Lipase immobilization onto Cellulose Nanocrystals (CNC) for catalyzing lipolysis of triglycerides

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Abstract. Over the past decade, the technology of enzyme immobilization has been developed because it is able to produce reusable immobilized enzymes to reduce production costs. This research aims to synthesize cellulose nanocrystals (CNC) from sugarcane bagasse. The CNC was then used as a matrix in immobilization of lipase to be applied in triglyceride lipolysis. Lipase was immobilized onto CNC through covalent bonding method at 25°C dan pH 7 with immobilization period variation of 0.5–4.5 hours. The immobilized lipase will be utilized to catalyze lipolysis reaction of triglyceride from palm oil, with lipolysis period 2–10 hours, reaction temperature of 25–60°C, and pH of 6.25–11.25. Lipase was successfully immobilized onto CNC so it can be utilized in lipolysis of palm oil with degree of hydrolysis about 10%. The optimum immobilization time and reaction time of lipolysis was 1.5 hours and 6–8 hours, respectively. Both free lipase and immobilized lipase had the optimum temperature at 40°C. In contrast to the optimum temperature, the optimum pH of the lipolysis reaction using free lipase is different from that of lipolysis using immobilized lipase. The optimum pH of lipolysis using free lipase is pH 7.25, while the optimum pH for reaction using immobilized lipase is shifted to pH 8.25.

1. Introduction

Free fatty acids (FFA) are aliphatic monocarboxylic acids with 4 to 28 carbon atoms. FFA are intermediate compounds that are widely used in industries such as the soap, cosmetics, pharmaceutical and bioenergy industries. During this time, FFA are produced from the hydrolysis of vegetable oils by the Colgate-Emery process which requires large amounts of energy (250°C, 4.82 MPa) [1]. To overcome these problems, enzymatic hydrolysis technology (lipolysis) was developed. The use of lipase in hydrolysis reactions can take place at low temperatures and pressures. However, the use of enzyme on an industrial scale tends to require high production costs due to expensive enzyme prices. This is because the enzyme purification process is difficult and tends to require expensive operating costs [2].
Over the past decade, the development of enzyme immobilization has been a great research topic. Immobilized enzymes exhibit high stability in high range of operation conditions, such as temperature, and pH. In addition, the immobilized enzymes can be reused because they can be easily separated from the product. Moreover, the reusability of immobilized enzymes might reduce the production costs in enzymatic process industry. One of the important factors in the immobilization of this enzyme is a matrix to support the enzyme. Most matrices tend to be difficult to decompose in nature, so their use is not environmentally friendly. There are even several types of matrices that exhibit high toxicity [3]. Choosing the right matrix material is important to be considered. Organic materials that are more environmentally friendly and low in toxicity are attractive to be used as a matrix. One of the potential organic materials to be used as a matrix or enzyme support is cellulose nanocrystallines (CNC) [4]. CNC exhibits some superior material properties, such as low density, high mechanical properties, high surface area, and high biocompatibility [5–7].

The development of current immobilization technology research tends to lead to the immobilization of enzymes by the covalent bonding method in inorganic supports. One of the study is conducted by Xie and Ma (2009), immobilization of the lipase from *Thermomyces lanuginose* by the covalent bonding method on Fe$_3$O$_4$ nanoparticles matrix. The results from the study is yield of immobilization obtained by 84% [8]. Along with the development of renewable-based materials, the researchers developed immobilization technology on organic supports. Research conducted by Kim et al. (2012), immobilization of the lipase from *Candida rugosa* by entrapment method on a 5% cellulose-biopolymer composite hydrogel buffer resulting in an immobilization yield of 24% [9]. Based on these two studies, it shows that immobilization of enzymes using the covalent bonding method is very potential. However, immobilization of the lipase in organic matrix by the covalent bonding method is still rarely performed. Therefore, this study was conducted to examine the covalent bonding method in the process of immobilizing lipase onto CNC for triglyceride lipolysis.

2. Methodology

2.1. Materials

The main raw material used in CNC synthesis is sugarcane bagasse. CNC was synthesized on a laboratory scale using the TEMPO ((2,2,6,6-Tetramethylpiperidin-1-yl) oxyl) oxidation method [10]. In TEMPO oxidation, the oxidant used was NaOCl to oxidize the alcohol group into carboxylic group. Lipase type VII originally obtained from *Candida rugosa*, N-hydrosuccinimide (NHS) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were from Sigma Aldrich. Other materials used were materials for enzyme activity analysis and lipolysis. The lipolysis substrate used was Refined Bleached Deodorized Palm Oil (RBDPO) (Bimoli, produced by PT Salim Ivomas Pratama Tbk.). For the titration analysis, potassium hydroxide in ethanol was used. Universal indicators used were phenolphthalein indicators (lipolysis) and thymolphthalein indicators (enzyme activity).

