into a foam, releasing $CO_2$ through the pores. Finally, the PUF was cut into squares measuring 0.5 cm × 0.5 cm × 0.5 cm.

### 2.4 Immobilization of Crude Lipase from *M. miehei*

The methods used for immobilization are adsorption and covalent bonding. In the adsorption method, PUF is attached to the lipase without the addition of support materials. In the covalent method, PUF is attached to the spacer arm, followed by the binding of lipase.

The covalent method involved immersing PUF squares measuring 0.5 × 0.5 × 0.5 $cm^3$ in a support solution for 1 h in an incubator at weight ratios of 1:10, 1:15, 1:20, 1:25, and 1:30, as detailed in Table 1. Subsequently, the immobilized PUF was drained and dried in an oven at 30 °C for 1 h. PUF impregnated with support materials is then soaked for 24 h in crude lipase at a ratio of 1:20, drained, and dried in the oven at 30 °C until all liquid has evaporated. The covalent immobilization technique represents an advancement of the previous method with a matrix-to-support ratio of 1:2.

The immobilization efficiency was assessed through activity and yield measurements. Activity analysis was calculated using Equation (1), replacing 1 mL of enzyme suspension with 1 g of immobilized lipase on PUF. This involved washing the PUF-immobilized lipase with 5 mL of sodium phosphate buffer solution at pH 7, followed by activity analysis. Moreover, immobilization was evaluated based on the PUF-immobilized lipase's capacity to absorb lipase as a protein. Protein content in the free lipase solution was determined using the Bradford method:

$$Lipase\ Activity\ (U/ml) = \frac{(A-B)\times N_{NaOH}\times 1000}{t} \quad (1)$$

where, $A$ represents the volume of NaOH needed for titration when using lipase, while $B$ represents the volume required without lipase, both measured in milliliters. The variable $t$ denotes the incubation time, which was set at 30 min. The determination of matrix activity followed a similar procedure, but instead of using a 1 milliliter crude sample, co-immobilized lipase-PUF 1 gram was utilized.

Post-immobilization, the supernatant was separated from the PUF, and the remaining unimmobilized lipase concentration was determined using the BSA standard curve. The modified immobilization % yield equation is expressed in Equation (2).

$$\%Immobilization\ Yield = \frac{Initial\ lipase\ (\text{mg/ml}) - Supernatant\ (\text{mg/ml})}{Initial\ lipase\ (\text{mg/ml})}\times 100\% \quad (2)$$

### 2.5 Hydrolysis by Immobilized Lipase of *M. miehei*

The hydrolysis of coconut oil by lipase immobilized on PUF yields free fatty acids (FFA) and glycerol. The hydrolysis reaction was conducted in a three-neck flask. Coconut oil and deionized water were mixed in a weight ratio of 1:1 (w/w). A total of 7.5 U of immobilized lipase per gram of PUF was added to 10 g of the coconut oil-water mixture. The reaction was maintained at 40 °C for various reaction times, up to 20 h. After incubation, the reaction was stopped by adding 15 ml of acetone-ethanol (1:1 v/v), and FFA were titrated with 0.05 M KOH [28].

The product of the hydrolysis reaction consisted of two layers: fatty acids in the upper

Table 1. Support material requirement at each support ratio.

| PUF (g) | PUF and Support ratio (w/w) | Support materials (g) | | | | |
|---|---|---|---|---|---|---|
| | | Lechitin | Gelatin | $MgCl_2$ | PEG | Total |
| 20 | 1:10 | 50 | 50 | 50 | 50 | 200 |
| 20 | 1:15 | 75 | 75 | 75 | 75 | 300 |
| 20 | 1:20 | 100 | 100 | 100 | 100 | 400 |
| 20 | 1:25 | 125 | 125 | 125 | 125 | 500 |
| 20 | 1:30 | 150 | 150 | 150 | 150 | 600 |

layer and glycerol in the lower layer, which were separated using a separating funnel. The chemical properties of the product were determined, including the acid value for the upper layer as the following Equation 3.

$$Acid\ value = \frac{V_{KOH}(ml) \times C_{KOH}(N) \times 56.1}{Oil\ sample\ (g)} \quad (3)$$

2.6 Esterification by Immobilized Lipase of *M. miehei*

FFA produced from the hydrolysis reaction of coconut oil were utilized as the substrate for further esterification reactions. The esterification reaction was conducted in a batch process using an erlenmeyer flask containing FFA, citronellol, and immobilized lipase. The substrates of FFA, such as lauric acid, and citronellol were prepared in a molar ratio of 1:1, and the immobilized lipase was added. The enzyme activity used in the esterification process was 7.5 U of immobilized lipase per gram of PUF. A quantity of 5 g of FFA containing 1.07% lauric acid, equivalent to 0.0003 moles, reacted with 0.0003 moles of citronellol (molar ratio 1:1) or 0.05 g in a glass vial bottle. Subsequently, incubation was conducted at 40 °C for 20 h, and the reaction was halted by adding 10 ml of an acetone-ethanol mixture (1:1 v/v). The reaction was maintained at 40 °C for 25 h.

2.7 Analysis and Characterization Methods

The determination of crude lipase involves two methods: enzyme activity and protein content analysis. Lipase activity, expressed in units (U), denotes the enzyme's ability to release 1 µmol of fatty acids per minute under specific conditions. For protein content analysis, the Bradford method was employed. Typically, 0.1 mL of crude lipase was mixed with 5 mL of Bradford reagent and incubated at room temperature for 10 min.

