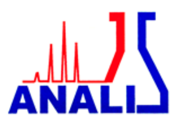
Malaysian Journal of Analytical Sciences, Vol 20 No 6 (2016): 1365 - 1372
DOI: http://dx.doi.org/10.17576/mjas-2016-2006-15



MALAYSIAN JOURNAL OF ANALYTICAL SCIENCES

Published by The Malaysian Analytical Sciences Society

ISSN
1394 - 2506

ENZYMATIC GLYCEROLYSIS OF METHYL LAURATE UTILIZING *Candida antarctica* Lipase b

(Gliserolisis Berenzim oleh Metil Laurat Menggunakan Lipase b *Candida antarctica*)

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Received: 1 February 2015; Accepted: 29 August 2016

Abstract

Enzymatic glycerolysis using *Candida antarctica* lipase b (CALB) as catalyst was studied. Production of monolaurin was optimized based on two selected types of CALB, varying enzyme concentrations and reaction time. Novozyme-435 (N-435) and *Aspergillus oryzae* were used in this study. About 47.6% of monolaurin was synthesized using N-435 at a 5% enzyme concentration within 24 hours of reaction time. Characterizations were performed using different techniques namely gas chromatography-flame ionization detector (GC-FID), thin layer chromatography (TLC) and Fourier transform infrared spectroscopy (FTIR) analysis.

Keywords: methyl laurate, enzymatic glycerolysis, monolaurin, Novozyme-435, *Aspergillus oryzae*

Abstrak

Tindak balas gliserolisis berenzim menggunakan lipase b *Candida antarctica* (CALB) telah dikaji. Penghasilan monolaurin dioptimumkan berasaskan dua jenis CALB, kepekatan enzim dan masa tindak balas. Novozym-435 (N-435) dan *Aspergillus oryzae* digunakan dalam kajian ini. Sebanyak 47.6% monolaurin telah berjaya disintesis menggunakan N-435 pada kepekatan enzim 5% dalam tempoh 24 jam masa tindak balas. Pencirian dijalankan menggunakan teknik analisis berbeza seperti kromatografi gas (GC), kromatografi lapisan nipis (TLC) dan spektroskopi inframerah transfomasi Fourier (FTIR).

Kata kunci: metil laurat, gliserolisis berenzim, monolaurin, Novozym-435, *Aspergillus oryzae*

Introduction

Enzymatic glycerolysis reactions were reported to be carried out in a mild reaction condition, low temperature with solvents that are not harmful towards the environment. With the proliferated interest in green and moderate chemical reaction, enzymatic reaction is attractively favored in researches that produce edible products such as monoacylglycerol (MAG) and diacylglycerol (DAG) [1].

MAG which is the glycerol monoester of fatty acids exhibits surfactant and emulsifying properties that advocates mixing of hydrophilic and lipophilic substances. MAG is extensively used in food, detergent, cosmetics, pharmaceutical formulations and plasticizer. Monolaurin (ML) is a medium-chain monoacylglycerol (MAG) with 12 carbons. The industry highly anticipates MAG of various fatty acids due to its unique feature that prevents microbial activity among diverse microorganisms including gram-positive, gram-negative bacteria, spore-forming bacteria, yeasts, filamentous fungi and developed viruses. In particular, ML compounds are able to dissolve and

penetrate into the phospholipid layer of bacterial cell and disintegrate the membrane layer. Thus, ML compounds inhibit bacterial cell reproduction by hindering cell replications [2]. A natural occurring ML exists as an active ingredient in breast milk, which acts as a booster to the infant's immune system [3].

So far, search on literature revealed studies which were focused on the synthesis reaction. MAG could be synthesized by promoting methyl oleate upon a strongly basic MgO via glycerolysis. The catalyst activity decreased with calcination temperature following a trend similar to the site density of the strong base [4]. Furthermore, 2-MAG can be produced from *Echium plantagineum* seed oil and marinol with three purification methods, silica gel column chromatography, liquids-liquids extraction and low temperature crystallization [5]. In addition, it can also be produced from canarium oil by enzymatic reaction using immobilized lipase from *Mucor miehei*, of which has a specific activity towards triacylglycerol (TAG)[6]. Besides that, 2-MAG was produced by enzymatic glycerolysis of omega-3-polyunsaturated fatty acid using short path distillation (SPD) of an acylglycerols mixture [7]. Additionally, the production of 2-MAG done via glycerol esterification of functionalized SBA-15 mesoporous catalyst has been carried out [8].

Lipases are stable enzyme and the general classes of enzymes that break down fat molecules. Lipases are often obtained from animals, plants and recombinant microorganisms. The physiological role of lipases (water-soluble triacylglycerol acylhydrolases) is the catalytic conversion of tri-glycerides into di- or mono-glycerides, fatty acids and glycerol [9]. Extracellular lipases from *Candida sp., Pseudomonas sp., Thermomyces lanuginosus and Rhizomucor miehei* have been investigated by a considerable number of studies to be employed in the biodiesel synthesis [10]. Lipases are hydrolases that catalyze the hydrolysis of fatty acid esters found in living organisms [11].

