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Thermal stability and reusability of home-made co-immobilized lipase from *Mucor miehei* in polyurethane foam for the production of bio-flavor

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Abstract. In this study, polyurethane foam (PUF) was used as a matrix to immobilize *M. miehei* lipase through a modified substance coating consisting of lecithin, gelatine, polyethylene glycol (PEG), $MgCl_2$, known as co-immobilized. Co-immobilized lipase application was carried out on bio-flavor manufacturing through hydrolysis and esterification reactions. Fatty acid was gained from hydrolysis of glyceride coconut oil (CNO) containing main components of C8 to C16. Furthermore, esterification of fatty acids and citronellol from citronella oil was employed. The remaining triglycerides (TAGs) in hydrolysis were analysed using LC-MS/MS. While bio-flavor was analysed using GC-FID. Maximum bio-flavor production was obtained at 40 °C and fatty acids lauric acid/citronellol mole ratio of 1:5. The remaining activity of free lipase, immobilized lipase on PUF, and immobilized lipase on co-coated PUF at 50 °C for 50 min was 0 %, 34 %, and 54 %, respectively. Immobilized lipase on co-coated PUF was able to reuse up to 5 cycles. The maximum conversion of bio-flavor was attained using lipase on modified co-immobilized. The use of co-immobilized lipase of the non-commercial source used in this study could be a promising method for a zero-waste bio-flavor production in a mild and solvent-free process to support low-cost and green technology.

1. Introduction

These Bio-flavor as an ester produced from plant extracts is rare in commercial scale production [1]. Until now, many food industries use artificial flavor as a food additive which is carried out through organic chemical synthesis. Even though, bio-flavor can be produced as an ester through an enzymatic reaction using lipase [2].

Lipases (triacylglycerol EC 3.1.1.3 hydrolases) are enzymes used for the reaction of lipid hydrolysis to fatty acids and glycerol. Lipase has the ability to catalyse several reactions such as esterification (alcohols and carboxylic acids), transesterification (esters and alcohols), interesterification (esters and acids) and transfer of acyl groups from esters to other nucleophiles (e.g. amines and thiols) [3–5]. Commonly, lipases are produced from a variety of microorganisms, but yeast and fungi are the primary sources widely used in industry. Commercial lipases obtained from *M. miehei* are able to catalyse the CNO which consist of lauric acid (C12) and myristic (C14) about 60% to produce ester flavor [6–9]. However, the low stability of free enzymes, especially the effect of temperatures above 40 °C for a long operational time, causes the use of this enzyme on a limited industrial scale [10].

There are three ways to increase enzyme stability. According to Cui et al. [6], *Y. lipolytica* lipase Lip2 was stabilized with improved thermal stability by adding a material for protective lipase and



immobilizing various matrixes. PUF was widely utilized as a lipase immobilization matrix because of commercially inexpensive, inert, and excellent mechanical properties for high resistance. Nyari et al. [12] used PUF as a matrix for immobilizing lipases in the esterification reaction of alcohols and carboxylic acids.

The use of immobilization techniques is a solution to the low stability of free enzymes through various carrier binding or crosslink and encapsulation methods in the inorganic/organic polymer matrix [13]. The immobilization of enzyme on various solids supports through physical and chemical mechanisms was utilized to recover, reuse and increase temperature stability (thermal, operational and storage) of the enzyme [14]. This biotechnology process can be economically an option for the development of enzyme applications [12,15]. Enhancing enzyme stability can also be prepared by adding material support such as glutaraldehyde or a combination of lecithin-gelatine / lecithin-PEG / gelatine-PEG and the addition of metal ions as cofactors so as to improve enzyme performance [16–18]. Based on literature review, the combination of lecithin-gelatine-PEG-cofactor Mg has never been studied related increasing stability and reusability for immobilization of lipase on the bio-flavor hydrolysis from CNO.

This study aims to prepare co-immobilized coating on PUF matrix, followed by covalent and crosslinking methods, and study thermal stability and reusability of immobilized lipase. The use of lipase immobilization in PUF matrix was applied to produce bio-flavor from CNO through hydrolysis process producing fatty acids then reacted with citronellol in the esterification reaction.