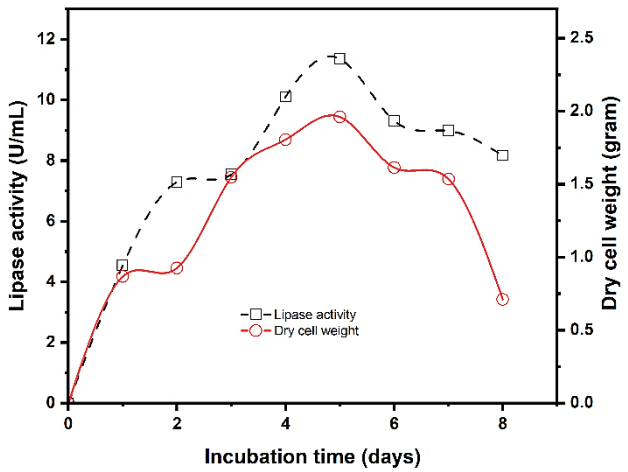


Figure 1. Lipase activity and dry cell weight on *M. miehei* growth

Coomassie Brilliant Blue (CBB) G-250 served as the substrate, with Bovine Serum Albumin (BSA) as the standard solution. Protein concentration was determined by measuring the absorbance at 595 nm using a spectrophotometer (Cecil CE 1011, Peterborough Cambridge, England).

The surface topography and elemental composition of the immobilized lipase on PUF were investigated using a Phenom Pro X (Phenom-World, Eindhoven, Netherlands) SEM/EDS. This analysis allows for the physical examination of sample structures and the determination of their elemental composition [29]. The element identification software package, in conjunction with a specially designed and fully integrated Energy Dispersive Spectrometer (EDS), facilitates the rapid identification of different chemical elements in a specimen.

The FTIR analysis of all samples, both before and after soaking in SBF, was conducted using a Shimadzu FTIR Prestige 21 spectrophotometer (Tokyo, Japan). The instrument enabled the recording of transmittance spectra in the 400 – 4000 $cm^{-1}$ region with a resolution of 2 $cm^{-1}$ (45 scans). The FTIR spectra were analyzed using Prestige software (IR solution) [30,31].

The product of esterification consisted of two layers: ester above and water below. The presence of natural flavor ester was confirmed using GC-FID analysis (HP 5890, Santa Clara, California, United States). The conversion of citronellol was determined based on the area under the chromatogram graph [32].

The content of coconut oil was analyzed using GC-MS-AGILENT 6980 N with a J&W Scientific HP 5% phenylmethyl siloxane column with 30 m × 0.32 mm i.d. × 0.25 µm film thickness. The specifications of the GC-FID HP 5890 used included an HPL 608 column with dimensions of 30 m length × 0.53 mm i.d. × 0.5 µm film thickness. The GC-FID oven temperature was adjusted to 125 °C for 3 min, with a rise of 7.5 °C $min^{-1}$ up to 250 °C. The injector and detector temperatures were maintained at 255 °C and 275 °C, respectively.

The experiment was conducted in triplicate. Analysis of variance (ANOVA) was employed to determine the significance level of variables using Minitab 16 (Minitab Inc., ITS Surabaya, Indonesia) with a confidence level of 95% [33].

## 3. Results and Discussion

3.1 Crude Lipase Production

Figure 1 illustrates the activity of lipase and dry cell weight during the growth of *M. miehei* incubated at 37 °C and 125 rpm for 8 days. As depicted in Figure 1, durin g the first 5 days, the dry cell weight increased significantly until it reached its maximum value, but then decreased

significantly until day 8 ($p < 0.05$). The highest activity, 17.75 U/ml, corresponded to the maximum dry weight of 1.96 g. The rise in both activity and dry cell weight implies the production of crude lipase as a secondary metabolite, likely released through the secretion process of *M. miehei*. Protein analysis using the Bradford method yielded a concentration of 0.2162 mg/mL, with an activity of 82.10 U/mg protein.

The lipase activity of homemade *M. miehei* was found to be comparable to that of *Candida cylindracea* lipase produced under similar conditions. Candida cylindracea lipase exhibited activities of 17.30 U/ml and 47.25 U/ml at a temperature of 27 °C over a period of 5 days when glucose and olive oil were used as carbon sources, respectively. Additionally, lipase produced by *Streptomyces sp.* displayed an activity of 121 U/ml at 35 °C over a 5 day period, utilizing glucose as a carbon source and supplemented with $Ca^{2+}$, $Cu^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ ions [34]. The incorporation of these ions as co-enzymes notably enhanced enzyme activity.

### 3.2 Immobilization of Lipase on PUF Matrix

Polyurethane (PUF) used in this study is a synthetic polymer containing a urethane group in the bond formed by di/poly isocyanate (RN=C=O) and di/poly polyol (R−OH). The polymerization reaction scheme of PUF was previously reported [35], comprising two reaction stages: the gelling reaction and the blowing reaction. In the gelling reaction, isocyanate and polyol react slowly at first, with the reaction rate increasing as polyurethane is formed (auto-catalysis). The blowing reaction involves the reaction of isocyanate with water, typically added in small amounts to polyol, to form unstable carbamic acid, which then decomposes into amines ($R-NH_2$) and pores/bubbles ($CO_2$). The amines subsequently react with excess isocyanates to form urea as hard segments. Additionally, excess isocyanate reacts with urethane or disubstituted urea to form allophanate and biuret, resulting in polymers with cross-linked structures.

