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Heterogeneous biocatalyst: Polyurethane foam coating technique with co-immobilized lipase for bio-flavor production

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Abstract. Bio-flavor is produced from the esterification reaction including free fatty acids and citronellol. Free fatty acids (lauric C_{12}) can be obtained from the hydrolysis of coconut oil substrate. Both of these reactions use heterogeneous polyurethane foam (PUF) matrix catalysts which immobilize the lipase enzyme. PUF is used as an immobilized matrix, because it has characteristics as open cell, rigid, high porosity, consists of of the ether-, carbamate-, amide-groups, so it has the ability to absorb water-oil. PUF has been modified its surface structure by co-immobilized lipase coating technique to make it more hydrophobic / oleofilic. Co immobilized consists of a mixture of gelatine, lechitin, polyethylene glycol (PEG), $MgCl_2$. The aim of this research is to compare the immobilization technique of lipase *Mucor miehei* using different carriers, that are zeolite, alginate and polyurethane foam (PUF) to produce bio-flavor at mild condition. Each of that was through the mechanism of adsorption, entrapment and covalent binding process. The research was done in specific condition and all of the carriers has gone through the esterification process using different mole ratio of FFA and citronellol. Optimum result of bio-flavor conversion was obtained at the temperature of 40^0C, highest thermal stability of 90% and the ability for reusage 4 times on PUF carrier. This results have proven to be cheaper on cost and environmentally friendly.

1. Introduction

Lipase or triacylglycerol acylglycerolases (EC 3.1.1.3) as a biocatalyst has been widely used in the food, pharmaceutical, textile, flavor, fragrance industries. Lipase enzyme works through the mechanism of hydrolysis, esterification, and transesterification reactions to produce several products, such as bio-flavor product as natural flavor. Enzymes are proteins that are sensitive to pH, temperature, and less soluble in organic solvents, so their use are limited. The effort to enhance stability of the enzyme is already developed through a several techniques, for example, immobilization on a carrier, where the protein enzyme attached in a semi permeable support material [1-4].

The immobilization method is divided into two group of process, first, chemical methods, through covalent bonds of enzymes – carrier and enzyme entrapment, and the second, physical methods, through the weaker bonds between enzymes and carriers. These two procedures are widely used, because of they are easy to work on. The improvement of enzyme properties will produce conformation changes after immobilization process is completed. If the lipase has been immobilized on hydrophobic carrier, the lid

will open, so the active site of the enzyme is become wider. This mechanism is known as interfacial activation and widely used to increase enzyme activity [4].

The adsorption method is simple and inexpensive, but generally not strong enough to attach the proteins and will cause some desorbtion on proteins during washing procedure. There are some carriers which are commonly used as adsorption agents, such as zeolite, silica gel, celite, alumina and ceramics.

Furthermore, in the entrapment method, the enzyme is trapped in a polymer or microcapsule polymer so that the enzyme is still retained while substrate and product flow. Some of common carriers which are used for entrapment method, are made from gels. Since gels can be made from natural and synthetic polymers and have ability to immobilize the enzyme in mild condition, like using silica aerogel and celite supported sol-gel and also several carriers other than gels, like carrageenan and alginate.

The immobilization using covalent bond has developed so that enzymes and carriers are very strongly bonded. However, it also has disadvantage side, because the native enzymes are easily denatured during the binding process. Polyurethane foam (PUF), magnetic particles, chitosan and epoxy-SiO2-PVA, are sort of examples of materials which are being used in the formation of covalent bonds between enzymes and carriers [2,5].

These three methods has commonly used in enzymatic reactions in the synthesis of biodiesel, hydrolysis of fish oil and the synthesis of flavor-esters by using chemicals and organic solvents. This is interesting to make further research regarding on the use of immobilized lipase on several carriers like zeolite, alginate and polyurethane foam (PUF) for producing a bio-flavor without presence of organic solvent, enviromentally friendly and waste-free process from lauric acid (FFA) which is obtained from coconut oil and also citronellol from citronella oil.

This study is conducted to compare the stability of zeolite, alginate, and PUF as a carrier in immobilized lipase to produce bio-flavor from natural substrates.