2. Experimental section

2.1. Materials

CNO was collected from the local Permata Agrindo Pendowoharjo (Sewon, Bantul, Indonesia) under the commercial name Laitco. The stock of *M. miehei* was cultured in Biochemical Technology Laboratory, Department of Chemical Engineering, Sepuluh Nopember Institute of Technology, Surabaya. Co-immobilized consisting of gelatin, lecithin, PEG, $MgCl_2$ was purchased from Merck (Germany).

2.2. Production of crude lipase from *M. miehei* through solid state fermentation

Media consisting of KH_2PO_4 , $FeSO_4 \cdot 7H_2O$, olive oil, palm oil, dried coconut grout (solid), and water was sterilized at 121 °C for 15 min. *M. miehei* culture stock was inoculated in the media and incubated through solid state fermentation at 37 °C for 5 days. Furthermore, crude lipase obtained by extraction was added phosphate buffer- solid media ratio of 4:1 (wt/wt), then incubated at 37 °C, for 135 min, 150 rpm. Crude/free lipase as a supernatant was filtrated and analysed the activity (U/ml) [19,20].

2.3. Assay of lipase activity

The activity of crude lipase was determined using olive oil as a substrate. Olive oil and Gum Arabic were emulsified for 2 min. Next, 5 ml of emulsified olive oil was mixed with 2 ml of 0.1 M phosphate buffer (pH 7) and 1 ml of enzyme suspension. The mixture was incubated at 37 °C for 30 min. After incubation, the reaction was stopped by adding acetone-ethanol of 1:1 (v/v) and the free fatty acid (FFA) was titrated by 0.05 M NaOH. One unit of lipase activity is defined as a number of enzymes capable of liberating 1 μ mol of fatty acids per minute [21]. Lipase activity was calculated by Equation (1), where V_s and V_b are the volume of NaOH needed to titrate the sample and blank (ml), respectively. The value of 1000 is the conversion from mmol to μ mol and t is the reaction time (30 min).

$$\text{Lipase activity (U/ml)} = \frac{(V_s - V_b) \times N_{NaOH} \times 1000}{t} \quad (1)$$

Matrix activity was determined in the same way as above, but a 1 ml crude sample was replaced with 1 g of PUF co-immobilized lipase. Hence, the co-immobilized lipase-PUF activity has units of U/g matrix

2.4. The procedure of lipase immobilization

2.4.1. Co-immobilized coating

Co-immobilized was prepared by making a mixture of solutions consisting of lecithin (50 g/l), gelatine (50 g/l), MgCl_2 (10 g/l), and PEG 6000 (20 g/l) with the same weight composition ratio. PUF was made by reacting isocyanates and polyols with the same volume ratio, then cut to size of 5 mm x 5 mm x 5 mm. The co-immobilized-coated PUF was prepared by soaking PUF in co-immobilized with a ratio of 1:20 (wt/wt) and then stored at 40 °C.

2.4.2. Immobilization of lipase on PUF

Immobilization of lipases on PUF was carried out by the following steps. First, physical adsorption on PUF was done by soaking 1 g of plain PUF into free lipase for 24 h at room temperature, washed and stored. Second, 20 gr of co-immobilized consisting of dispersed lecithin, gelatine, PEG, MgCl_2 was dispersed cross-linking through the reaction of each material. Lastly, covalent lipase binding in PUF was activated by co-immobilized.

2.5. Hydrolysis and esterification reaction

2.5.1. Hydrolysis of CNO by immobilized lipase of *M. miehei*

Hydrolysis of CNO by lipase immobilized on PUF produced FFA and glycerol. The hydrolysis reaction was carried out in a three-neck flask. CNO of 10 g and water of 50 g was mixed and added 26.24 U of immobilized lipase on PUF. The reaction was maintained at 40 °C during 900 min. The product of hydrolysis reaction consisting of two layers, the fatty acid and glycerol layer, was separated using a separating funnel. Chemical properties of the remaining TAGs were determined by LC-MS/MS.

