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Immobilized Lipase Technology for Making Natural Flavor

Dwina Moentamaria*, Zakijah Irfin, Sigit Udjiana
Chemical Engineering Department – State Polytechnic of Malang (POLINEMA), Malang – 65141, Indonesia
** Corresponding author. Tel.: +62-341-404424; fax: +62-341-404420.* E-mail address*:*

Abstract:- Solid waste of coconut oil industry, approximate 40% of the raw materials was solid waste, it has been utilized optimally up to now. To increase its added value and, by transforming solid waste to high value lipase commodity as an natural-flavor based which is safer for food, through immobilized lipase techniques. Currently, chemical based synthetic flavor are still the main choice in food industry and there is no successful research and efforts to replace it. Lipase is a wide range biocatalyst used in various bioconversion reactions, such as hydrolysis, inter-esterification, esterification, trans-esterification, alcoholysis, acidolysis and aminolisis. Previous research has been established by Chumaidi, Moentamaria (2009), lipase crude extract can be produced by Bacillus substilis and Mucor miehei in a liquid medium Potato Dextro. In this research, the flavor was made from natural materials, citronellol, isolated from lemongrass oil and free fatty acids from hydrolyzed triglycerides of coconut oil. Purifying of crude lipase of Mucor miehei by ammonium sulfate fractionation method at 30%, 45%, 60%, 75%, and at the various percentage of waste of coconut cake. Furthermore, do immobilization of lipase at Polyurethane foam (PUF) 0.5 cm3 and 0.25 cm3 used in the esterification reaction of citronellol and free fatty acid at 400C at various reaction times of 30, 60 ,90, 120, 150 minutes . It uses also the variable weight of triglycerides to citronellol ratio are 1 : 1 and 1 : 3. Purification of lipase reaches 6.9 times increase from crude lipase by using ammonium sulfate 75%. The indication of esterification results is observed by presence of mix product of orange and strawberry flavor, with PUF 0.5 cm3 , reaction time of 150 minutes and ratio 1 : 3.

Keywords:- immobilized lipase , natural flavor, PUF, esterification

I. INTRODUCTION

Today the use of food/beverage additives, especially synthetic flavor increasingly dominate in Indonesia. Almost all of food in the market using artificial flavor ingredients. Usage of such flavor should be controlled strictly during its consumptions. To solve this problem, the natural flavor shall be made. The flavor is considered natural if the materials used are natural and use of enzymes as biocatalysts. The enzymes expected to catalyze the esterification reaction is lipase.

Production of this natural flavor use lipase enzymes is considered as an alternative to reduce the negative impact usage of chemicals in synthetic flavor. The advantages of lipase as biocatalyst are: (1) it is specific, so side products generation can be avoided, (2) low temperature and pressure required during it processes, therefore lower cost impact, (3) more environmental friendly, (4) glycerol separation process can done without purification and (5) bio-catalyst is very effective to improve the speed of specific chemical reactions real time, where the reaction without enzyme will be slower . In this research, flavor will be made of coconut-oil based natural flavor and citronellol /geraniol are derived from lemongrass oil with lipase biocatalyst.

Lipase (tri acylglycerol hydrolase , EC 3.1.1.3) is an enzyme that is applied to the process of hydrolysis of triacylglycerol into fatty acids, di acyl-glycerol, mono acyl-glycerol and glycerol, in certain conditions it can catalyze the reverse reaction (Sharma et al , 2013; Badgujar et al, 2014). Lipase be able to catalyze the ester synthetic and trans-esterification in organic media containing a low concentration of water (Pera et al , 2006). This various capabilities, lipase can be applied to a variety of industries and chemicals such as drugs, food additives, biopolymer synthesis , cleaning and clinical reagents . In addition lipase can also be applied to wastewater treatment and cosmetics (Sharma , 2001), also in the fuel processes use lipase as biocatalyst in the esters synthesis and trans-esterification of oils for biodiesel (Pera et al , 2006). Lipase used in this process is the product of *Mucor miehei*.

Produced crude lipase purified by salting in and salting out method and known as a salt fractionation. In this study we use ammonium sulfate salt. Fractionation is done base on differences of protein solubility in the solution. Effect of neutral ammonium sulfate salts on the solubility of proteins is a function of its ionic strength, a concentration measure and total electrical charge cation and anion of salt. Salting effect it is caused by dissociation tendency of side-chains groups of proteins to be ionizing. But when the capability of the ionic strength is increased, the protein solubility will decline and on sufficient high ionic strength, protein will precipitate or salting out .Enzymes immobilization is a process in which the movement of enzyme molecules keep in specific catalyst place in a chemical reaction chamber.