Lipase activity at the PUF-immobilized ratio of 1:25 was lower than at 1:20 (see Table 2). The observed lower lipase activity at the PUF-immobilized ratio of 1:25 compared to 1:20 and 1:15 can be attributed to several factors. Firstly, at higher PUF-immobilized ratios, there may be a decrease in the accessibility of the immobilized lipase to substrate molecules due to overcrowding or steric hindrance, leading to reduced catalytic activity despite higher protein loading [36]. Secondly, changes in the immobilization ratio may influence the orientation and distribution of immobilized lipase molecules on the PUF matrix, affecting the efficiency of enzyme-substrate interactions and subsequent catalytic activity [37]. Additionally, higher immobilization ratios may result in increased diffusion limitations for substrates and products within the porous structure of the PUF matrix, leading to decreased enzyme activity due to mass transfer limitations [13]. It is essential to note that the discrepancy between lipase activity and protein loading at different PUF-immobilized ratios underscores the complexity of enzyme behavior upon immobilization. Factors, such as enzyme orientation, conformational changes upon immobilization, and substrate accessibility, may also significantly influence enzyme activity [24], highlighting the importance of comprehensive analysis beyond protein loading alone.

The appropriate amount of immobilized addition in this study was achieved with the ratio of 1:20, leading to increased enzyme stability through multipoint covalent attachment. Support materials, acting as a short spacer arm, bound to the enzyme, enhancing the rigidity of the lipase structure [38]. The higher enzyme activity increased the number of active lipase sites interacting with the coconut oil substrate to produce FFA, reflected in the acid number increase. The use of support materials, such as *β-glycosidases* crosslinked aggregates, can impart a distinct aroma in wine [39]. The results demonstrated increased stability of the immobilized enzyme compared to free lipase.

Table 3 depicts the effects of support coatings on protein loading, immobilization yield, and changes in the diameter of PUF pores. From Table 3, it is evident that the pore diameter of the matrix decreased after coating, as it became filled with

Table 2. Effects of various immobilization techniques on the activity of enzymes and acid numbers.

| Immobilization Techniques | Activity (U/g PUF) | The final acid number after hydrolysis (mg KOH/g oil) | The increase of acid number (%)*) |
|---|---|---|---|
| Adsorption | 9.4 | 1.571 | 180 |
| *Crosslink-covalent* | | | |
| PUF : *immobilized* lipase 1:15 | 11.941 | 1.483 | 164 |
| PUF : *immobilized* lipase 1:20 | 15.197 | 2.929 | 422 |
| PUF : *immobilized* lipase 1:25 | 11.345 | 1.477 | 163 |

*) Calculated based on the acid number equation, where the initial acid number is 0.561 mg KOH/ g oil

immobilized lipase. The highest immobilization yield of 94.62% was achieved with the smallest PUF diameter of 56.33 µm. The phenomenon of shrinking the initial PUF diameter occurred because support materials acted as spacer arms. PUF bounds lipases through crosslink-covalent spacer arms, forming amino bonds and peptide bonds. Enzyme molecules were directly attached to the matrix's reactive groups (hydroxyl, amide, amino, carboxyl groups) or to spacer arms artificially bound to the matrix through various chemical reactions. This state enhanced interfacial lipase activity on the matrix surface. The reactive groups in the matrix reacted with

