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Bulletin of Chemical Reaction Engineering & Catalysis, 14 (2) 2019, 391-403

Research Article

A Performance Study of Home-Made Co-Immobilized Lipase from *Mucor miehei* in Polyurethane Foam on The Hydrolysis of Coconut Oil to Fatty Acid

Dwina Moentamaria, Maktum Muharja, Tri Widjaja, Arief Widjaja*

Department of Chemical Engineering, Institut Teknologi Sepuluh Nopember, Jl. Arief Rahman Hakim, Kampus ITS Sukolilo, Surabaya 60111, Indonesia

Received: 7th December 2018; Revised: 11st February 2019; Accepted: 15th February 2019; Available online: 30th April 2019; Published regularly: 1st August 2019

Abstract

Bio-based fatty acids (FAs) produced through hydrolysis of natural oils and fats are promising chemical feedstocks for increasing the economic value of renewable raw materials. In this work, lecithin, gelatin, PEG, and $MgCl_2$ were employed as the co-immobilized material of crude lipase *Mucor miehei* immobilization on the polyurethane foam (PUF) matrix for hydrolysis of coconut oil to Free Fatty Acid (FFA). The unconventional immobilized technique was used through cross-linking and covalent bond. Single factor analysis and response surface method were utilized to determine the optimum conditions of the hydrolysis reaction. After optimization, co-immobilized lipase was examined for storage stability at a temperature of 4°C and reusability performance. The optimum conditions for coconut oil hydrolysis were obtained on the co-immobilized-PUF ratio, water-oil ratio, and reaction time of 20.17 w/w, 4.45 w/w, and 20 h, respectively. Under these conditions, the acid value as lauric acid enhanced 573% to 3.21 mg KOH/g oil. Storage stability attained through remaining activity on free lipase, PUF-lipase, PUF-co-immobilized-lipase were 9.89%, 42.3%, and 91.88%, respectively. In this study, the application of PUF-co-immobilized lipase in hydrolysis reactions can be reused up to 5 times. Characteristics of the addition of co-immobilized lipase have been analyzed using Fourier Transform Infra Red (FTIR) and Scanning Electron Microscope (SEM), showing the presence of functional groups binding and the changes in the surface matrix structure.

Keywords: co-immobilized lipase; coconut oil; free fatty acid; polyurethane foam; response surface methodology

How to Cite: Moentamaria, D., Muharja, M., Widjaja, T., Widjaja, A. (2019). A Performance Study of Home-Made Co-Immobilized Lipase from *Mucor miehei* in Polyurethane Foam on The Hydrolysis of Coconut Oil to Fatty Acid. *Bulletin of Chemical Reaction Engineering & Catalysis*, 14(2): 391-403 (doi:10.9767/bcrec.14.2.3848.391-403)

Permalink/DOI: https://doi.org/10.9767/bcrec.14.2.3848.391-403

1. Introduction

The production of fatty acids through hydrolysis of natural oils and fats is important to increase the economic value of renewable raw materials. Fatty acids are widely used in the food, cosmetic, pharmaceutical, and chemical industries such as flavor, omega three fatty acids, emulsifiers, coatings, adhesives, and fragrances. Oils and fats, which are composed of fatty ester or triglyceride components, can produce free fatty acids (FFA) and glycerol by using chemical or enzymatic hydrolysis reactions [1-3]. The enzymatic reaction using lipase as biocatalyst in mild condition is an attractive green process be-

* Corresponding Author.
Email: arief_w@chem-eng.its.ac.id (A. Widjaja);
Telp: +62-31-5946240, Fax: +62-31-5999282

cause it is not only able to increase energy efficiency but also eliminate by-product reactions compared to steam splitting process [2,4-6].

The use of free enzymes on enzymatic methods is still limited due to the high cost of isolation and purification and has a labile nature leading to denaturation and reduction of enzyme activity. Moreover, it is necessary to separate dissolved products from the free enzymes. Therefore, immobilization techniques by which the enzyme is not free to move are required. Although immobilized enzyme have limited space for their catalytic activities, they can be reused repeatedly, and the stability of the enzyme can be increased against temperature and organic solvent as well as an increase conversion yields [2,7,8].

The development of immobilization lipase technology has been broadly applied to hydrolysis, esterification, transesterification, interesterification by using various matrix. The use of lipase immobilization has been shown to provide higher stability, reusability, practicability and activity response than lipase-free [3,9-11]. On the other studies, a combination of two immobilization techniques, namely crosslinking-covalent, adsorption-crosslinking, and cross-entrapment, showed higher stability compared to using only one immobilization technique [9,12,13]. Among all available immobilization matrix, polyurethane foam (PUF) has higher commercial prospects since it is inert, rigid, porous, and inexpensive. PUF has been widely used as a matrix to covalently immobilize lipase [14-16].

In some processes, co-immobilization agents have positive functions and clear synergies in enzymatic hydrolysis where the enzyme binds to the matrix functional group to be more stable [13,17,18]. In the previous study, mixtures of additives, surfactants, emulsifiers, and cofactors were used as co-immobilized lipases [19,20]. Some of these substances can be synergized together and serve as penetration of substrate entry because they are well dispersed, resulting in low turbidity and excellent stability. Also, the co-immobilization agent can protect from inactivation, increasing the enzyme activity several times after the initial activity of the enzyme [4,21].

Generally, hydrolysis is carried out using chemical substrates and commercial lipases. The phenomenon occurred in the reaction process conditions using a co-immobilized lipase mixture on the PUF matrix using natural substrates, non-commercial as home-made lipases, free solvents that have an impact on yield (as acid value) has never been discussed and reported before.

From aforementioned problem above, the home-made co-immobilized lipase of *M. miehei* was utilized to catalyze the hydrolysis process of coconut oil into FFA through an unconventional crosslinking and covalent bonding. Response surface methodology (RSM) was used as a tool to optimize the hydrolysis process. The effect of some important parameters such as co-immobilized lipase-PUF ratio, water-oil ratio and incubation time of hydrolysis was investigated and discussed comprehensively.