Candida Antarctica lipase b (CALB) is one of the most effective lipases in the synthesis of ester [12]. The structure as shown in Figure 1. It is an enzyme extracted from yeast. Compared to bacteria, yeasts are easier to handle and grow. This enzyme is commonly used in organic synthesis reactions and as a biocatalyst that is associated to its stereoselectivity and thermal stability. CALB does not show interfacial activation compared to other lipases. This enzyme is optimized at pH 7. CALB generally convert straight-chain acid, which prefers 5 to 12 carbon atoms. This is because bulky acyl groups restrict the size of the binding pocket. CALB is an effective biocatalyst for promoting high FAME production yield [13].

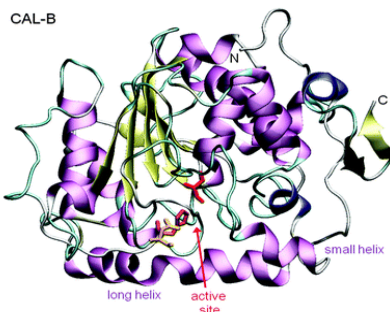

Figure 1. Crystal structure of the lipase CAL-B [17]

Novozyme-435 (N-435) is a heterogeneous biocatalyst system that consists of lipase b from CALB immobilized on a hydrophobic carrier. During esterification and transesterification of sugars, nucleosides and steroids, N-435 has an extraordinary ability to provide high regioselectivity [14]. Previously, a research found that the enzyme showed low activity in the presence of water and proposed a reaction to be done in an anhydrous medium for efficient catalyst activity. N-435 can be used ten times without loss of significant activity [11].

Aspergillus oryzae is a filamentous fungus that has ability to secrete large amounts of hydrolytic enzymes [15]. It is used as a host because it has high protein productivity in the expression of heterogeneous gene using improved

promoters [16]. In this research, we aimed to produce ML with the highest composition through enzymatic glycerolysis of methyl laurate with two selected *Candida antarctica* lipase b *(CALB),* Novozyme-435 (N-435) and *Aspergillus oryzae* with differences concentration of enzyme and reaction time.

Materials and Methods

Raw materials

Glycerol 99%, methyl laurate $C_{13}H_6O_2$, Novozyme-435, CALB *Aspergillus oryzae*, sodium chloride, sodium hydroxide, sodium bicarbonate, chloroform, diethyl ether and ethyl-ethanol were purchased from Sigma Aldrich.

Enzymatic glycerolysis of methyl laurate

Reaction ratio between glycerol and methyl laurate was ratio 1:1 (mol/mol). About 5 g of glycerol reacted with 11.64 g of methyl laurate. Concentrations of enzyme were tested at 3%, 5% and 10% based on the weight of glycerol. Mixtures were heated at 50 °C for 6, 12, 18, 24 and 30 hours in oven shaker with a speed of 250 rpm. Once the reaction ended, the sample was post-treated with 60 mL of sodium bicarbonate solution, 60 mL of sodium chloride solution, 60 mL of sodium hydroxide solution, 60 mL of distilled water, 80 mL of chloroform and 40 mL of ethanol. Unreacted glycerol and water as by-products were dissolved in ethanol, while the desired product was dissolved in chloroform. Products were separated using separating funnel and sample was further purified to isolate monolaurin from by-products (dilaurin and trilaurin) using solid phase extraction (SPE) method. Schematic reaction for enzymatic glycerolysis of methyl laurate was illustrated in Figure 2.