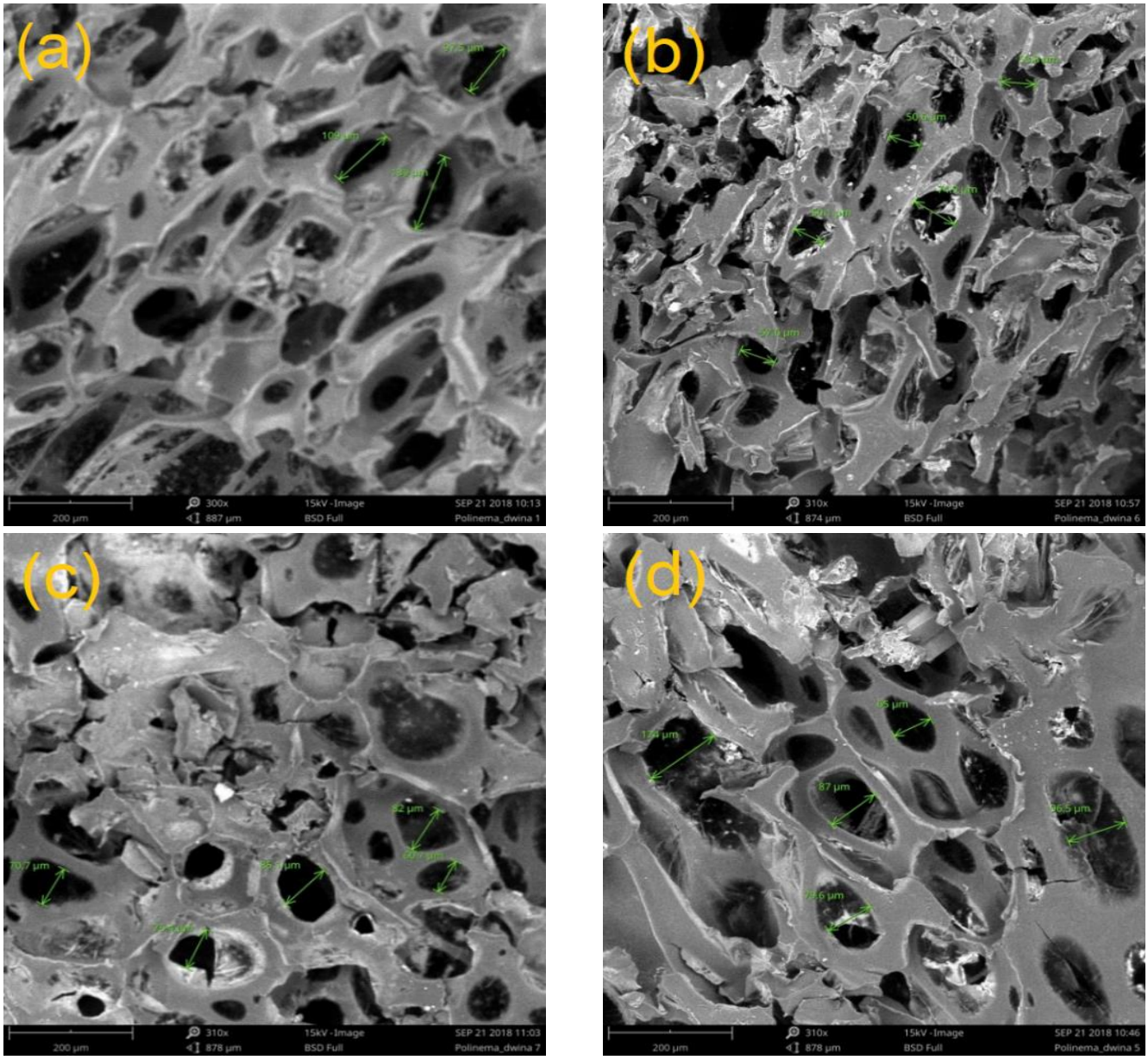


Figure 2. SEM images of (a) native PUF, PUF: immobilized lipase ratio of (b) 1:15, (c) 1:20, and (d) 1:25.

Table 3. Effects of PUF:immobilized lipase ratio toward protein loading, immobilized yield, and average pore diameter of PUF

| Protocols | Protein loading (mg/mL) | Immobilized yield (%) | Average diameter PUF (µm) |
|---|---|---|---|
| Free lipase | 0.2162 | - | - |
| PUF | - | - | 129 |
| PUF: immobilized lipase 1:15 | 0.2005 | 92.75 | 72.77 |
| PUF: immobilized lipase 1:20 | 0.2046 | 94.62 | 56.33 |
| PUF: immobilized lipase 1:25 | 0.2012 | 93.06 | 72.37 |

those on the enzyme's surface, providing stability to the enzyme matrix during prolonged reaction times. The optimal immobilization condition was achieved with a crosslink-covalent ratio of PUF to immobilized lipase of 1:20. Xie *et al.* [40] found that the crosslinking-covalent method resulted in the highest protein loading yield. Incorporating gelatin into the PUF matrix increased the protein loading from 8.2 to 9.4 mg protein/g PUF.

### 3.3 Characteristics of Immobilized Lipase

The impact of support modification on the immobilized lipase's influence on changes in the matrix surface morphology was analyzed using SEM [41]. Figure 2 illustrates that PUF layers immobilized with ratios of 1:15, 1:20, and 1:25 appeared thicker and brighter than native PUF. This disparity indicates that support materials could coat the surface and inner cavities of PUF pores through crosslink-covalent binding. At ratio of 1:15, thickening of the pore diameter resulted in unevenly distributed layers. Conversely, at ratio of 1:25, the SEM results depicted a rupture of the pore matrix structure due to the inability to absorb all the support materials (refer to Figure 2). This circumstance affected PUF's inability to be entirely coated. Changes in pore diameter due to matrix modification through immobilization techniques have been observed by several researchers. Following the immobilization process, the diameter of the polymer membrane for lipase immobilization decreased compared to before [42]. The modification of the PUF matrix surface using gelatin and glutaraldehyde in immobilized lipase also led to a reduction in PUF pore size.

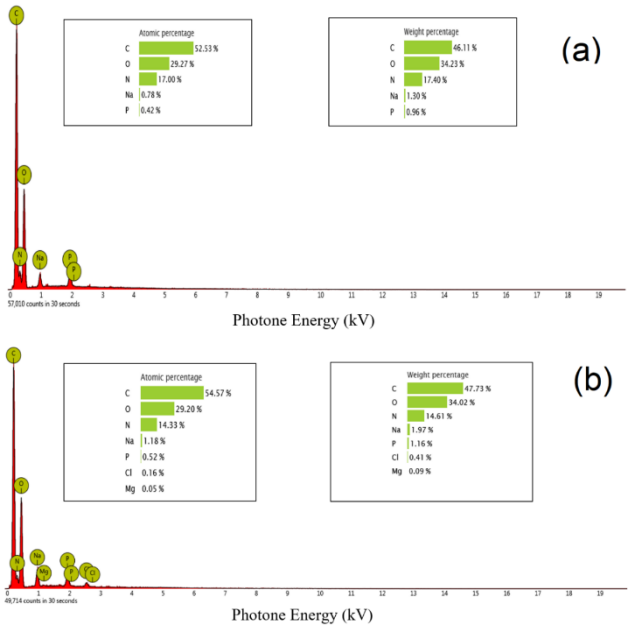


Figure 3. Spectrum of EDS on the PUF conditions; (a) initial and (b) coated with support materials.