2. Materials and Methods

2.1 Materials

Coconut oil (purity of 100%) was obtained from local Permata Agrindo Pendowoharjo (Sewon, Bantul, Indonesia) with the commercial name Laitco. The stock culture of *M. miehei* is obtained from Biochemical Technology Laboratory, Department of Chemical Engineering, Sepuluh Nopember Institute of Technology Surabaya. Co-immobilized consisting of gelatin, lecithin, polyethylene glycol (PEG), $MgCl_2$ was purchased from Merck (Darmstadt, Germany). PUF was made by reacting isocyanate and polyol with the same volume ratio [22,23].

2.2 Methods and Techniques

2.2.1 Production of crude lipase from *M. miehei*

M. miehei culture stock in Potato Dextrose Agar (PDA) was mixed with 10 mL of sterile water. The 10^7 spores/mL was inoculated on sterile media containing KH_2PO_4, $FeSO_4.7H_2O$, olive oil, palm oil, dried coconut grout (solid), and water at pH of 7. Cells were incubated at solid state fermentation at 37°C for 5 days.

Crude lipase was obtained by extracting with phosphate buffer with a buffer to media ratio of 4: 1 (w/w) in incubator shaker at 150 rpm, 37°C, for 135 min. The filtration was carried out to separate solid to crude lipase liquid. The supernatant was then analyzed for its lipase activities using olive oil as substrate [24,25].

2.2.2 Preparation of unconventional immobilization lipase through cross- linking and covalent methods.

Matrix PUF is a cuboid shaped porous matrix measuring 0.5 cm × 0.5 cm × 0.5 cm. Previously prepared co-immobilized solution contained a) lechitin (50 g/L), b) gelatin (50 g/L), c) $MgCl_2$ (10 g/L), d) PEG 6000 (20 g/L)

[22,23]. PUF and co-immobilized were prepared with a ratio of 1:15, 1:20, 1:25 (w/w). Then the PUF and co-immobilized were immersed for 30 min and incubated at 30 °C. Immobilized PUF was filtered, dried in a 30 °C oven for 1 h. Then it was soaked in crude lipase for 24 h and then dried at room temperature.

2.2.3 Hydrolysis of coconut oil

The hydrolysis of coconut oil to FFA was determined by its acid value. Coconut oil of 10 g was hydrolyzed with water under a certain ratio (w/w), incubated at 40 °C with a certain time. The hydrolysate was then separated and analyzed using the titrimetric acid value based on following Equation (1).

$$Acid\ value = \frac{v\ KOH\ (ml) \times C\ KOH\ (N) \times 56.1}{Oil\ sample\ (gr)} \quad (1)$$

2.2.4 Single factor experiment

The effect of stirring was determined using orbital shaker velocity of 0, 60, 90, 120, 150, and 180 rpm in coconut oil hydrolysis process on water oil ratio 5:1, hydrolysis incubation time 15 h, co-immobilized lipase to PUF 20:1 . The influence of water to oil has been determined by different ratio of water: oil of 0.6:1, 1:1, 3:1, 5:1, and 7:1 at 10 h incubation time hydrolysis, co-immobilized lipase to PUF = 20:1, 120 rpm. Effect of hydrolysis incubation time of 2, 5, 10, 15, 20, and 25 h in co-immobilized ratio against PUF of 15:1 (w/w), water oil ratio of 5:1 (w/w) was studied. The effect of co-immobilized lipase on PUF has been determined by varying ratio of co-immobilized: PUF of 10:1, 15:1, 20:1, 25:1, and 30:1.

2.2.5 RSM design and statistical analysis

Based on the initial experimental data generated on the single factor experiment, a Box-Behnken design was utilized to determine the effect of the three independent variables of co-immobilized lipase:PUF ratio, water-oil ratio, and hydrolysis incubation time as shown in Table 1.

Table 1. Independent variables and their levels use in the response using Box-Behnken design

Independent variables	Code	Factor levels		
		-1(-α)	0	+1(+α)
Co-immobilized lipase : PUF ratio (w/w)	A	15	20	25
Water : oil ratio (w/w)	B	1	3	5
Hydrolysis incubation time (h)	C	10	15	20

Table 2. Box-Behnken design and the acid value observed

Run	Actual variable			Acid value (mg KOH/g oil)		
	A (co/PUF)	B (W/O)	C (h)	Experimental	Predicted	Residue
1	20	3	15	2.4678	2.7581	-0.2903
2	20	3	15	2.4678	2.7581	-0.2903
3	15	3	10	1.2266	1.2286	-0.0020
4	20	3	15	2.4678	2.7581	-0.2903
5	25	5	15	1.4618	2.2493	-0.7875
6	15	3	20	1.4830	1.9645	-0.4815
7	15	1	15	0.9652	1.0164	-0.0512
8	20	5	10	1.9612	2.9780	-1.0168
9	25	1	15	0.9935	0.9476	0.0459
10	15	5	15	1.2199	2.1045	-0.8846
11	20	3	15	2.4678	2.7581	-0.2903
12	25	3	10	1.1246	1.2236	-0.0990
13	20	1	20	2.7401	2.5620	0.1781
14	20	3	15	2.4678	2.7581	-0.2903
15	20	1	10	1.4728	1.7742	-0.3014
16	20	5	20	3.2107	3.7480	-0.5373
17	25	3	20	1.4670	2.0455	-0.5785

Complete design which consists of 17 experiments including 6 repetitions on the center point was run randomly. Table 2 shows the run order, variable conditions, and experimental and predictive values. After determining the preliminary range of hydrolysis through the single factor test, the relationships between the response and three selected variables were approximated by the following second order polynomial equation as shown in Equation (2).

$$Acid\ value\left(mg\ KOH/gram\ oil\right)=\beta_0+\sum_{i=1}^{3}\beta_i X_i^2+\sum_{i=1}^{3}\beta_{ii}X_i^2+\sum_{i<j=2}^{3}\beta_{ij}X_iX_j \quad (2)$$

Where X_i is the corresponding actual value of variable, β_0 is the estimated regression coefficient of the fitted response at centre point of design, β_i is regression coefficient for liner effect terms, β_{ij} is interaction effects, and β_{ii} is quadratic effects.