2.5.2. Esterification of FFA and citronellol by immobilized lipase from *M. miehei*

The FFA produced from hydrolysis reaction of CNO was used as the substrate for the further esterification reaction. The esterification reaction was carried out in a batch process using erlenmeyer containing FFA, citronellol and immobilized lipase. The substrates of FFA and citronellol were prepared in a molar ratio of 1:1 and the immobilized lipase was added in 6 U. The reaction was maintained at 40 °C for 5, 10, 15, 20, and 25 h. The product of esterification consisting of two layers, bio-flavor as ester and water, were separated by a separating funnel.

2.6. Thermal stability and reuse analysis

Stability of free lipase, immobilized lipase on PUF, immobilized lipase on co-coated PUF were determined through thermal stability test at an incubation temperature of 50°C. During the incubation period, the remaining activity was analysed every 10-minute interval and defined by the Equation (2).

$$\text{Remaining activity (\%)} = \frac{[(\text{initial activity}) - (\text{activity after storage})]}{\text{initial activity}} \times 100 \quad (2)$$

The relative percentage of hydrolysis was calculated by the Equation (3), where n denotes to a number of cycles.

2.7. Analytical method

The remaining TAGs from the CNO hydrolysis reaction were analysed using LC-MS/MS. ACCELLA 1250 LC-MS/MS (Thermo Scientific-Waltham, MA USA) was equipped by Hypersil Gold column (50 mm x 2.1 mm x 1.9 μm), vacuum degreaser, quartener pump, thermostatic autosampler controlled by a personal computer through x-calibre program 2.1. The mobile phase of 2 μL was employed at a flow rate of 300 $\mu\text{L}/\text{min}$. The column and autosampler compartment were controlled at 30 °C and 16 °C, respectively. The use of MS/MS Triple Q TSO QUANTUM ACCESS MAX mass spectrometer with Electrospray Ionization was controlled by TSQ Tune software and operated in positive mode.

Bio-flavor was examined using GC-FID (HP 5890, Santa Clara-California, United States). GC FID was equipped by HPL 608 column in dimension of 30 m length x 0.53 mm i.d. x 0.5 μ m film thickness. The temperature of the GC-FID oven was adjusted to 125 °C for 3 min elevating of 7.5 °C/min up to 250 °C. The temperature of the injector and detector were kept at 255 °C and 275 °C, respectively. Conversion of citronellol was determined based on the area under the curve [22,23].

3. Results and Discussion

3.1. Effect of thermal stability free and immobilized activity on hydrolysis reaction

The operating conditions of the hydrolysis and esterification reactions using co-immobilized lipase in the PUF matrix were carried at 40 °C and pH 7 based on an optimum condition of previous work by Patel et al. [24]. The thermal stability of the lipase activity was examined at 50 °C from free lipase, immobilized lipase on PUF, and immobilized lipase on co-coated PUF in the hydrolysis of olive oil [6].

Figure 1 demonstrated the remaining activity of the enzyme on hydrolysis using free and immobilized lipase. As presented in Figure 1, in the temperature above the optimum condition, the thermal stability of the three immobilization methods shows different performance. Free lipase has lower stability compared to the others. At 50 min of hydrolysis, the remaining activity of each free lipase, immobilized lipase on PUF, immobilized lipase on co-coated PUF was 0 %, 34 %, 54 %, respectively. The composition of co-immobilized would affect stability remarkably. These results are owing to gelatine and lecithin as emulsifiers are dispersed into small particles which can penetrate the substrate entry into the immobilized lipase on co-coated PUF. Moreover, PEG would reinforce the polymerization of gelatine lecithin through its hydroxyl bond and $MgCl_2$ as a cofactor metal ion [18]. Perez-Miller and Hurley [25] also showed the covalent bonding between Mg and phospholipid owned by lecithin.