The impact of support modification on changes in its constituent elements was analyzed using EDS. Figure 3, as the EDS spectrum, presents the atomic and weight percentages of elements under various PUF conditions. EDS, integrated with SEM, revealed alterations in atomic percent and weight percentages from the initial PUF to after support modification. Initially, the constituent elements comprised carbon (C), oxygen (O), nitrogen (N), sodium (Na), and phosphorus (P). After the addition of the support material $MgCl_2$, there was a notable increase in the atomic percent and weight of Mg and Cl by 0.16% and 0.05%, respectively. Additionally, the weight percentage of Mg and Cl changed by 0.09% and 0.41%, respectively.

The effects of support materials on changes in the characterization of functional groups were analyzed using FTIR [43]. Figure 4 illustrates the results of FTIR for various adsorption protocols and crosslink covalent with PUF: immobilized lipase ratios of 1:15, 1:20, and 1:25. The FTIR spectrum of native PUF and its modifications were interpreted based on their functional groups. The urethane spectrum of PUF served as the basis of this study, indicated by the carbonyl −C=O group at 1730 $cm^{-1}$ and the isocyanate in the −NCO group at 2270 $cm^{-1}$. The addition of support materials, consisting of gelatin, lecithin, PEG, and $MgCl_2$, coated onto PUF, contained N−H amide groups at 3400 – 3250 $cm^{-1}$ and carbonyl −C=O at 1730 $cm^{-1}$. Lipase, being a protein composed of amino acids, exhibited a carboxylic terminal −C=O at 1730 $cm^{-1}$ and primary amine N−H primary amine at 1650 $cm^{-1}$. The increase in the amount of support materials led to the weakening/widening of the N−H spectrum at 3400 – 3250 $cm^{-1}$, indicating the attachment of PUF. Similarly, the latter lipase addition via covalent N−H bonding at 1650 $cm^{-1}$ showed a weakened

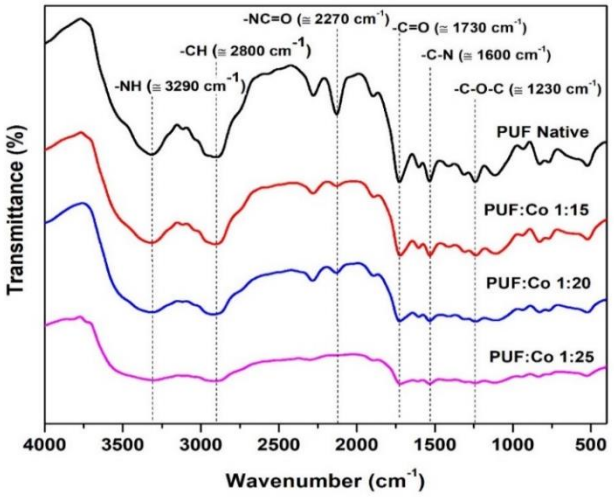


Figure 4. FTIR spectra of various ratios of immobilized lipase and PUF.

peak. Ratio of 1:25 resulted in a weak peak, supporting the previous SEM analysis (see Figure 2), which revealed weathering in the PUF morphology, leading to a weakened structure.

Figure 5 presents the proposed reaction mechanism involved in the process of modifying the PUF layer with support materials, including gelatin, lecithin, PEG, and $MgCl_2$. The activation of the PUF surface by the support material acts as a short spacer arm, strengthening the bond between PUF and lipase. Several mechanisms explain these phenomena. First, during the synthesis of PUF from polyols and isocyanates, a reaction takes place between the alcohol and isocyanate groups, forming urethane. Second, in the adsorption method, hydrogen bonding occurs between PUF and its urethane group and the –NH group of lipase. Lastly, a covalent bond occurs.

In the covalent bonding, several reaction steps occur as follows. First, the NHCOO reactive group of PUF would bind to gelatin, which contains a reactive $-NH_2$ group. These two