Data were analyzed by ANOVA to determine the effect of linear, quadratic, and interaction variables on the hydrolysis of coconut oil using co-immobilized lipase. Data analysis and RSM are carried out with a commercial statistical package, Design-Expert version 9.0.4.1 (Minneapolis, USA). The surface and contour plot revealed the effect of all factors at a central point in the design space.

2.2.6 Characterization of substrate and product

Acid value of coconut oil and fatty acid were analyzed by AOCS method [1]. Fatty acid composition was determined using GC-MS. To 4 drops of the sample in a test tube, 4 mL of hexane (p.a) was added, vortexed for 2 min. The clear hexane extract was transferred to a derivatization tube and dried with nitrogen bursts. Then 2 mL of 2% NaOH in methanol was heated at 90 °C for 5 min. After cooling, addition of 2 mL of BF3 in methanol, and reheat for 30 min. After cooling the sample was extracted with 3 mL n-hexane (p.a). The upper phase (n-Hexane) was taken for analysis with GC-MS (Agilent 6980 N, Santa Clara United States). The detector was Inert MSD Agilent 5973 detector. The column J&W Scientific HP-5% phenylmethylsiloxane 30 m length, 0.32 mm i.d, 0.25 μm film thickness. The detector temperature was 280 °C; the column temperature was held at 170 °C for 1 min increased to 180 °C at 2 °C/min, increased 270 °C at 5 °C/min, and held at 270 °C for 3 min. The carrier gas was helium at a flow rate of 1 mL/min.

2.2.7 Analytical method

The activity of crude lipase was determined using olive oil as a substrate. 25 mL of olive oil and 75 ml of 7% solution of gum arabic were emulsified for 2 min. Furthermore, 5 mL of the emulsified olive oil was mixed with 2 mL of 0.1 M phosphate buffer (pH 7) and 1 mL of the enzyme suspension. The mixture was incubated at 37 °C for 30 min with an orbital shaker. After incubation, the reaction was stopped by the addition of 15 mL of acetone-ethanol (1:1 v/v) and the FFA was titrated with 0.05 M NaOH. A unit of lipase activity is defined as a number of enzymes capable of liberating 1 μmol of fatty acids per min [25,26]. Lipase activity can be calculated by the following Equation (3).

$$Lipase\ Activity\left(U/ml\right)=\frac{(a-b)\times N\ NaOH\times 1000}{t} \quad (3)$$

Where, a and b is the amount of NaOH required for the sample titration using lipase and without lipase (ml), respectively, and t is the incubation time (30 min). The matrix activity was determined in the same manner as above, but the 1 ml crude sample was substituted by co-immobilized lipase-PUF 1 g.

Residual TAGs were analyzed using LC MS/MS. Accella 1250 LC-MS/MS (Thermo Scientific-Waltham, MA USA) was equipped by Hypersil Gold column (50 mm × 2.1 mm × 1.9 μm). The mobile phase of 2 μL was employed at a flow rate of 300 μL/min. The column and autosampler was controlled at 30°C and 16°C, respectively. The MS/MS Triple Q TSO Quantum Access Max mass spectrometer was operated in positive mode.

2.2.8 Characterization of co-immobilized lipase-PUF

Morphology of PUF matrix with co-immobilized lipase were evaluated by SEM - JEOL JSM-7800F (Pleasanton, California, United States). FTIR is a technique used to obtain an infrared spectrum of absorpsion of solid, liquid or gas**.** An FTIR spectrometer simultaneously collects high-spectral-resolution data over a wide spectral range FTIR spectra was analyzed using a Shimadzu IR Prestige 21 (Tokyo, Japan). The IR Prestige-21 uses a bright ceramic light source, high-sensitivity DLATGS detector, and high-throughput optical elements.

2.2.9 Storage stability and reusability calculation

The storage stability of the PUF-co-

immobilized lipase was determined by measuring the activity at the time of week storage of 4 °C, using Equation (4).

Activity analysis was carried out on free lipase, PUF-lipase and PUF-co immobilized lipase every week to analyze its degradation. After hydrolysis reaction, the acid value of fatty acid was determined, and PUF-co-immobilized lipase was subjected to enzyme assay. The immobilized enzyme was then can be reused for subsequent reactions. The reusability of co immobilized lipase on PUF was determined by following Equation (4).

$$Remaining\ activity(\%) = \left[\frac{(activity\ at\ t_0) - (activity\ at\ t_r)}{(activity\ at\ t_r)}\right] \quad (4)$$

Where, activity at t_0 was defined as initial activity and activity at t_r is activity after storage/ hydrolysis.

3. Result and Discussion

3.1 Single Factor Analysis

Figure 1a shows the effect of orbital rotation on the acid value of coconut oil hydrolysis. As exhibited in the figure, the acid value increased significantly by increasing rotation speed from 50 to 150 rpm. Moreover, at the orbital rotation speed of 180 rpm, the acid value was constant. These results indicated that the mass transfer barrier would increase when there was no effect of the mass transfer diffusion at high rotation speed [27]. These results match those observed in earlier studies on the study of citronellyl acetate esterification using *Burkholderia cepacia* immobilized lipase (BCL) [28,29]. They revealed that the external mass transfer limit at various rotation speed gave a significant impact for chemical or enzymatic reaction.