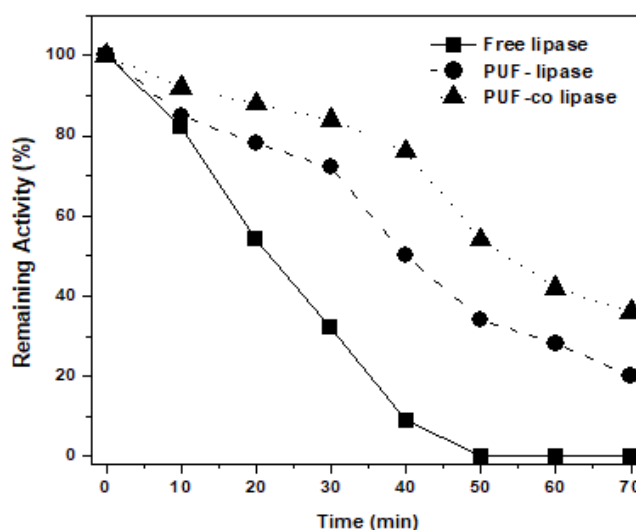


Figure 1. Thermal stability of free and immobilized lipase on PUF

3.2. Reusability on hydrolysis reaction

Generally, CNO contains saturated fatty acids in the form of 92 % TAGs and 0.1-0.3 % FFA. Most of the TAGs (about 70 %) are saturated fatty acids known as medium chain fatty acids (MCFA), with the lauric acid content of 45-56 % [9]. the FFA content before and after the hydrolysis process which analysed using the titration method were 0.2 and 0.85 %, respectively. Residual TAGs analysis using LCMS/MS was observed as TAGs in the form of C8, C8, C10; C8, C10, C10 or C8, C8, C12; 3×C10; C10, C10, C12; C10, C12, C12 or C10, C10, C14; 3×C12, C12, C12; C14, C12, C14, C14 or C12, C12, C16; 3×C14; C14, C14, C16; C14, C16, C16 or C14, C14, C18.

TAGs, also known as lipids, need a lipase biocatalyst to dissolve into the water on the hydrolysis reaction. The surface actives work on the interface area of water and lipid phase. Immobilization of lipase was done by combined encapsulated-coating, crosslinking and covalent binding to increase the enzyme performance. The advantages of using immobilized enzyme techniques are due to highness of stability and reusability. In this immobilization technique, lipase was bounded to the solid matrix PUF facilitating separation between the product and immobilized lipase directly [26].

The hydrolysis reaction was carried out on the water-oil ratio of 5:1 (w/w) using immobilized lipase-PUF resulting in FFA and glycerol (see Figure 2). Immobilized lipase-PUF in this study can be reused up to 5 times. As shown in Figure 2, the remaining TAGs show an unreacted composition of fatty acids. As the example in reuse 1, from mass spectra analysis shows BM 527 which is identified as C8, C10, C10 or C8, C8, C12, while reuse 4 is identified as C14, C14, C16.

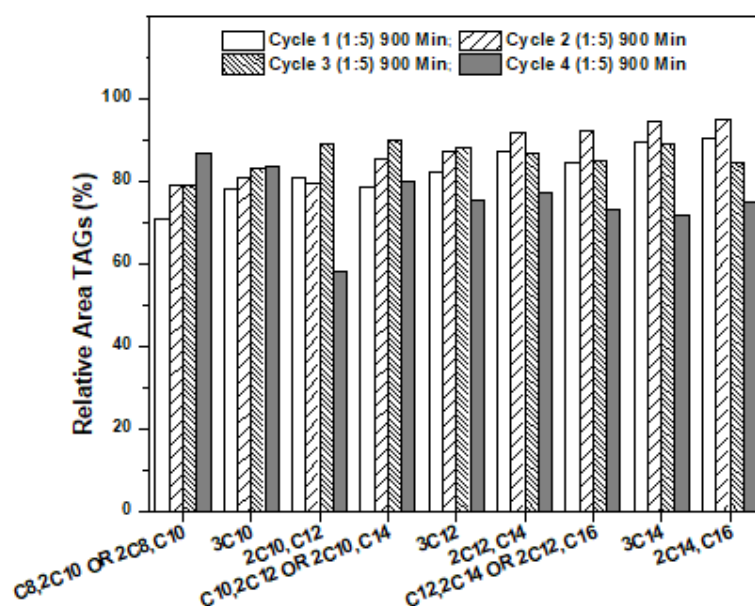


Figure 2. The relative area of fatty acids in immobilization of lipase for CNO hydrolysis

In this study, after completing the hydrolysis reaction, the enzyme was separated from the product then immediately reused in the reaction with the same variables to compare. The immobilized enzyme was washed without solvent before reuse. This step was confirmed by Nyari et al. [12] that using washing stages without hexane solvents.