2.2. Preparation of activated cellulose nanocrystals

Before being used to immobilize the lipases by covalent binding, the CNC needed to be activated. The inactivated CNC as amount of 2.5 mg was added into 9 mL of 0.1 M phosphate buffer (pH 7.0) and dispersed under digital ultrasonic for 60 min. The suspension was mixed with 2.3 mL of NHS (or equivalent to 50 mg/mL) and 1.2 mL of EDC (or equivalent to 10 mg/mL) and then the mixture was stirred for 30 min. pH was maintained in the range 7.5–8.0 by dropping 1.5–2.0 mL of 0.5 M NaOH/HCl. After centrifugation for 10 min at 5,000 rpm, the supernatant was removed. The product was washed with distilled water three times.

2.3. Immobilization of lipase on cellulose nanocrystals

For the preparation of lipase solution, 1 mg of lipase powder was added into 0.125 mL of 0.1 M phosphate buffer (pH 7.0). Activated CNC was mixed with 9.9 mL of 0.1 M phosphate buffer (pH 7.0) and dispersed under digital ultrasonic for 60 min. The suspension was mixed with lipase solution at 320
rpm in the shaker for certain time (0.5 h, 1.5 h, 2.5 h, 3.5 h, and 4.5 h). The mixture was centrifuged at 2,000 rpm for 5 min and then the supernatant was removed to remove unbound enzyme. Additionally, the celluloses were washed with 0.1 M phosphate buffer (pH 7) two times. Prepared immobilized lipase were stored at 4°C until use.

2.4. Lipolysis triglycerides

Immobilized lipase was added with 0.5 mL of Ammonia-NHCl₄ buffer (pH 8.25). The solution was mixed with 0.5 g of palm oil. The reaction mixture was agitated for 6 h at 45°C and 200 rpm in incubator shaker. For analysis, lipolysis product was settled for 5 min and two layers are formed. Sample was pipetted from upper layer for ± 1 g. Sample was solved in 0.5 mL of ethanol and 0.5 mL of chloroform. Sample was titrated with 0.1 N ethanolic KOH. Degree of hydrolysis was determined with Equation (1) [11].

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\text{Degree of hydrolysis} (\%) = \frac{V_{KOH} \times 10^{-3} \times N_{KOH} \times MM}{m \times f} \times 100
\]

\(V_{KOH}\) is volume of ethanolic KOH needed for titration (mL), \(N_{KOH}\) is normality of ethanolic KOH (N), \(MM\) is molar mass of fatty acid in palm oil (g/mol), \(m\) is sample mass (g) and \(f\) is fraction of fatty acid in palm oil.

3. Result and discussion

3.1. The effect of mixing time in lipase immobilization through EDC/NHS crosslink method

The mixing time between the enzymes dissolved in the phosphate buffer and the CNC during the lipase immobilization process can affect the amount of enzymes bound in the CNC [12]. The amount of bound enzymes will affect the enzyme activity. The effect of mixing time between soluble enzymes and CNC during the lipase immobilization on the enzyme activity was investigated. The enzymatic activities of lipase immobilized in CNC that was observed at each different mixing time are shown in Figure 1.

![Figure 1. The effect of immobilization time to the lipase activity.](image)

The mixing time of 0.5 hours between lipase and CNC during the immobilization process indicated the presence of enzymes attached to the CNC or in other words, enzyme immobilization occurred. It was proved that immobilized lipase activity was detected at 0.33 U/mL for the enzyme that had been immobilized for 0.5 hours (see Figure 1). A longer immobilization time can result in more enzyme binding to CNC. Immobilization time of 1.5 hours showed higher enzyme activity (i.e. 0.54 U/mL) than immobilization time of 0.5 hours. There was an increase in enzyme activity by 64% with an increase in
time for one hour. However, an increase in immobilization time longer than 1.5 hours did not indicate an increase in enzyme activity. This is because the longer the immobilization time, the more lipases are bound to CNC, but the lipases that are bound close to each other cause steric hindrance on the active site of the lipase [10]. Steric hindrance on the lipase active site may cause some of the substrate has no direct contact with the lipase active site so that the interfacial activation does not occur [13]. According to the results, it is concluded that the optimum immobilization time is obtained at a duration of 1.5 hours. This is consistent with the results reported by Chen et al., (2016). They reported that a favorable immobilization time for lipases derived from Candida rugosa was between 1 hour and 2 hours [14].