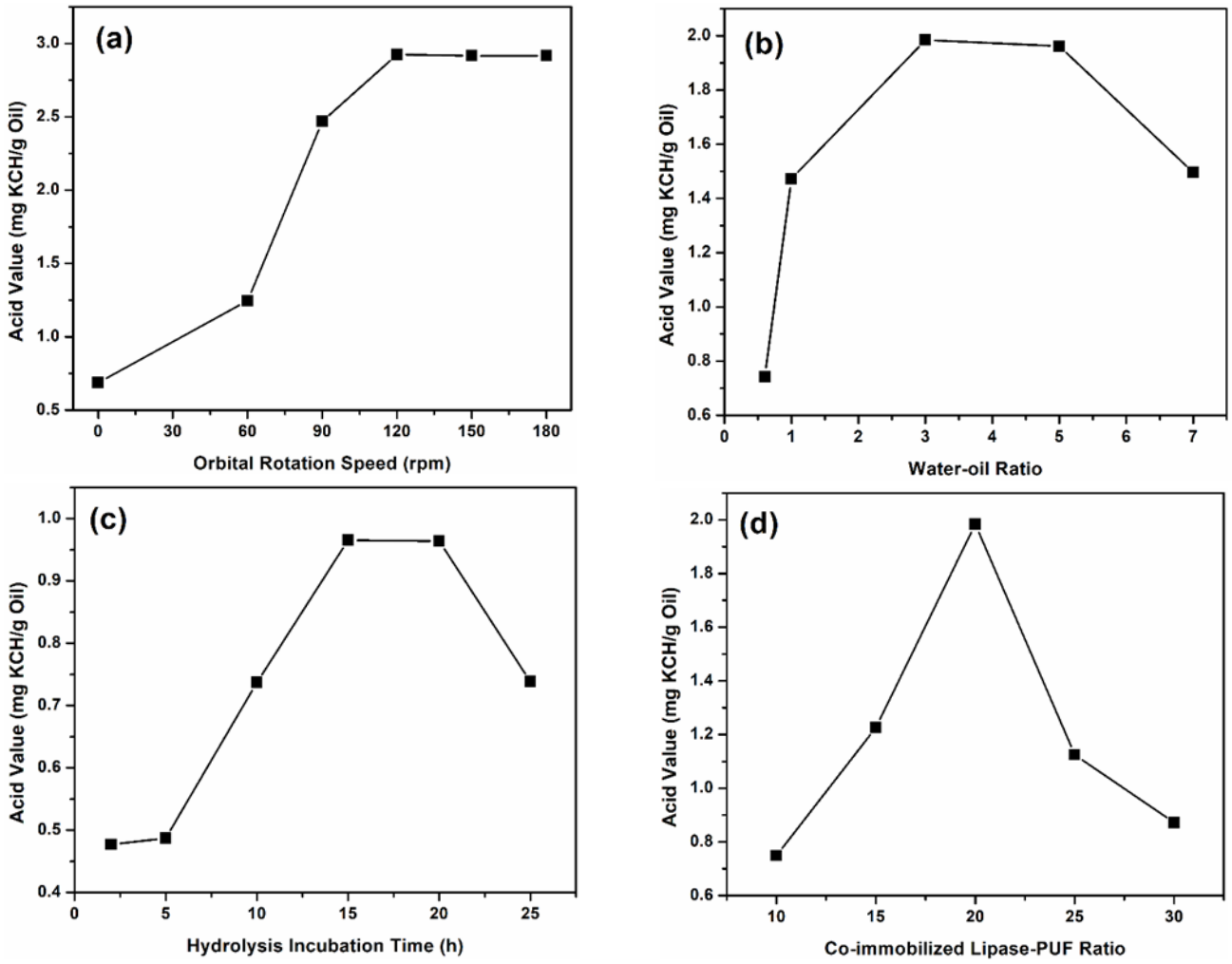

Figure 1. The effect of (a) orbital rotation speed, (b) water-oil ratio, (c) incubation time, and (d) co-immobilized-PUF ratio on the acid value of coconut oil hydrolysis reaction.

The effect of water-oil ratio on the acid value of hydrolysis was presented in Figure 1b. The figure demonstrates that the acid value escalated by increasing water-oil ratio from 0.6:1 to 3:1, followed by slight decreased at ratio of 5:1, and finally decreased remarkably at 7:1. From the results, the optimum water- oil ratio was obtained at ratio of 3:1 (w/w), which has the same tendency as some previous studies [1,30]. Increasing water-oil ratio was proportional as increasing the acid value which related to the amount of FFA as the product of hydrolysis. This phenomenon may due to enlargement of the interfacial area at the higher ratio. The high amount of water in the hydrolysis reaction causes equilibrium shifting to the product side during the process. Nevertheless, adding continuously the water would initiate diminishing the lipase enzyme activity. In the water-oil ratio greater than 5:1, lipase competes with FFA produced to occupy the interfacial region. Hence the contact between lipase and oil becomes smaller [31].

The effect of hydrolysis incubation time was illustrated in Figure 1c. The acid value increased considerably to a maximum value at 15 h of hydrolysis, then slight decreased after 20 h, and lastly declined notably following 25 h. The increase of acid value indicates a high interaction between triglycerides and lipases as increasing reaction time. FFA produced acts as inhibitors that block the interaction between oil and enzyme so that the resulting FFA became less [9].

The effect of co-immobilized-PUF ratio is shown in Figure 1d. There was a significant increase of acid value as an increase co-immobilized lipase-PUF ratio from 10:1 to 20:1. Co-immobilized consisted of lecithin, gelatin, and PEG that has the properties as a surfactant/emulsifier and $MgCl_2$ as a cofactor. The surfactants and lipases which both work on the interfacial surface requires proper comparison with the PUF matrix leading to the excellent role of the active site of enzymes. Lecithin and gelatin as thymol emulsifiers are used as food preservation antimicrobials [32]. Thymol as lipophilic is needed as a bacterial inhibitor in milk, carrot juice, ground beef. Gelatin and lecithin as an emulsifier were added so that thymol dispersed well. However, on the co-immobilized lipase: PUF ratio higher than 20:1, the acid value extremely decreased. These results revealed that the excessive addition of co-immobilized lipase would result in incomplete binding between the active site and PUF.

3.2 Second-order Polynomial Model

The acid value based on the RSM is shown in Table 2. The regression coefficients of intercept, linier, quadratic, and interaction terms of model were calculated using the least square technique and are presented in Table 3. The obtained second-order polynomial equation was found well to represent the experimental data ($R^2 = 0.9390$). The model p-value of 0.0018 imply that the model was statistically significant and show the reliability of the models.