2. Material and methods

Lauric acid (Free Fatty Acid) is obtained from hydrolysis process of Indonesia's coconut oil. Citronellol from citronellal oil, zeolite and PUF were purchased local market. Alginate solution was purchased from Sigma. *M miehei* from Bioprocess Laboratory State Polytechnic of Malang. The enzyme which is used is a crude lipase from *M miehei* through solid state fermentation method.

2.1. Procedure of immobilization

2.1.1. Physical Adsorption. Zeolite of a certain size were washed with water, at the temperature of 70^0C, for 1 hour. Then soaked with 1 M NaCl for 12 hours. Zeolite later was heated at 300 ^{0}C, for 3 hours in the oven, and cooled at room temperature. Immobilization was carried out by mixing 30 ml of lipase with 10 grams of zeolite which was previously soaked in phosphate buffer. After that, the mixture is filtered and then heated for 48 hours at 37 ^{0}C in the oven.

2.1.2. Entrapment. 3 ml of Lipase is added with 10 ml of sodium alginate solution. The solution later was put through syringe into 0,2 M sodium chloride [6].

2.1.3. Covalent-crosslink. PUF was soaked with co immobilized substances (lecithin, gelatin, PEG, $MgCl_2$) with the ratio of 1: 20 (w/w). After that PUF was dried. And would be soaked with lipase with a ratio of PUF: lipase = 1: 20 (w/w) [7,8].

2.2. Determination of lipase activity

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 minutes. After that, 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 minutes with orbital shaking. After incubation, the reaction was stopped by adding 15 ml of acetone-ethanol (1: 1 v / v) and the free fatty acid was titrated with 0.05 M NaOH. One

unit of lipase activity is defined as a number of enzymes capable of freeing 1μmol of fatty acids per minute [6,9]. Lipase activity can be calculated by formula (1).

$$Lipase\ Activity\ (U/ml) = \frac{(A\text{-}B) \times N_{NaOH} \times 1000}{60} \quad (1)$$

Where, A = The amount of NaOH needed to titrate the sample (ml)
B = The amount of NaOH needed to titrate the blank (ml)
30 = 30 minutes incubation time [10]

2.3. Thermal stability

Thermal stability of free and immobilized lipase was determined at 50°C, at 10-70 minutes. The remaining activities of free and immobilized lipase on zeolite, alginate, PUF are measured by modification of coconut oil hydrolysis [11].

2.4. Esterification

FFA and citronellol with a 1:1; 1:2; 1:3; 1:4; 1:5 mole ratio, were placed in a round bottom flask, and being reacted at the temperature of 40°C, 20 h, pH 7 using immobilized lipase on each zeolite, alginate, PUF and free lipase.

2.5. Gas Chromatography (GC) analysis

Bio-flavor which was produced later was analyzed 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 μm 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 [12,13].

3. Results and discussion

3.1. Thermal stability studies

Thermal stability of the enzymes were tested in the condition above their optimum working temperature, at 50^0C for 10 -70 minutes, with the pH of 7, using olive oil as a substrate [11]. Free lipase as a protein would denature quickly and after 50 minute had remaining activity of 0 % (Figure 1.). When it was compared with immobilized lipase, after 50 minutes of heating, the immobilized lipase still had remaining activity, with zeolite, alginate and PUF as carriers, had remaining activity of 60%, 72% and 90% respectively.

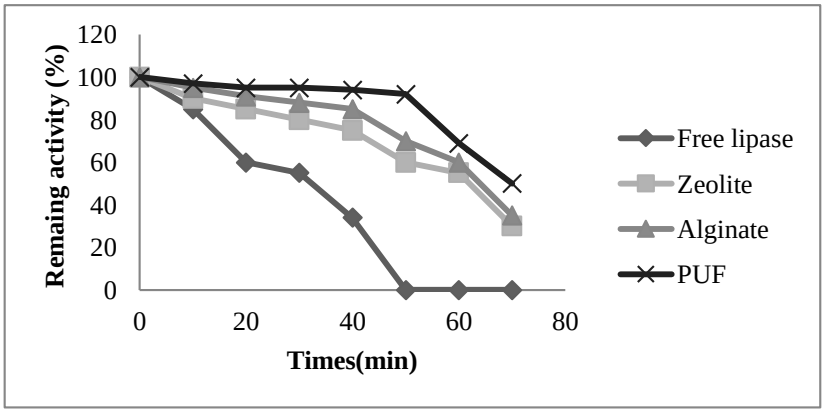

Figure 1. The thermal stability on activity of free lipase and immobilized lipase - alginate, zeolite, PUF.

