Enhancement in synthesis of citronellyl laurate flavour by combined effect of ultrasound and immobilized lipase as heterogeneous biocatalyst

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Abstract. This study aims to improve the performance of heterogeneous biocatalysts by using the combination of immobilized and ultrasonic lipases in the esterification reaction for the synthesis of citronellyl lauric flavors. Synthesis was conducted out through the reaction of citronellol with lauric acid by using immobilized lipase and ultrasonic. The results showed that immobilized lipase provided stability for up to 9 weeks and could be reused for 5 cycles of reactions. Immobilized lipase 473 U / gram was applied to the synthesis of citronellyl laurate with a reaction time of 90 minutes, 45°C, with and without ultrasonic. The result of citronellol conversion with ultrasonic is 3.6 x higher than conventional method. It shows that the immobilization method provided stability to the biocatalyst, but because it had a limited internal mass transfer so the rate of reaction of ester formation reduced. On the other hand, ultrasonic contributed to increase the dispersion and collision of substrate molecules, reducing reaction time, and intensifying catalytic processes. Thus, the combination of immobilized enzyme and ultrasonic methods can be applied to biochemical processes widely.

1. Introduction
Ester is one of the most important natural flavors and can be applied in various fields, including in the food, beverage, jam, candy, and dairy-based, cosmetics industries [1,2]. Short chain esters with fruity flavors have been widely used in the food industry as a flavor component. The short chain of aliphatic ester is produced from short chains of alcohol and short chains of carboxylic acids. Its constituent carbon atoms is less than 10 and produces a fresh and fruity odor [2,3].

However, the industry still requires a lot of flavor components with high molecular weight, because it has a strong and durable odor [1]. The high % conversion of esterification is correlated with the length of the carbon free fatty acid chain [4]. The results showed that lauric acid C12 had a higher ester conversion when compared to capric acid (C10), caprylic acid (C8), 79%, 43%, 25% respectively.

Natural flavors which are conventionally obtained by direct extraction from plant material are low yield and often too rare and expensive for commercial scale. Meanwhile, industry to obtain artificial flavor components using chemical synthesis methods 303K-533K [5]. The market value that is more desirable is the ester of the natural flavor, so it needs to be developed sustainably [2,6].

In a commercial scale, natural flavors can be produced by enzymatic reactions using lipases from various microbes including Mucor miehei, Candida rugosa, Candida Antarctica B (CALB), Bacillus aerius, Thermomyces lanuginosus, Penicillium crustosum, Aspergillus oryzae. This enzymatic (free)
lipase reaction is an option because it uses mild conditions, specific reactions and works on the interface area between the insoluble substrate and water in hydraulic reactions and esterification of the hydrophobic matrix [2,4,6-10]. However, the use of this free enzyme is unstable over a wide temperature and pH range and is only used one reaction. This causes the enzymatic synthesis of natural flavor with free lipase to be very expensive [11,12].

Lipase immobilization technique in making natural flavor uses matrix hydrophobic, the enzyme is more stable and can be used repeatedly, so it is cheaper than free lipase. Various matrices, including polyurethane foam (PUF), Amberlite MB-1, Celite, Silica gel, Exfoliated Graphene Oxide (EGO), Silica-epoxy, have been used for flavor making at reaction times of 10 - 72 hours, resulting in a conversion of 30% -90 %.

On an industrial scale, the length of reaction time in the enzyme immobilization method becomes an obstacle for large production [2,4,6-9,13]. So comprehensive efforts need to be made to overcome the problem of long reaction time. One solution that can be used is by utilizing ultrasonic technology. Ultrasonic apparatus is a device that can convert electrical energy into mechanics in the form of ultrasonic sound waves above 20kHz. The basic mechanism that occurs in the use of ultrasonic is acoustic cavitation, which after formation occurs, growth and microbubbles collapse in the liquid medium. This occurs due to a microscopic implosion which causes very high local turbulence and releases heat energy. This condition causes a significant increase in temperature and pressure, such as the phenomenon that occurs hydrodynamic cavitation which causes mechanical damage to propellers and pumps [14]. In acoustic cavitation, sound waves form a sinusoidal at various pressures during cavitation in solution (Figure 1).

![Oscillating sound wave]

**Figure 1.** Phenomena of acoustic cavitation cycles in ultrasound.

Phenomena of acoustic cavitation cycles in ultrasound, which is the formation, growth and collapse of microbubbles. The bubbles oscillate through sound waves in the presence of compression followed by rarefaction and eventually will burst and produce energy that can produce vibrations and can emulsify a material [14].

Ultrasonic has been widely used in various industries, as shown in Figure 2.
Figure 2. The use of ultrasonic in the industry [15].