Figure 3 exhibits the relative conversion of hydrolysis (%) on the reuse of immobilized lipase. As illustrated in Figure 3, on the cycles number of 2 and 3, the relative conversion decreased from 17.48% to 12.65%. These results were confirmed by another previous work [24,27]. However, on the number of cycles of 4 and 5, relative conversion increased gradually. This phenomenon was also attained by Ferraz et al. [20] using home-made immobilized lipase from *P. crustosum* for the esterification of geraniol laurate. In the study, it was found that the percentage of relative activity and conversion tends to fluctuate with increasing the number of cycles. This trend was related to the commercial and non-commercial use of lipases from various sources and reaction systems.

The utilization of commercial lipases commonly presented the consistency of decreasing the percentage of conversion and enzyme activity during a number of cycles. In the immobilization of commercial lipase, enzyme activity efficiency decreases with increasing number of reuse [4]. As revealed by Mohammadi et al. [27] on the study of the hydrolysis of fish oil using immobilized lipase *R. miehei*, the remaining activity decreased from 100% to 83% after 5 cycles. Moreover, Patel et al. [24] also showed a decrease in residual esterification percentage from 100% to 50% on the production of ethyl caprylate after 29 times cycles.

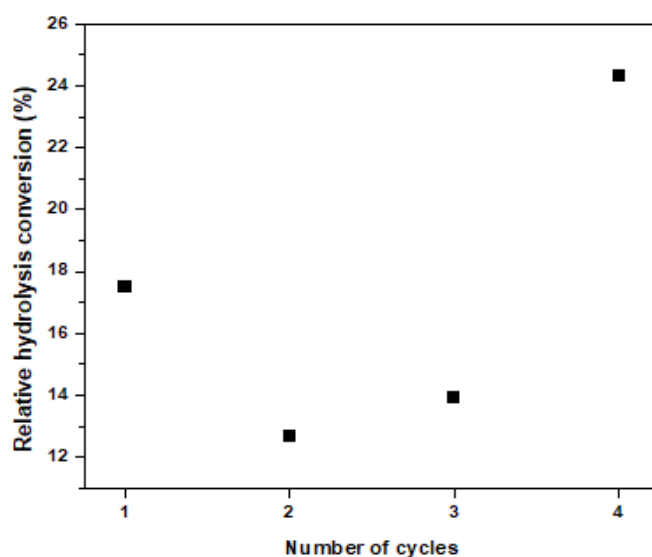


Figure 3. Relative hydrolysis conversion (%) of the immobilized lipase reuse

3.3. Application immobilized lipase in esterification for bio-flavor production

Immobilized lipase on PUF which has been examined the stability and reusability performance was applied to the esterification reaction with the FFA substrate produced by hydrolysis (in section 3.2) and citronellol, resulting in bio-flavor. The content of citronellol before and after the esterification process was analysed by GC-FID to determine the conversion percentage. The conversions of citronellol (%) in producing bio-flavor after 5, 10, 15, 20 and 25 h were 32.5, 42.6, 46.4, 51.9 and 52.1, respectively.

The results of the optimum bio-flavor conversion by employing immobilized lipase and free lipase were 52.1 and 44.4 %, respectively. Applying immobilized lipase provides a higher conversion than the free lipase because, after immobilized, the enzyme will be a restructuring of the "lid" lipase structure yielding configuration changes. This configuration changes may be due to contact of the enzyme into the interfacial region which causes hydrophobic exposed outside and the hydrophilic inside (becomes an open form) leading to high contact between the enzyme and substrate [4,24]. As a comparison, the highest yield produced by the esterification reaction using immobilized lipase enzyme was 85 % for 48 h (higher than the yield of this study). However, this study uses non-commercial *M. miehei* lipase, while Patel et al. [24] used commercial lipase *C. rugosa* so that giving different performance.

4. Conclusion

Lipase immobilized on co-coated PUF gave the highest thermal stability compared to immobilized on PUF only and free lipase. The immobilized lipase could retain its hydrolytic activity after 5 times cycles without organic solvent washing. The immobilized enzyme prepared in this study also gave better performance than free lipase in the production of bio-flavor.

Conflict of interest

No conflict of interest.

Acknowledgments

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