Table 3. The significance of each response variable effect showed by using F-value and p-value in the non-linier second order model

	Variables	DF	SS	MS	F-value	p-value
Model		9	7.48	0.83	11.98	*0.0018*
Linier effect	A	1	0.002896	0.002896	0.042	0.8439
	B	1	0.35	0.35	5.1	0.0585
	C	1	1.21	1.21	17.49	*0.0041*
Quadratic effect	A^2	1	5.71	5.71	82.27	*0.0001*
	B^2	1	0.087	0.087	1.25	0.3008
	C^2	1	0.002001	0.002001	0.029	0.8699
Interaction effects	AB	1	0.011	0.011	0.16	0.6972
	AC	1	0.001849	0.001849	0.027	0.8749
	BC	1	0.00007921	0.00007921	0.001142	0.9740

Note. DF: Degree of freedom, SS: Sum of square, MS: Mean of square. The bold letter denoted statistically significantly different at $p = 0.05$

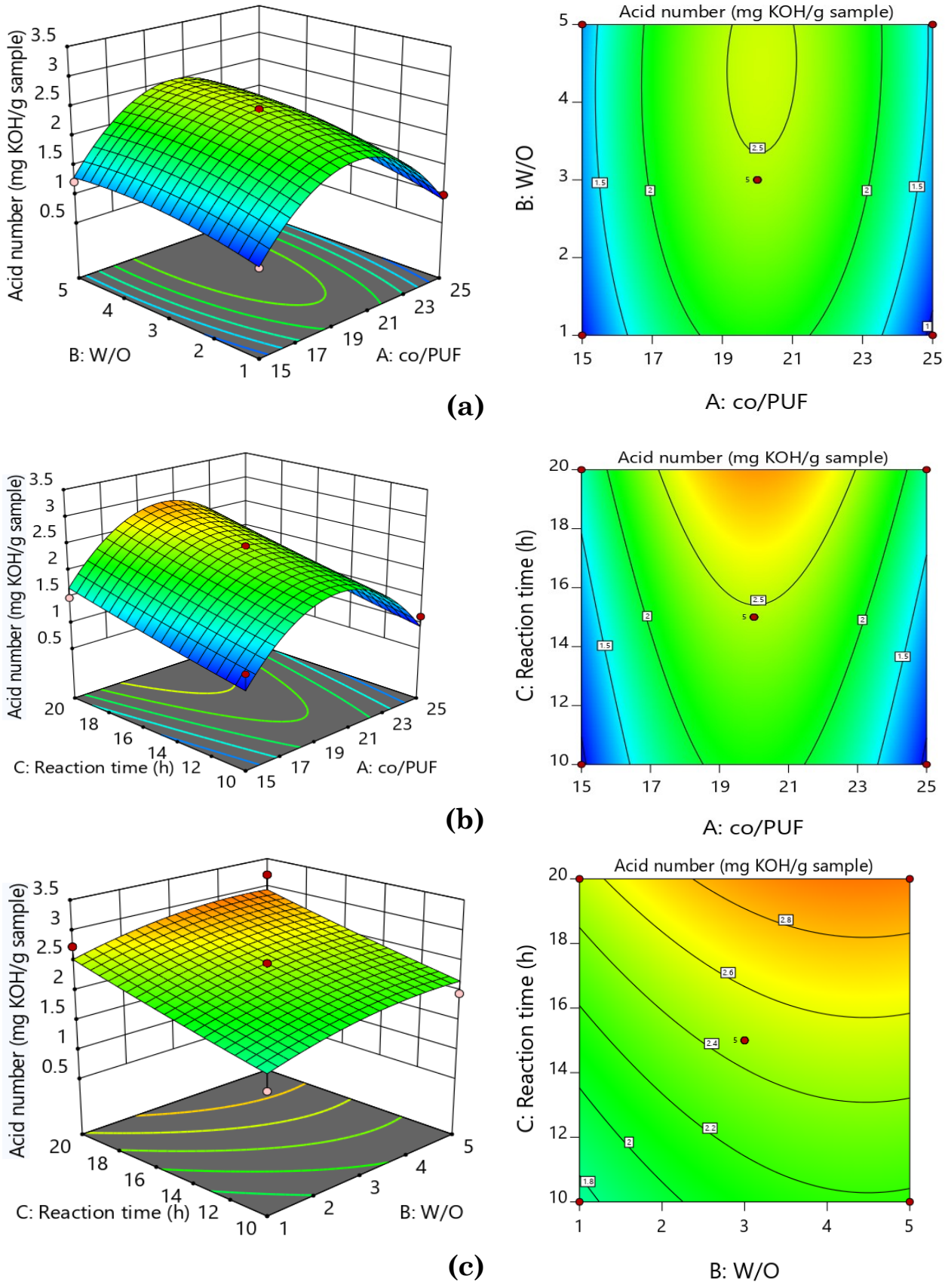

Figure 2. Surface and contour plot of (a) fixed hydrolysis incubation time of 15 h, (b) fixed water–oil ratio = 3:1, and (c) fixed co-immobilized lipase:PUF ratio = 20:1

$$Acid\ value = -17.2889 + 1.83776\,A + 0.2201\,B + 0.035865\,C + 0.00534\,AB + 0.00086\,AC - 0.000445\,BC - 0.046572\,A^2 - 0.003585\,B^2 + 0.000872\,C^2 \quad (5)$$

The *p*-values were used as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interactions between the variables. As shown in Table 3, the linear coefficients (A,B), quadratic coefficients (B^2,C^2) and interaction coefficients (AB, AC,BC) had no significant effect ($p > 0.05$). On the other hand, the effect of linier C and quadratic A^2 was highly significant ($p << 0.05$). The predicted values calculated from Eq.(5) were in very good agreement with the experimental values, as shown in Table 2. Hence, this quadratic model is well suited for this experimental set up.

In order to compare the predicted result with the practical value, retrial experiment was performed using some different hydrolysis condition. The values obtained from real experiments demonstrated the validity of the RSM model, since there were no significant differences of residue (See Table 4). The strong correlation between the real and the predicted results confirmed that the response model was adequate to reflect the expected hydrolysis condition.