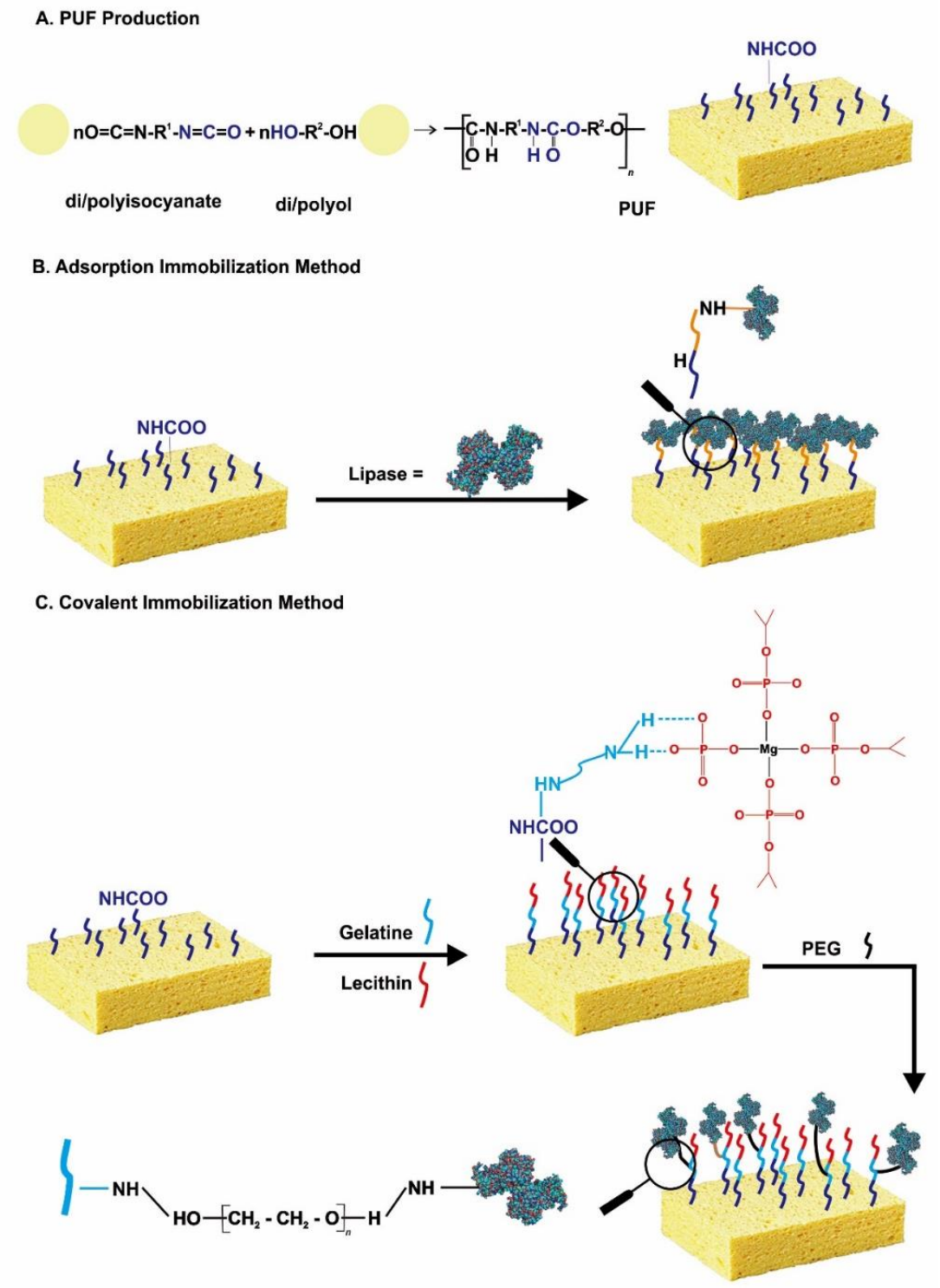


Figure 5. Proposed reaction mechanism on the modifying PUF process by using support materials.

reactive groups interact as peptide bonds. PUF containing of the constituent groups of amide, ether, and carbamate, absorbs water and oil. Gelatin, as a macromolecule with excellent biocompatibility, was used to coat the surface structure, transforming it into a biomimetic layer capable of altering the matrix's characteristics. Previous studies have reported on PUF modified with gelatin [44,45].

Second, gelatin comprises 18 types of amino acids, while lecithin consists of phospholipids, glycolipids, carbohydrates, triglycerides, and FFA [46]. Mixing gelatin and lecithin is related to the turbidity nature and physical stability in the dissolution system. This mixture forms nano dispersions with a particle diameter characteristic of <100 nm. Gelatin and lecithin addition are widely used as food stabilizers/emulsifiers, such as when added to thymol (a component of essential oils) as an antimicrobial ingredient. The best results are achieved when gelatin and lecithin are mixed, producing three beneficial characteristics: clarity, stability at pH 5 – 8, and particles that do not settle easily. Therefore, the choice of blended gelatin-lecithin through hydrogen bonds $-NH_2$ and $-CH-$ in the production of support materials is justified [47].

Third, the phosphate group in lecithin binds to $Mg^{2+}$ to form complex compounds. $Mg^{2+}$ metal ions, widely used as enzyme cofactors, play an important role alongside phosphates in forming $Mg^{2+}$ complexes. Magnesium has broad capabilities in electrostatic stability and electrophilic activation of the substrate through polarization and the hydrolysis pattern of P–O and C–O bonds in lecithin. Furthermore, PEG with OH bonds acts as a crosslinker and can bi-functionally bind to amino gelatin through hydrogen or covalent bonds. By using PEG, all support materials form short spacer arms to increase lipase stability [48].

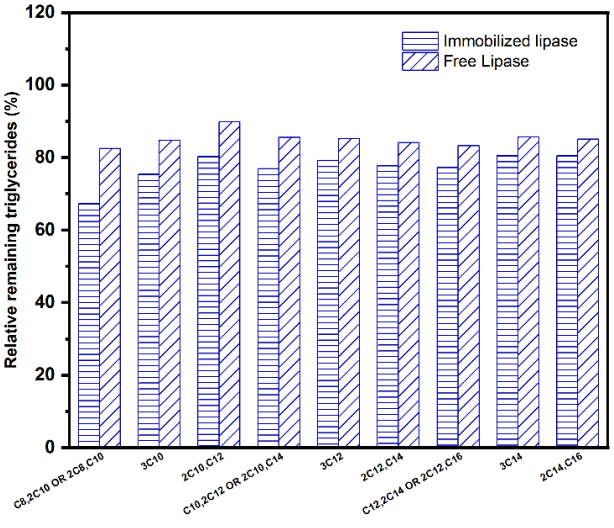


Figure 6. Relative percentage of remaining coconut oil triglycerides after hydrolysis at 40 °C.

Fourth, lipase, with a reactive terminal group $-NH_2$, would be attached to the short spacer arms-support materials formed through the covalent bonds. The presence of these bonds caused an increase in activity and stability, leading to an increase in the reaction of active sides reacting with the substrate.