There were because of the stability effect from immobilized lipase which attached to the composite particle using multipoint attachment, which had an ability to whit stand deformation effect in the higher temperature [14,15]. Micro-environtment between enzyme and carrier is an important reason for the carrier's affinity against water which would have an effect on the catalytic activity of the enzyme.

Adsorption interaction between lipase enzyme and zeolite were weaker rather than encapsulated alginate interaction and covalent bonds of the enzyme and PUF. The addition of polymers as a co-immobilized layer (lechitin, gelatin, polyethylene glycol (PEG) and $MgCl_2$) on PUF, alginate also capable to stabilize and absorb lipase enzyme, thus produced higher stability on immobilized lipase [11].

3.2. Application carrier for bio-flavor production in molar ratio citronellol-FFA

After reviewed the effect of pH, temperature and thermal stability, each carrier were applied for esterification process between citronellol and FFA substrates. High substrate concentration could drive higher reaction rate toward product formation. Further research was needed to determine optimum mole ratio for producing bio-flavor products.

This research used several mole ratio of citronellol to FFA to produce bio-flavor, there were 1:1, 3:1 and 5:1 at a fixed temperature of 40^0C and the pH of 7 for 20 hours. As shown in Figure 2, all of the carriers showed similar profile between each other, with velocity rate increased in line with the increasing mole ratio from 1:1 to 3 :1 but decreased at mole ratio 5:1. This was due of increasing concentration of substrate which could act as an inhibitor in the process of esterification. The initial reaction was occurred by the binding interaction between acid molecules and the enzyme. The acyl transfer, was affected by condition without citronellol as an alcohol. The maximum conversion of citronellol in each carrier were shown at mole ratio of 3:1 with the conversion rates of 42%, 47% and 55% for zeolite, alginate and PUF respectively. It also indicated situation where acyl transfer occurred.

In higher mole ratio of citronellol and FFA (5:1), the higher mole ratio could bind citronellol to the lipase enzyme which would have a competition with acid molecule at initial stage of reaction. This caused a decreasing in reaction rate, because reaction was limited due to the presence of acid molecules around the enzyme [2,5,14,15].

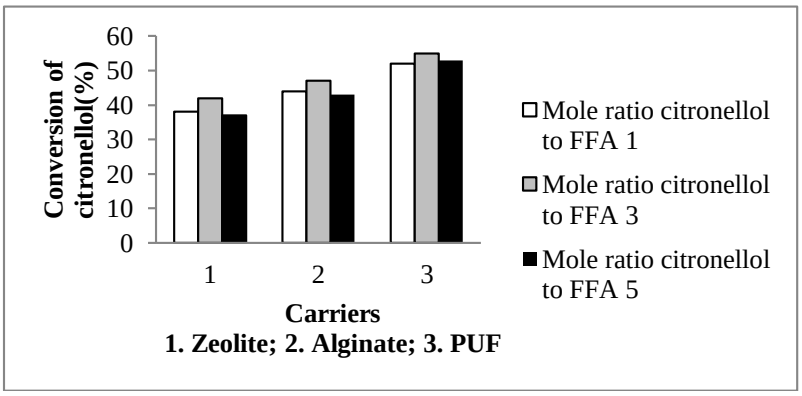

Figure 2. The effect of mole ratio citronellol to FFA for bio-flavor production using immobilized lipase - alginate, zeolite, PUF.

3.3. Reusability studies

The reuse capability was carried out to determine potential stability of immobilized lipase in working condition on several carriers. Esterification reaction was done in cycles (20 hours for each cycle) and later was reused for subsequent reactions without washing procedure. As shown on figure 3, after fourth cycle, the remaining activity of immobilized lipase on majority of the carriers, were still above 50 % as in alginate and PUF are 51% and 71% respectively. However, in zeolite, the remaining activity of immobilized lipase decreased drastically until 36%. The differences of remaining activity of each carrier after certain cycles, showed stability of lipase – carrier bonding, depending on their immobilization technique [14,15].