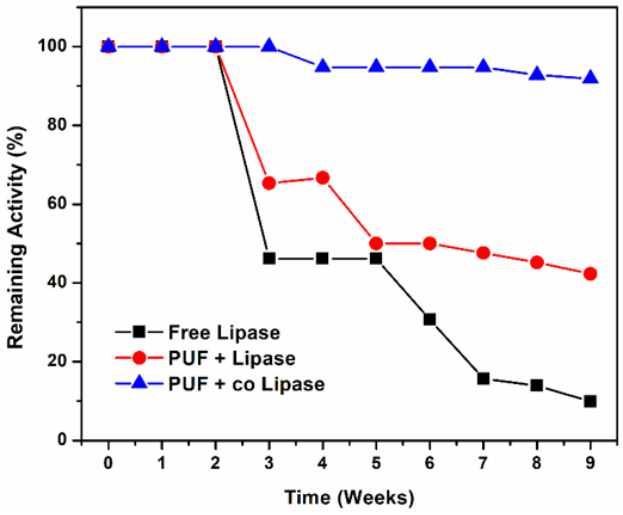

Figure 3. Time course of remaining enzyme activity (%) on free and immobilized lipase at the storage temperature of 4 °C

3.3 Response Surface Methodology Analysis

Lipase is an enzyme that works in the interfacial area of water and oil in a two-phase system. Lipase acts to cut the acyl group ($RCOO^-$) in triglycerides and hydrogen ions (H^+) in water to form free fatty acids (RCOOH). Figure 2a presents the effect of water-oil ratio, and co-immobilized lipase: PUF ratio towards the acid value at fixed hydrolysis incubation time of 15 h. The acid value is typically decreased from the water-oil ratio of 1:1 to 5:1. The steep curvature in water: oil ratio behavior demonstrated the response of acid value was very rapid to these factors. The results of this study have the same tendency as earlier work that the increase in the water-oil ratio was proportional to the increase in the percentage of FFA yield [31]. This result may occur because of at the higher water ratio, the interfacial area of water oil was also getting greater. This high interfacial area caused the equilibrium shift to the product of hydrolysis. On the contrary, in low interfacial conditions, the lower area will cause a decrease in the effective of the lipase enzyme activity [31].

The contour plot in Figure 2b shows that the acid value increased remarkably from Co: PUF ratio of 15 to 20, then decreased significantly on Co: PUF ratio of 25. Pretreatment using the organic polymer as initial wetting could increase the amount of the loaded enzyme, as seen on the Co: PUF ratio of 20 [33]. Co-immobilized consisted of blending of surfactant, lecithin, gelatin with PEG as crosslinker and $MgCl_2$ as a cofactor. The blending of lecithin and gelatin can reduce the size of the dispersed particles significantly. This condition allows for more bonding with the active site of lipase, leading to an increase in the conversion of hydrolysis reactions to fatty acids [32,34,35]. The combination of these ingredients can also take a role as a membrane for immobilization of lipase [23]. However, the decrease in acid value above co: PUF ratio of 20 may due to the

Table 4. Predicted and experimental values of responses at some hydrolysis condition

Run	Actual variable			Acid value (mg KOH/g oil)		
	A (co/PUF)	B (W/O)	C (h)	Experimental	Predicted	Residue
1	15	5	20	1.6012	1.6832	-0.0820
2	20	3	20	2.9293	2.8795	-0.0503
3	25	3	15	1.2388	1.3224	-0.0836
4	25	5	10	1.1115	1.0580	0.0535

ability of PUF to adsorb Co-immobilized has been saturated. Furthermore, $MgCl_2$ which was not adsorbed by PUF would become an inhibitor because it will compete with enzymes [36].

Figure 2b demonstrates response surface and the contour plot for hydrolysis of coconut oil by varying co-immobilized: PUF ratio and hydrolysis incubation time at a fixed water-oil ratio of 3. In the contour plot shown in Figure 2b, the acid value escalated significantly from the reaction time of 10 to 20 h. Prolong the reaction time gave a significant curvature effect on the speed of the hydrolytic lipase reaction yielding the increase of FFA production. The reaction time was limited to 20 h which intend to prevent FFA produced did not play a role as an inhibitor that can block the interaction between oil and water (see Figure 1c) [30].

Figures 2c shows the effect of the water-oil ratio and hydrolysis incubation time in the hydrolysis process at a fixed co immobilized lipase: PUF ratio of 20. The acid value increased evidently as the increasing of water-oil ratio and nearly reached a maximum value at the highest hydrolysis incubation time. It can be seen that the maximum acid value of FFA obtained at the water-oil ratio and hydrolysis incubation time was 4.4 and 20 h, respectively.

From *F*-value in Table 3, the significant parameter was determined. It can be seen that linear effect of hydrolysis incubation time and quadratic effect of co-immobilized lipase: PUF ratio was the significant parameter. The optimal hydrolysis condition was achieved at co immobilized: PUF ratio, water: oil ratio, and hydrolysis incubation time of 20.17, 4.45, and 20 h, respectively. At optimized condition, the predicted acid value of FFA was 3.21 mg KOH/g sample oil.

3.4 Storage Stability of Co-immobilized Lipases

The storage stability of the enzyme was carried out by examining the enzyme activity of free lipase, PUF-lipase, and PUF-co-immobilized lipase at a storage temperature of 4 °C for 9 weeks. As presented in Figure 3, the remaining activity of 100% of the three immobilization methods was still stable until the two weeks. However, after the 3rd week, there was a decrease in stability in free lipase and PUF-lipase to 46.15% and 65.31%, respectively. At week 4, the stability of PUF-co-immobilized

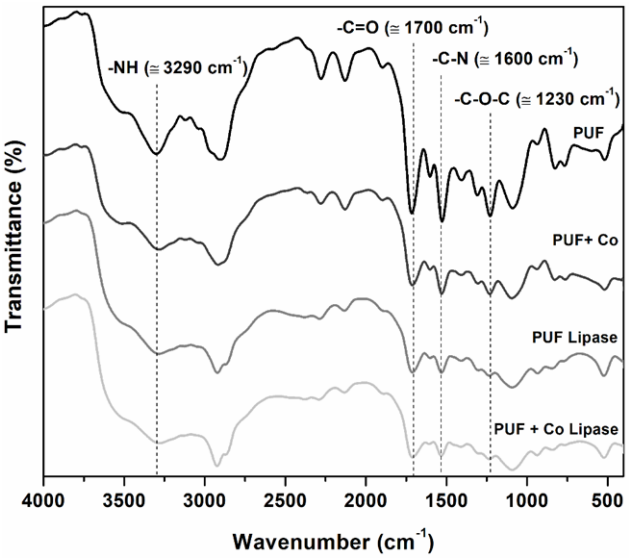

Figure 5. FTIR spectra of (a) PUF, (b) PUF-Co, (c) immobilized lipase on PUF and (d) co-immobilized lipase on PUF.