This process will be done by tying the enzyme molecules on a certain support material PUF (matrix) by specific chemical bond or physically hold in a cavity of the support material. In this system, the enzyme retained to changing conditions such as pH or temperature. This system also helps enzymes keep in place during reaction running, and facilitate the separation process and it be able to used more in another reaction (Awang , 2007). PUF is a mixture of two chemicals (isocyanate and polyol) were stirred so that the reaction runs and foam formed. Based on the chemistry PUF is any polymer consist of organic chains tied in urethane carbamate. PUF is excellence, it has lighter specific gravity, approx 36 kg / m3 and has a heat transfer coefficient of polyurethane is only about 0,017 generate high conversion , withstand at acidic conditions. (Awang , 2007).

Natural flavor produced by reacting coconut oil with citronellol / geraniol derived from isolated lemongrass oil. This process is called esterification reaction with immobilized lipase. Esterification is the reaction between carboxylic acid and alcohol to form esters. Carboxylic acid derivatives produces a carboxylic acid ester .

II. MATERIAL AND METHODS

2.1**.** Material

Coconut oil was obtained from was obtained local market of Malang. *Mucor miehei* was obtained in Bioprocess Laboratory of State Polythecnic of Malang and citronellol from isolation of lemongrass oil with vacuum distilation.

2.2. Lipase Activity determination

Trigliseride kapok oil was used as substrate for lipase activity determination. The method used is titrimetric by preparing 2 ml kapok oil, 1 ml of buffer phosphate pH 7 and add 1 ml of the enzyme. The mixture was incubated for 30 minutes in a shaker incubator. Add pp as indicator. Then titrate with 0.1 M NaOH and the product was analyzed for the quantity of FFA formed. Lipase activity was expressed as the amount of lipase required to release one µmole of fatty acids per min in 40^0C (Khaskheli, 2015).

2.3. Growth cell

Whatman paper is weighed then dried into constant weight. Residual substance called mycelium was dried on a paper at 105 °C ,for 12 hours. Weigh up to a constant weight. Cell was incubated for 8 days .

2.4. Enzyme production

Medium culture containing 1 litre, 5 % peptone, 1 % KH_2PO_4, 0.001 % $FeSO_4.7H_2O$, 10 % olive oil , 10 % palm oil , 20% waste coconut cake and distilled water. The media is added 1 ml of inoculum and stored in the shaker incubator for 5 days , 40°C at 120 rpm . Then separate the culture and its mycelium used in this growth study, cell-free filtrate is a source of extra cellular lipase (crude lipase).

2.5.Enzyme purification

Fractionation is done by adding ammonium sulfate to the crude lipase 0-30 % , 30-45 % , 45-60 % and 60-75 % . Let precipitate for 1 hour and sediment of enzyme was centrifuged for 30 minutes at 3000 rpm. At every stage do the activity test to determine peak activity in every related stage .

2.6. PUF production

Mixed isocyanat and polyol with the desired ratio. Stirred it to get complete reaction and formed CO_2 and produce a rigid hard segment. Repeat stirring until homogenized color to PUF production. PUF is immersed in co-immobilizer with composition: lecithin, n - hexane, gelatin, MgCl2 and PEG. Soak PUF for 24 hours and dried at room temperature for 24 hours. Then add crude lipase for 24 hours. Dried PUF at room temperature for 24 hours .

2.7.Ester flavor production

Isolate citronellol in the essential oil using vacuum distillation fractionation . Amount of lipase used is 5% of the substrate . The esterification process runs in desired time variation with temperature of 40^oC. Take the product and let it a while to form two layers. The upper layer is the desired product ester. Then this esterification product was centrifuged for 15 minutes at 3400 rpm . Analyze the composition using GC-MS and acid number with Food Chemical Codex (FCC) standards.

III. RESULTS AND DISCUSSION

Result of 4 days *Mucor miehei* incubation, as in the Figure 1.

Figure 1. *Mucor miehei*, grey color mycellium in 4 days incubation

Growth cell

Dry cell weight for 8 days was obtained in Figure 2.