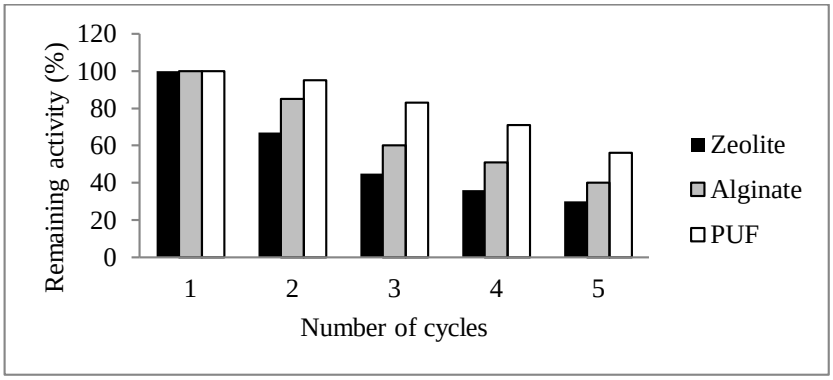

Figure 3. Operational stability for synthesis of bio-flavor using lipase immobilized on zeolite, alginate, PUF at pH 7 and 40^0C.

3.4. The influence of immobilization techniques on carriers for various parameters

The selection of carrierfor immobilized was based on several characteristic, such as easiness for recovery and reuse ability, adaptability on continuous reactions, pH and thermal stability, highly tolerance for reactants and products and also low costing. After all of the above, including pH and thermal stability and also conversion rate, showed that the immobilization technique which was used, had an important influence. That was why in this research, the result of percentage of remaining activity and percent of conversion tend to be similar.

Immobilized lipase using zeolite, alginate and PUF, as a carrier, each of them was using different technique, namely, physical adsorption, encapsulated and covalent bonding, respectively. The result obtained, physical adsorption of zeolite had the weakest bond with the enzyme, because protein was absorbed via non-specific force to the carrier, like, Van Der Waals force, hydrogen bonds and hydrophobic interactions (Figure 4). Because of the majority of protein-carrier interaction was based on electrostatic and hydrophobic interactions that was why it would be easier to stripped off from the carrier.

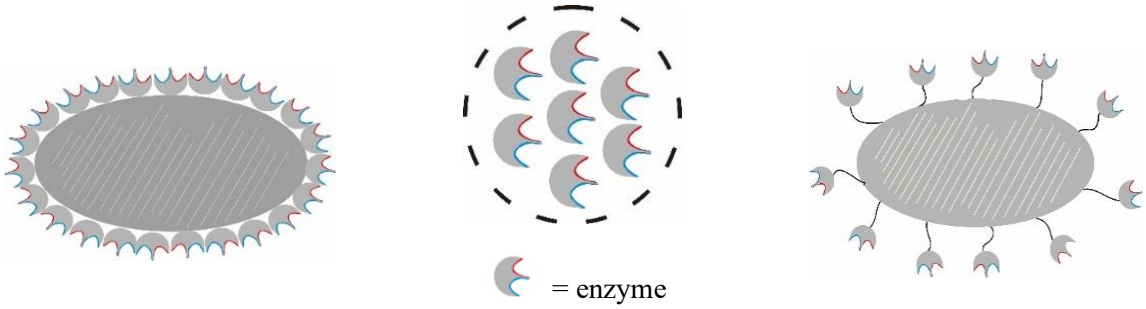

Figure 4. Various techniques of immobilized lipase a) adsorption, b) encapsulated, c) covalent binding.

If it compared with alginate carrier, which was using encapsulated technique, it provided more stable interaction than zeolite, because the enzyme will be entrapped inside the polymeric networks or microcapsules, where the substrate and product could flow but the enzyme was still retained.

The use of PUF as a carrier using co-immobilized layer, proved to have stronger bond with enzyme by forming crosslink and covalent bond. Co-immobilized as spacer was able to strengthen the bonding between amine group of the enzyme and carrier functional group, making it became more stable.

4. Conclusion

The use of zeolite, alginate and PUF as immobilized carrier for lipase enzyme can increase thermal stability of the enzyme because it has wider range of pH and temperature rather than free lipase. The highest stability is obtained by covalent binding and crosslink interaction through coating technique of lipase on PUF carrier compared to zeolite's adsorption and alginate's encapsulated. Immobilized lipase on the carriers as heterogeneus biocatalyst can be reused, thus reducing operational costs.

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