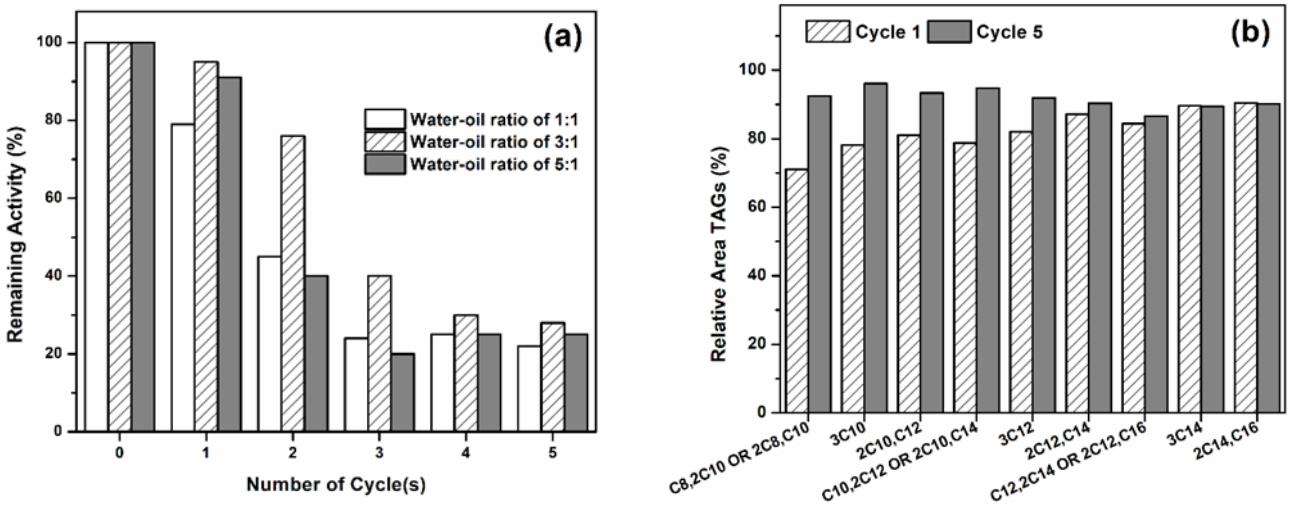

Figure 4. Plot of (a) remaining activity on reuse of Immobilized Lipases and (b) relative percentages of residual TAGs to fatty acids in various uses of immobilization of lipase for coconut oil hydrolysis.

lipase began to decline to 94.73%. After 4 weeks, the enzyme stability by all methods have decreased, especially free lipase. Until the storage time of 9 weeks, the stability of free lipase, PUF-lipase, and PUF-co-immobilized-lipase decreased to 9.89%, 42.3%, and 91.88%, respectively. The same phenomenon was found on the esterification reaction using PUF as a matrix for lipase immobilization through polyethyleneimine (PEI) coating and the addition of glutaraldehyde (GA) [16]. At an incubation condition of 50 °C for 30 min, remaining activity of free lipase, immobilized lipase on PUF, and immobilized lipase on PEI-coated PUF was 0%, 20%, and 50%, respectively. Great remaining activity from lipase immobilization due to covalent bonds that can increase storage stability [4,12,16]. The addition of matrix material in this study namely gelatin, lecithin, PEG, $MgCl_2$ can act as spacers to extend and strengthen covalent bonds between matrix and lipase. The enzyme molecules that have been activated with the addition of co-immobilized are therefore bound to be more stable to the PUF matrix [4,16].

3.5 Reusability Studies

One of the advantages of the immobilization method is the reusability of enzymes leading to minimizing operational costs. Figure 4a depicts the remaining activity of the reuse lipases *M. miehei* on the various water-oil ratio in the hydrolysis of coconut oil into FFA. After 3 cycles, the activity remained about 50% of the initial activity. Similar results were shown by [15,37,38]. The acid values of this study were relatively low compared to literature which uses commercial immobilized lipase with a remaining activity of 50% after 5 times cycles. A water-oil ratio of 3:1 and 5:1 have a relatively better ability than 1:1. However, after 4, 5, and 6 times cycles, there was a significant decrease in remaining activities up to 22, 28, and 25%, respectively.