In the field of sonochemistry, ultrasonic is used in various industries (Table 1).

| Industry        | Product          | Application                                      | Reference |
|-----------------|------------------|--------------------------------------------------|-----------|
| Flavor          | Ethyl butirate   | Mixing of butyric acid and ethanol               | [4]       |
| Water treatment | Clean water      | Mixing, inactivate *Escherichia coli*             | [16]      |
| Chemical        | Wetting agent    | Mixing dichloromethane with ethanolamine         | [17]      |
| Cosmetic ester  | Skin care        | Mixing butyl alcohol and acetic acid             | [18]      |
| Medicine        | Bioactive anticancer compounds of | Extraction of *Cuminum cyminum* | [19]      |
| Fuel            | Biodiesel        | Mixing soybean fatty acid and ethanol            | [20]      |
| Oil and gas     | Oil recovery     | Extraction oil dan gas                           | [21]      |

Natural flavour- citronellyl laurate is a high –value processed product from long chain carbocyclic and alcohol because it has a strong and long lasting odor [1]. The objective of this study was to know the effect of ultrasound on the reaction between lauric acid and terpene alcohol citronellol, not using solvent, catalyzed by immobilized lipase *Mucor miehei* on polyurethane foam.

The synergy of ultrasound and immobilized enzyme is an attractive solution to reduce reaction time and increase the performance of lipases so that they remain stable and can be used repeatedly. This condition supports the achievement of green technology process, especially the existence of free solvent and washing is not carried out in repeated reactions [10].

2. Material and methods

Lauric acid, citronellol, lipase Mucor miehei are obtained from Sigma. Co-immobilized consisting of gelatin, lecithin, polyethylene glycol (PEG), MgCl2 was purchased from Merck (Darmstadt, Germany). PUF is obtained from local market, the isocyanate and polyol reaction with a volume ratio of 1: 1. PUF cut into sized cubes 0.5 x 0.5 x 0.5 cm3 [22].
2.1. Procedure of immobilization lipase on PUF
We made a co-immobilized solution from a mixture of lecithin, gelatin, PEG, MgCl₂ solutions. PUF was immersed in a co-immobilized solution in a ratio of 1:20, for 1 hour and then dried to 30°C. The result is soaked in lipase for 24 hours then dried to 30°C. drying is carried out in the oven [22-24].

2.2. Determination of lipase activity
Olive oil as a substrate was used to determine lipase activity. An amount of 25 ml of olive oil and 75 ml of a 7% solution of gum arabic was emulsified for 2 minutes. Next, 5 ml of emulsified olive oil was added to 2 ml of 0.1 M phosphate buffer (pH 7) and 1 ml of lipase. Then, it was incubated in lipase at 37 °C for 30 minutes using an orbital shaker. Furthermore, to deactive the lipase activity, 15 ml of acetone-ethanol (1: 1 v / v) was added and free fatty acids were titrated with 0.05 M NaOH. One unit of lipase activity is defined as the number of enzymes capable of releasing 1μmol of fatty acids per minute [22,25,26].

Lipase activity can be calculated by formula.

\[ \text{Lipase Activity (U/ml)} = \frac{(A-B) \times N_{NaOH} \times 1000}{30} \]

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 [27]

2.3. Determine of storage stability storage
The immobilized lipase in PUF was stored at 4°C and its remain activity was examined every day.

2.4. Esterification
Lauric acid and citronellol 1:1 mole ratio, were placed in erlenmeyer 100 ml, and being reacted at the temperature of 40°C, 20 h, pH 7 using immobilized lipase on Digital ultrasonic cleaner. The specifications of this equipment are tank size 300 x 240 x 150 mm (L x W x H), transducer 4 pcs, power 240 W, 40 KHZ.

![Figure 3. Digital ultrasonic cleaner.](image)

GC FID (HP 5890, Santa Clara-California, United States) was used to analyze the natural-flavor resulting from the experiment. GC FID uses an HPL 608 column with dimensions of length 30 m x 0.53 mm i.d. x film thickness 0.5 µm. The GC-FID oven temperature was set to 125 °C for 3 minutes in increments of 7.5 °C / min to 250 °C. The injector and detector temperatures were kept at 255 °C and 275 °C, respectively. Then the area under the curve is calculated to determine the conversion of citronellol [28-31].
3. Results and discussion

3.1. Storage stability free and immobilized lipase

Storage stability was used to test the performance of free and immobilized lipase against long storage time at 4°C. Figure 4 showed, until the storage time of 9 weeks, immobilized lipase on PUF still had high stability of 91.69%, while free lipase dropped to 9.63%. This significant difference was due to i) the surface of the PUF matrix became hydrophobic after being coated with co-immobilized MgCl2, PEG, lecithin, gelatin, ii) the strong covalent bond between PUF and lipase became stronger because it was co-immobilized as a spacer arm that extended the bond. The starting from weeks 1-3, free and immobilized lipase were still the same as the initial conditions, with 100% remaining activity. However, after week 4, there was a significant decrease in activity at 45.98% free lipase, while immobilized lipase was still stable as indicated by the remaining activity of 94.81%.