3.2. The effect of lipolysis condition using immobilized lipase

The results of experiments on the effect of lipolysis condition can be seen in Figure 2. Constant variable used in this study were 40°C, pH 8.25 and 6 hours lipolysis time. Generally, the reaction rate will increase with increasing the reaction temperature. However, reactions that utilize enzymes as biocatalysts may have limited activity in high operating temperature [15]. Based on Figure 2 (a), free lipase and immobilized lipase had the optimum temperature at 40°C. Babaei et al. (2014) reported that the optimum temperature for immobilized lipase from Candida sp. in magnetic nanoparticles and free lipase were at 40°C [16]. At a temperature of 50°C, the decrease in activity on free lipase is greater than immobilized lipase, which is 9.53%, while the decrease in activity on immobilized lipase is 1.69%. Meanwhile the reaction temperature at 60°C also showed an indication of decreased in the degree of hydrolysis of the free lipase, which was 8.63% and 3.92% for immobilized lipase. This is because there is a CNC matrix that prevents changes in protein structure or reduces the flexibility of enzyme conformation so that the denaturation process will only occurs when the enzyme receives sufficient energy such as heating treatment [17]. But some of the active sites of the lipase were blocked or covered by the CNC matrix and the substrate did not reach the active sites of enzyme which is covered by CNC matrix. Thus, the activity for immobilized lipase is lower than the free lipase.

![Figure 2](image_url)

**Figure 2.** The effect of (a) reaction temperature, (b) pH, (c) reaction time to degree of hydrolysis of free lipase and immobilized lipase.
The general profile of the pH dependency of free lipase and immobilized lipase was very similar above pH 8.25 based on observations shown in Figure 2 (b). At pH 8.25, the degree of hydrolysis using free enzymes is 1.83 times higher than immobilized enzymes. Free lipase was optimal at pH 7.25 while immobilized lipase was optimal at pH 8.25. This is consistent with the results reported by Atiroglu (2018) that free lipase was optimal at pH 7 while immobilized lipase was optimal at pH 8 [18]. The shifted optimum pH for immobilized lipase was probably caused by covalent bonds between the enzyme and matrix [19]. Covalent bonds cause ionic interactions between the anion compounds from CNC and cation compounds from lipase. Meanwhile carboxyl anion compounds from CNC are more dominant at basic pH [20]. Based on Figure 2 (b), the degree of hydrolysis of lipase immobilized at CNC is more stable than free lipase at acidic and basic pH. So, the advantage of lipase immobilized onto CNC is that it can be used in variety of chemical reactions that require a variety of pH conditions [21].

The effect of reaction (lipolysis) time was analyzed by measuring the degree of hydrolysis in various time and it was illustrated in Figure 2 (c). Based on Figure 2 (c), the degree of hydrolysis produced using free lipase was higher than immobilized lipase. This is probably caused by the differences in the amount of enzymes that react in lipolysis and due to the modification of amino acids from lipase which is not preferred [22]. Based on the results in Figure 2 (c), the longer the reaction time, the greater the degree of hydrolysis produced. The optimum degree of hydrolysis is showed after 6–8 hours lipolysis time. This optimum time range of lipolysis was also reported by Fadiloglu & Soylemez (1998) and Knezevic et al. (2002) [23, 24]. After 8 hours, the degree of hydrolysis of free lipase and immobilized lipase tend to be constant. This may due to chemical equilibrium between the substrate and the product or the accumulation of glycerol on the CNC surface, thereby reducing the active phase of the lipase. In addition, this can also be caused by the low rate of hydrolysis of monoglycerides by the lipase [25].

4. Conclusion

Lipase was successfully immobilized onto CNC so it can be utilized to obtain degree of hydrolysis of 10.1%. Optimum immobilized lipase activity was obtained in 1.5 hours for immobilization at pH 7. The proportion of arginine was lower than lysine in the amino acid of Candida rugosa lipase, which causes the lipase tend to be more active and more stable at low temperatures. There is an optimum pH shift from pH 7.25 for free lipase to pH 8.25 for immobilized lipase. This was because the carboxylic anion from CNC is more dominant at basic pH. The chemical balance between the substrate and the product in the lipolysis reaction was obtained after 6–8 hours.

5. Acknowledgments

Authors wish to acknowledge Program Riset ITB 2019 for financial support and the Asahi Glass Foundation, Japan year 2019–2020.

6. References

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