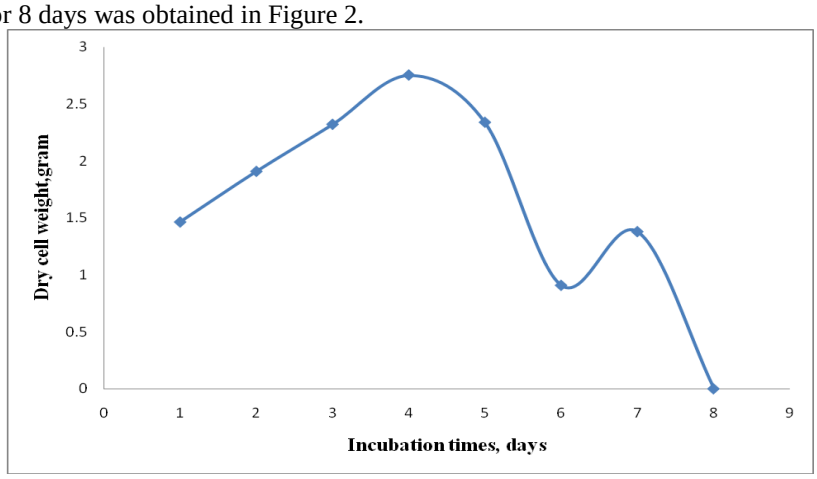

Figure 2. Growth cell of *Mucor miehei*

From the above growth curve it shows that four days incubation period is the highest number of mycelium. On the seventh day there is an increase mycelium mass, it caused by some dead cells become a source of food for other microbes.

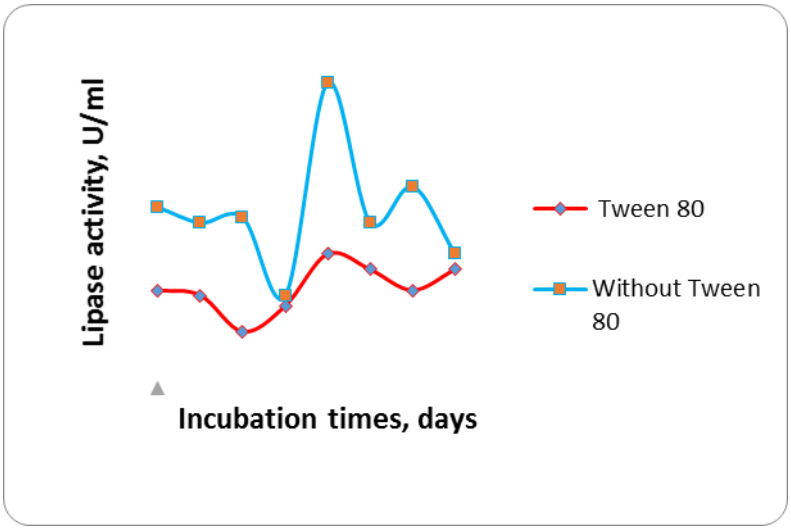

Figure 3. Lipase Activity Growth Study Results.

The highest activity generated on the five days incubation, with Tween 80. Tween 80 is a nonionic hydrophilic surfactant. Tween 80 has the ability to reduce the surface tension of the liquid. The surface tension of the liquid was formed by the attractive forces between molecules in a liquid with molecules of air above it. Tween 80 could break the force that holds the liquid molecules at the interface so that the surface tension will be reduced. This ability is due to the high value hydrophilic -lyophilic balance (HBL) of Tween 80. Tween 80 has a HBL value of about 15, which led Tween 80 is more soluble in water . 0.05% of tween 80 was used in this study was able to attract the extracellular lipase enzymes from the surface and disperse into solution, so that the highest of lipase activity can be obtained (Moentamaria, 2013). This result agrees with the studies of Nahas, 1987 and Dominiquez, et. al (2003), who used Tween 20, Tween 80 and Tween 100, and found the improvements on lipase production, although the results depend on the type of Tween used.

Production and Purification of lipase.

Lipase is produced twice . First production without Tween 80 and the second one use Tween 80. The first production was used as a sample to measure the stage of the purification process using ammonium sulfate fractionation. Here is lipase activity data produced using Ttween 80 before (crude lipase) and after purification by ammonium sulfate fractionation.

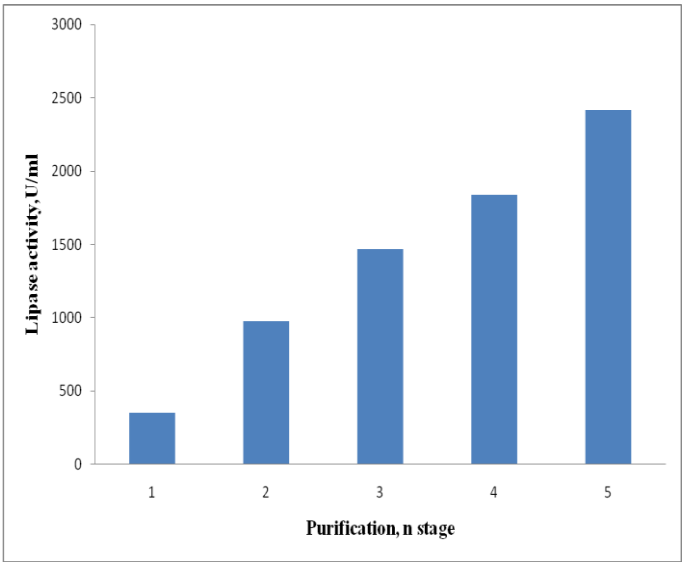

Figure 4. Purification crude lipase with $(NH4)_2SO_4$

In the above graph shows that the purified lipase increasingly to purification fractions 4 $(NH4)_2SO_4$ 75% (stage 5). The increase of lipase activity in fraction 4 is 6.9 times compared with the crude lipase.