### 3.4 Testing of Immobilized Lipase for Hydrolysis Reaction

The support-modified immobilized lipase, acting as a heterogeneous biocatalyst, was used to hydrolyze coconut oil (triglycerides) with water at a ratio of 1:3 and a temperature of 40 °C. Table 2 displays the effect of the protocol on immobilized lipase activity. In the first protocol, lipase was directly attached to PUF without using support materials. As shown in Table 2, the activity of *M. miehei* lipase immobilization through the physical adsorption method at an incubation time of 20 h was 9.4 U/g PUF. Using this method, the fatty acids bound in triglycerides increased by 180% from 0.561 to 1.571 mg KOH/g oil. The bond formed in this method is van der Waals. The low catalytic ability at the active site resulted in a slow hydrolysis reaction. This result is consistent with the previous report on the study of fish oil hydrolysis to cis-5,8,11,14,17-eicosapentaenoic acid and cis-4,7,10,13,16,19-docosahexaenoic acid, which revealed that lipases adsorption-bound to silica-epoxy exhibited low strength [49].

In the second protocol, the immobilization method used was cross-covalent. As shown in Table 2, the lipase activity was greater than that in the adsorption method. The highest increase in acid number, by 422%, was observed alongside the highest increase in activity, reaching 15.197 U/g PUF. This phenomenon may be attributed to the occurrence of intermolecular cross linkages on the surface and pores of the PUF matrix with support materials consisting of gelatin, lecithin, PEG, and $MgCl_2$. In this condition, enzymes formed a three-dimensional structure, altering the shape of lipases into spacer arms [50]. The bonding occurring in the crosslink-covalent method was more stable than in adsorption. This finding demonstrates that lipase activity was greater in the crosslink-covalent approach than in adsorption.

The utilization of support-modified immobilized lipase on PUF for the hydrolysis reaction is presented in Table 3 and Figure 6. From Table 3, it is evident that the highest increase in acid number, with reference to lauric acid (C12), was 422% at the ratio of 1:20. However, the relative hydrolysis percentage using immobilized lipase in PUF and free lipases was only 23.46% and 14.84%, respectively. This low percentage of hydrolysis may be attributed to the immobilized lipase being capable of hydrolyzing

only a small amount of triglycerides, which initially had a high content of 90%.

The notable enhancement of enzyme activity observed with the incorporation of $Ca^{2+}$, $Cu^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ ions as co-enzymes can be attributed to several mechanisms. Firstly, these metal ions play a crucial role in stabilizing the enzyme structure by interacting with specific amino acid residues in the enzyme's active site [51], thereby promoting optimal enzyme-substrate interactions. Secondly, they act as cofactors that bind to the enzyme-substrate complex, facilitating substrate binding and catalysis [52]. Additionally, these metal ions can modulate the enzyme's kinetic properties, such as substrate affinity and turnover rate, leading to increased enzyme activity [53]. Furthermore, they can regulate enzyme activity by affecting conformational changes or directly participating in catalytic reactions [54]. Overall, the incorporation of $Ca^{2+}$, $Cu^{2+}$, $Mg^{2+}$, and $Mn^{2+}$ ions as co-enzymes enhances enzyme activity by stabilizing enzyme structure, facilitating substrate binding, modulating enzyme kinetics, and regulating enzyme activity. This multifaceted effect underscores the importance of metal ions in optimizing enzyme performance and highlights their potential applications in various biotechnological processes.

### 3.5 Testing of Immobilized Lipase for Esterification Reaction

The esterification reaction involves the interaction between carboxylic acids and alcohols. In this study, the carboxylic acid utilized was FFA, which were obtained through the enzymatic hydrolysis of coconut oil. The alcohol substrate employed was citronellol, isolated from lemongrass oil. The objective of this reaction, facilitated by both free and immobilized lipase catalysts, was to produce natural flavor esters.

From Figure 7, the highest esterification conversion rate of 52.18% was achieved after 25 h of reaction time. Comparing the esterification conversion rates of citronellol at 20 and 25 h, they were 51.74% and 52.18%, respectively, with no significant difference observed ($p > 0.05$). This trend aligns with previous findings where a similar lack of significant difference in ester conversion rates was noted after 6 h of reaction time using 50% crude lipase by weight at 40 °C and 60 °C [55]. This increase in conversion rate is associated with the restructuring of the lipase enzyme's "lid" structure upon immobilization. Following immobilization, there is a reconfiguration of the lipase enzyme's "lid" structure, leading to changes in its conformation. These changes occur due to the interaction of the enzyme with the interfacial region, resulting in the exposure of the enzyme's hydrophobic sites to the substrate. Additionally, the hydrophilic sites of the enzyme/substrate complex transition to an open form, facilitating increased contact between the enzyme and the substrate [56]. However, increasing the concentration of the substrate (alcohol) used may decrease the reaction rate as the substrate begins to act as an inhibitor.

In the case of free lipase, as depicted in Figure 7, the highest esterification conversion rate of 44.16% was achieved after 25 h of reaction time. Interestingly, the esterification yield using immobilized lipase surpassed that of the free lipase, with the conversion of esters from immobilized and free lipase to ethyl caprylate ester products being 85% and 62%, respectively [57]. The matrix utilized for lipase immobilization is exfoliated graphene oxide.