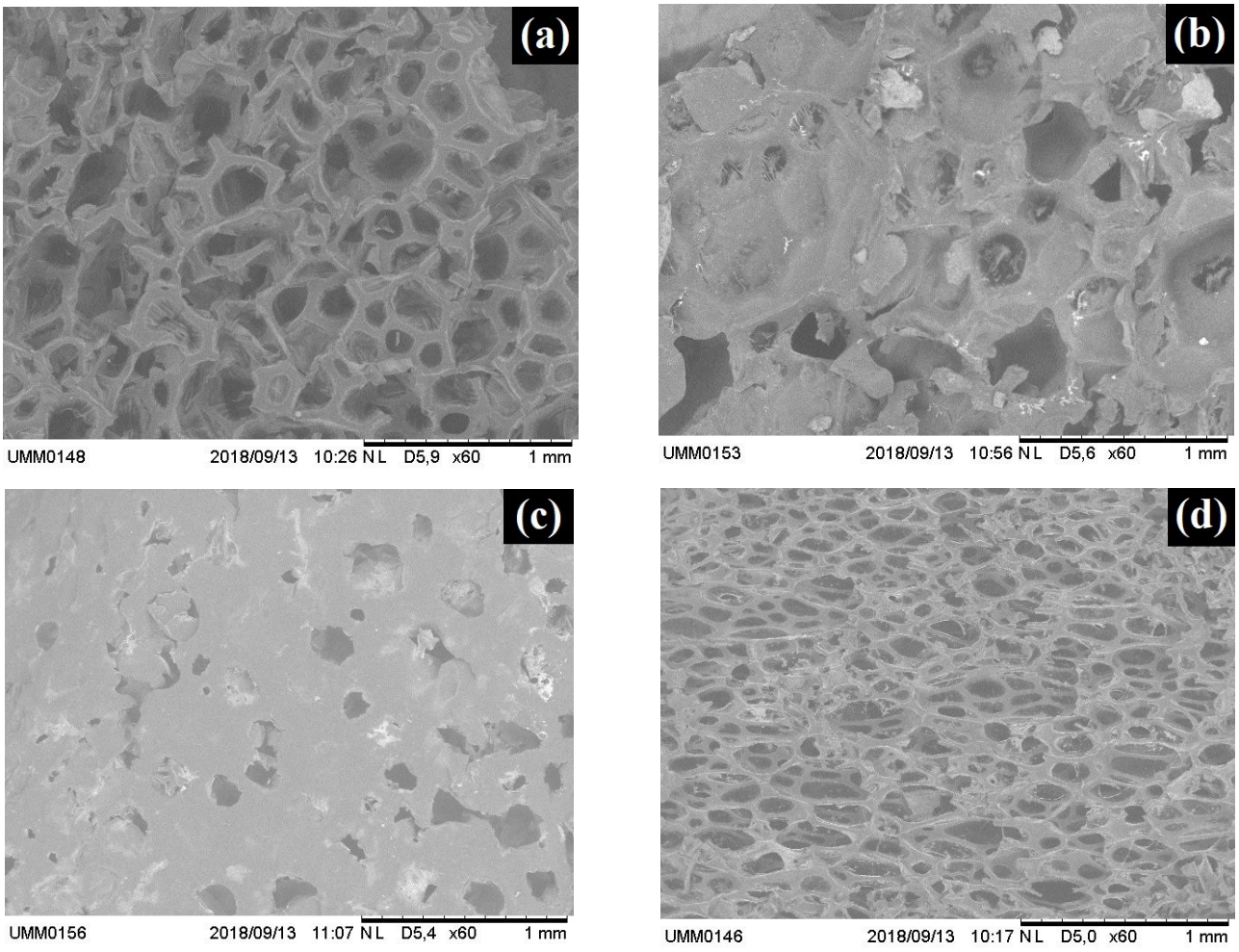

Figure 6. Scanning electron micrograph of a) PUF, b) PUF-Co immobilized, c) PUF-lipase, and d) co-immobilized lipase on PUF

The coconut oil contains TGAs of 92%, fatty acids as lauric acid (C12) of 0.1-3.5%, and sterols tocol of 0.5-1.5%. TGAs components may consist of a combination of 3 fatty acid molecules, including a combination of C8, 2 C10 or 2 C8, C10; 3 C12, and others [39]. In the initial condition, the combination of the TAGS components was 100% (see Figure 4b). In the cycle of 1 and 5, the amount of TAGs was less than 100%, which means TAGs was converted to FFA. For example, the initial TAGs components of C8, 2 C10 or 2 C8, C10, after hydrolysis in cycle 1, and cycle 5 were 100%, 71.07%, and 92.09%, respectively. While the initial TAGs component of 3 C12, after hydrolysis in the cycle 1, and cycle 5, were 100%, 82.07%, and 91.85%, respectively. The same trend was shown by other constituent components of TAGs. This phenomenon shows that in the cycle 1, hydrolysis using immobilized lipase on PUF could convert TAGs higher than cycle 5. These results were confirmed by remaining activity of cycle 1 which was higher than cycle 5 (Figure 4 (a)).

3.6 Characterization of Native PUF and Its Variation of Immobilization Techniques

3.6.1 FTIR analysis

In Figure 5, spectrum of native PUF were illustrated as the peaks of amide group –NH (± 3290 cm^{-1}), carbonyl urethane group –C=O (± 1700 cm^{-1}), carbamate group –CN (± 1600 cm^{-1}) and –COC (± 1230 cm^{-1}) [40,41]. The addition of co-immobilized agents affected the intensity of the peaks. There were changes of native PUF intensity. The co-immobilized which consist of gelatin, lecithin, PEG, and $MgCl_2$ contains a lot of amide, and carbonyl group. Whereas the lipases which are suspended from amino acids have primary amide N-H regions at wavelengths of around 1650 cm^{-1} and –C=O [41,42]. Because of the immobilization of lipase on PUF through coating these materials, the native PUF intensity in the four wavelengths become weaker. This phenomenon presented the real effect of co-immobilized and lipase coating on native PUF.

3.6.2 SEM analysis

The morphological structures of native PUF, PUF-Co immobilized, immobilized lipase on PUF and co-lipase immobilized on PUF are shown in Figure 6. As presented in Figure 6, the addition of lipase to PUF demonstrated a smoother surface than native PUF. This phenomenon indicates that the lipase, through its covalent bond, can attach to the porous PUF surface. On the addition of co-immobilized lipase (see Figure 6d), there was an agglomeration of these materials. This phenomenon was proven by the measurement of the average diameter of co-immobilized lipase on PUF (104 μm) which smaller than native PUF (221 μm). Moreover, this result reveals that lipase attached to the surface of PUF which has been coated with co-immobilized. The SEM figures of this study is in line with the previous work on the immobilization of lipase *C. Antarctica B* using PUF for geranyl propionate and ethyl oleate synthesis [43]. The SEM graph of PUF immobilized lipase *Y. lipolytica* via polyethyleneimine coating and glutaraldehyde coupling was also smoother than the native PUF [16].

4. Conclusions

The co-immobilized lipase on PUF can be used effectively to hydrolyze coconut oil to FFA. The optimum condition of hydrolysis reaction through RSM was obtained on the co-immobilized lipase: PUF ratio, water-oil ratio and hydrolysis incubation time of 20.17, 4.45, and 20 h, respectively. In the optimum condition, co-immobilized lipase can be reused for 5 reactions and increase storage stability at 4 °C for 9 weeks. Remaining activity after 5 times hydrolysis from co-immobilized lipase, immobilized lipase, and free lipase was 91.88%, 42.3%, and 9.89%, respectively. The optimal condition resulted in a FFA of 3.2107 mg KOH/g oil-sample.

Acknowledgments

The authors thank the Directorate General of Resources for Science, Technology & Higher Education, Ministry of Research, Technology and Higher Education of Republic Indonesia for financial support (205/SP2H/LT/DRPM/III/2016) of this study.

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