Coconut Oil Hydrolysis by Lipase. Hydrolysis is used to breaksubstrat of esterification. Here are the results of the conversion of hydrolysis using immobilized lipase in Table 1

Table 1. Persentase hydrolysis conversion coconut oil using immobilized lipase

Mass of Coconut oil (g)	Mass of water (g)	Mass of fatty acid after washing (g)	Conversion (%)
110,5	330,67	100,28	90,75
110,46	330,16	100,78	91,24
110,74	330,69	100,97	91,18
110,32	310,07	100,01	90,65

Immobilized Lipase Application in Esterification.

In this application, the esterification reaction is done by reacting fatty acids of coconut oil with citronellol of lemongrass oil. The amount of ester production can be observed by % yield. Below is the results of % yield, Figure 5 and 6.

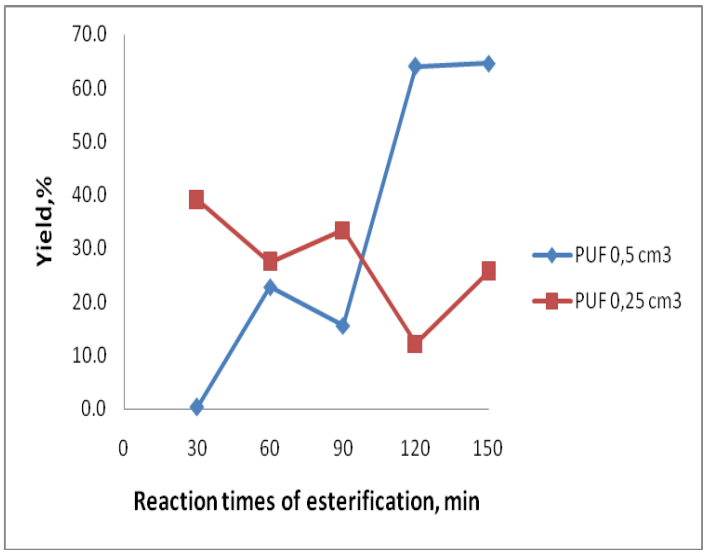

Figure 5. %Yield of ester production with feed ratio 1:1

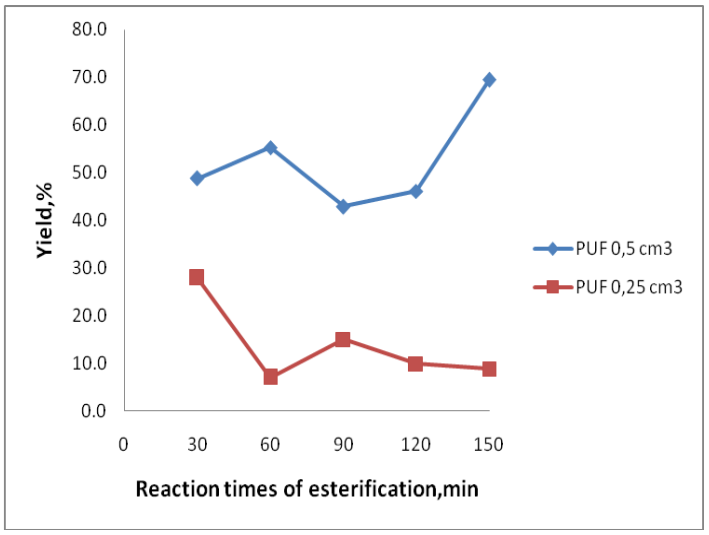

Figure 6. %Yield ester production with feed ratio 1:3

Based on the both above graphs, its show that the % yield with the feed ratio of 1: 1 and 1 : 3 either PUF size 0.5 cm3 or 0.25 cm3 are incline and decline experienced. It is caused by the nature of the esterification reaction, that are reversible and very rapid reaction so thus causing the amount of ester decreases due to the length of reaction time (Leung , 2010) .

Repeatedly use of immobilized Lipase.

To determine the frequency of lipase which can be used for esterification; the immobilized lipase used for five times. Here are the results of immobilized lipase used five times

IV. CONCLUSION

The best conditions of lipase produced by *Mucor miehei* with the highest activity is on five days incubation period. Lipase activity purified by fractionation of ammonium sulfate is 6.9 times compared to the crude lipase. At substrat ratio 1 : 3, PUF size 0. 5 cm3 give greater % yield than PUF 0,25 cm3 at 150 minutes for reaction of esterification .

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