Figure 8 illustrates the effect of lipase addition on citronellol esterification with both free

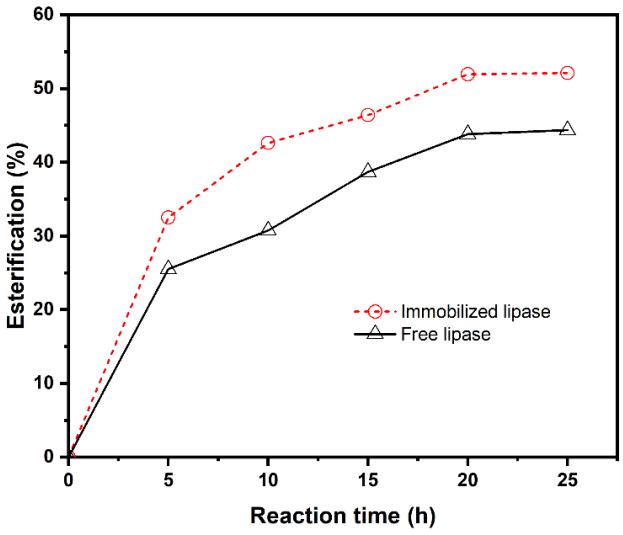


Figure 7. Esterification percentage during various reaction times.

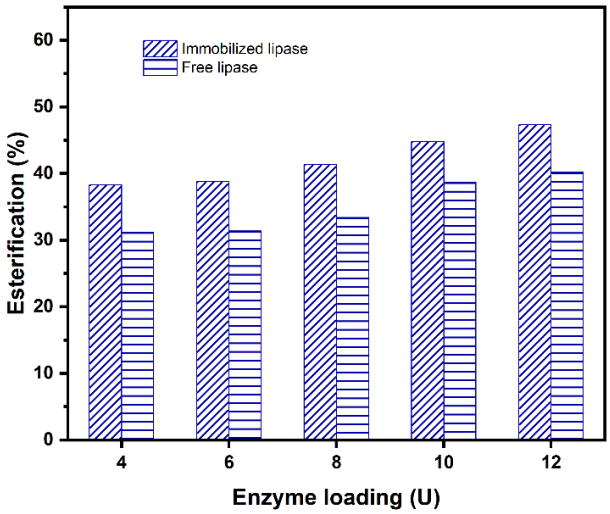


Figure 7. Esterification percentage during various reaction times.

lipase and immobilized lipase. As depicted in Figure 8, the percent esterification of the immobilized enzyme exhibited a significant increase compared to the free lipase at low enzyme activity levels (4 and 6 U) ($p < 0.05$). However, with the use of 12 U of lipase, the percent citronellol esterification did not show a significant difference ($p > 0.05$). Patel *et al*. [57] reported that the addition of excess enzymes could lead to protein accumulation, resulting in decreased enzyme activity. Similarly, Zhang *et al*. [58] found that excessive concentrations of *Candida antarctica* lipase during the synthesis of phenyl ethanol caused a decrease in percent conversion. Additionally, Garlapati and Banerjee [32] revealed that excessive enzyme concentrations made it challenging to maintain a uniform suspension of biocatalyst, leading to accumulation on immobilized lipases. However, in this study, the maximum yield for the addition of enzyme loading has not yet been achieved.

At the end of the discussion in this study, a relevant literature survey was conducted, as shown in Table 4. From Table 4, it is evident that the reusability of the covalent crosslink and adsorption methods remains relatively low [55,59,60]. This low reusability may be attributed to the low crude lipase activity of *M. miehei*. Despite the low yield, this study discovered that immobilization of lipase using edible support modification has the potential to be applied in the food sector because it consists of ingredients that are safe for the body and solvent-free.

## 4. Conclusion

Edible support modification of immobilized lipase from *M. miehei*, consisting of gelatin, lecithin, PEG, and $MgCl_2$, has been successfully applied to the surface of PUF. The support materials act as spacer arms between PUF and lipase through crosslink-covalent bonding, resulting in increased enzyme stability. The results showed that this immobilization technique increased the hydrolysis of triglycerides in coconut oil into FFA by 422%. Characterization utilizing SEM, FTIR, and EDS analysis demonstrated an increase in protein loading and immobilization yield, as well as a decrease in the pore diameter of PUF. Comprised of safe and healthy ingredients, this edible immobilized lipase is highly suitable for applications in the food industry. The results of this study offer an alternative process that promotes environmentally friendly and safe conditions, as it is free of chemical solvents.

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## CRediT Author Statement

Author Contributions: *Dwina Moentamaria*: Conceptualization, Methodology, Investigation, Resources, Writing; *Zakijah Irfin*: Writing, Validation, Review and Editing; *Achmad Chumaidi*: Review and Editing, Writting Draft Preparation, Project Administration; *Arief Widjaja and Tri Widjaja*: Supervision, Review and Editing Formal Analysis; *Maktum Muharja and Rizki Fitria Darmayanti*: Writing, Validation, Data Curation, Visualization, Software. All authors have read and agreed to the published version of the manuscript.

Table 4. Literature studies of various immobilization techniques of lipase.

| Products | Matrix | Source of lipase | Conditions | Reusability | Techniques | Conversion (%) | Ref. |
|---|---|---|---|---|---|---|---|
| Sitronelol acetate | Polylactic acid, chitosan, polyvinyl alcohol | *Burkholderia cepacia* | 55 ℃, 4 h | 6x | Adsorption | 90 | [59] |
| Geraniol propionat | Sodium alginate | *Penicillium crustosum* | 40 ℃, 24 h | 2x | Entrapment | 53 | [55] |
| Ethyl butyrate | PUF | *Candida rugosa* | 30 ℃, 47 h | 12x | Adsorption | 85 | [60] |
| Ester | PUF | *Rhizomucor miehei* | 40 ℃, 20 h | 5x<br>3x | *Crosslink*<br>adsorption | 51 | This study |

## Competing Interests

The authors have no competing interests to declare.

## Data Availability

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

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