![Figure 4. Performance storage stability free and immobilized lipase, 4°C.](image)

The same characteristics were shown in PUF coating using co-immobilized polyethilenimin (PEI) and glutaraldehyde (GA), which had higher stability when compared to free lipase, each remaining activity was 50% and 0%, after incubating 30 minutes, 50°C [28]. The use of co-immobilized in matrix coating can significantly increased enzyme stability, so this method was often used in enzymatic reactions [7,28,31].

3.2. Effect of ultrasound irradiation

Ultrasound has been used in the esterification reaction of fatty acids and alcohol. The results of data analysis showed that ultrasonic could increase enzyme activity and increase substrate transfer on the active side of the enzyme [33,34]. The same phenomenon was shown in the enzymatic reaction of esterification of lauric acid and citronellol to natural-flavor citronellyl laurate below (Figure 5). Ultrasonic with a power of 240 W, 40 KHZ, was used to produce citronellyl laurate at a reaction time of up to 90 minutes. The citronellol conversion was calculated based on [(initial citronellol – citronellol at t) / (initial citronellol)] x 100% by using GC FID [31].
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Figure 5. Effect of ultrasound irradiation on conversion of citronellol by using immobilized lipase for esterification reaction in 45°C.

The esterification reaction has been carried out using immobilized lipase on PUF, resulting in 55% conversion of citronellol, with a reaction time of 20 hours [31]. So by looking at the trend in Figure 5, it can be seen that there were significant differences for each method of immobilized lipase (without ultrasonic) and immobilized lipase with ultrasonic respectively. Without using ultrasonic, the citronellol conversion changes were not very significant, from 30-90 minutes. This was different from the conversion rate of citronellol using ultrasonic, there was a very significant change, so that the remaining citronellol after the esterification reaction was less or the number reacts more, which was influenced by the reaction rate. After esterification reaction time of 90 minutes, the conversion of citronellol with and without ultrasonic was 75% and 20.8%, respectively or 3.6 x. Ultrasonic plays an important role through mixing technique in fast esterification, so it could reduce time. The use of ultrasonic was also applied to carry out the enzymatic synthesis of butyl acetate, which was an increase of 7.5 x times compared to conventional stirring methods. The reaction rate in ultrasonic increased 2.85 times higher than conventional [18]. The use of high frequencies in ultrasonic caused small bubbles to occur, causing the ratio of surface area to volume to increase, so as to increase mass and heat transfer through the interface of the bubble. This would increase the emulsification reaction and reaction rate of soybean, canola and corn oil to produce biodiesel. The reaction results increased with increasing output power from 150W to 450 W, producing 95% fatty acid methyl ester, reaction time of 30 minutes [20].

In this study, a heterogeneous biocatalyst immobilized lipase on PUF was used, with lauric acid and citronellol as a substrate. There was an increase in substrate reactions and mass transfer due to ultrasound irradiation, causing bubble bursts and cavitation which would increase enzymatic reactions, through ultrasonic liquid media. In this reaction, the bubble collapsed near the surface of heterogeneous solids which could cause high intensity shock waves which would increase the substrate enzyme reaction [5].

3.3. Reusability

One of the advantages of immobilized enzyme is the reuse of heterogeneous biocatalysts, thereby reducing operational costs on an industrial scale [5]. In this study, to obtain heterogeneous biocatalysts again, washing was not performed because the co-immobilized ones (lecithin, gelatine, MgCl₂, PEG) used were edible. If it is not used immediately for reuse, it can be stored at a temperature of 4°C.
Figure 6. Reusability of the enzyme on % remaining activity of citronellyl laurate as natural flavor.

From Figure 6, using ultrasonic it can be shown that in 1st and 2nd cycle, there has been no change in immobilized lipase activity. Furthermore, in 3rd to 5th cycle, there was a significant decrease. In the 5th cycle, the remaining activity using ultrasonic and without ultrasonic was 80% and 51.18%, respectively. A decrease in remaining activity means that the enzyme activity decreases to carry out the esterification reaction. This is possible due to the release of enzymes from the matrix and the occurrence of deactivation during the process [5].

4. Conclusion

Process productivity can be increased through a combination of ultrasonic and immobilized lipase, into a synergy. Ultrasonic provides a reliable mixing technique on the substrate and reduces the formation of substrate / product layers in the porous PUF matrix. Meanwhile, immobilized lipase in the porous matrix PUF provides stability, so that the substrate enzymes can react properly. The use of ultrasonic can be used in all fields widely.

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