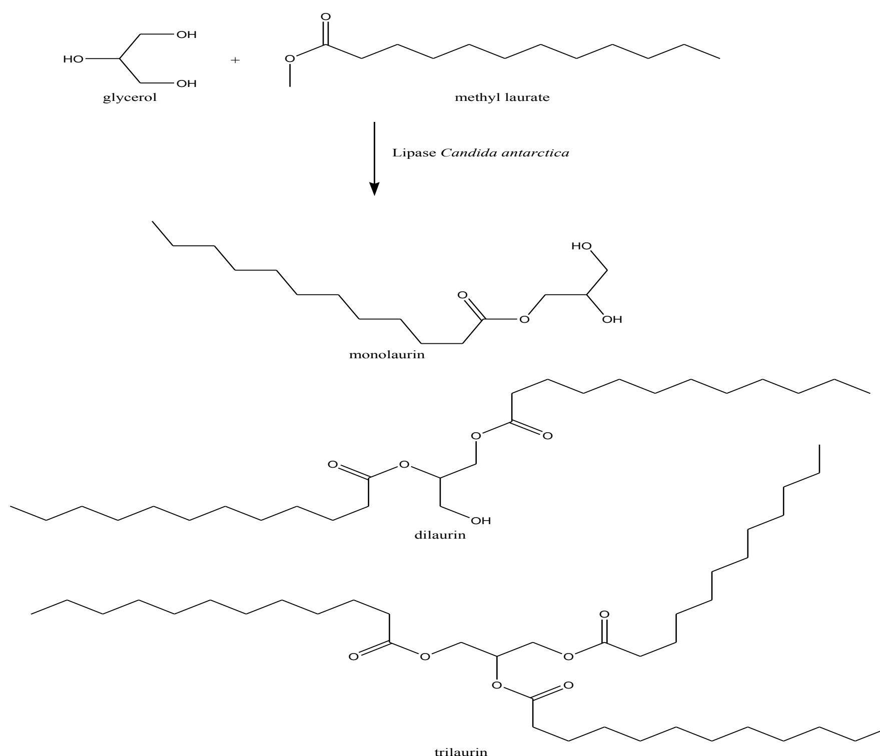

Figure 2. Enzymatic glycerolysis of methyl laurate

Characterization

A series characterization was performed using three different techniques namely thin layer chromatography (TLC), gas chromatography-flame ionization detector (GC-FID) and Fourier transform infrared spectroscopy analysis. A total of 100 mL of TLC mobile phase used toluene: chloroform: acetone with the ratio 70:20:10 (v/v) [18]. GC-FID

analysis is carried out using a capillary column DB-5HT column (30 m × 0.25 mm × 0:10 m). The operator setting GC-FID as follow; the temperature of the injector 250 °C, a temperature of detector 400 °C, initial temperature of 100 °C (1 minute), the final temperature of 380 °C (25 minutes) with a rate of increase of 5 °C/minutes and the carrier gas is nitrogen gas. The sample was prepared by adding 0.03 mL of reaction sample with 1.0 mL ethyl acetate.

Results and Discussion

Figure 3 and 4 represents the production of ML at different enzyme concentration (3%, 5% and 10% by weight) using N-435 and CALB *Aspergillus oryzae*. It is apparent that reaction using N-435 5%wt exhibits higher ML production with 47.6% of conversion in 24 hours of enzymatic glycerolysis. The lowest composition of ML production was 14.4% using CALB from *Aspergillus oryzae*. Hence, it could conceivably be inferred that N-435 is more effective for the synthesis of ML than *Aspergillus oryzae*. It is relevant to hypothesize that N-435 was immobilized on an acrylic resin. In general, acrylic resin is a robust support material for the enzyme which increased thermo stability [14]. Robust material was required to prevent the enzyme from entering the organic media. The lipase hydrolyzed not only produce ML but also dilaurin (DL) and trilaurin (TL). N-435 has higher specificity production toward ML and DL while *Aspergillus oryzae* specificity toward TL.

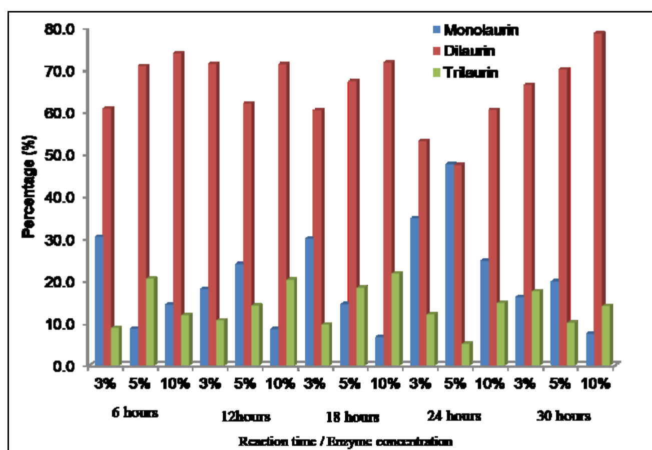

Figure 3. Product composition against concentration of Novozyme-435 and reaction time

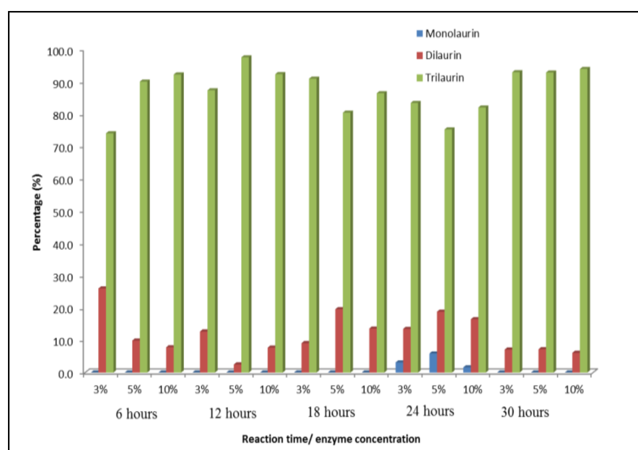

Figure 4. Product composition against concentration of *Candida Antarctica* lipase b from *Aspergillus oryzae* and reaction time

Thin layer chromatography
Separation using TLC was explained by the differences in affinity degree on the plates among the occurring components (Figure 5). Therefore, adsorption depended on the interaction of certain components with the stationary phase [19]. Sample dripped on TLC plates was known as the substrate. Substrates with higher polarity were adsorbed first because the interaction between the substrate and the stationary phase was very strong. This study chemically produced ML, DL and TL from enzymatic glycerolysis. Theoretically, ML has two OH groups and was higher in polarity than DL and TL. Both DL and TL moved slower than ML during elution process due to the hydrogen bonds that formed with the stationary phase. Sample chromatogram was compared with the reference [20].

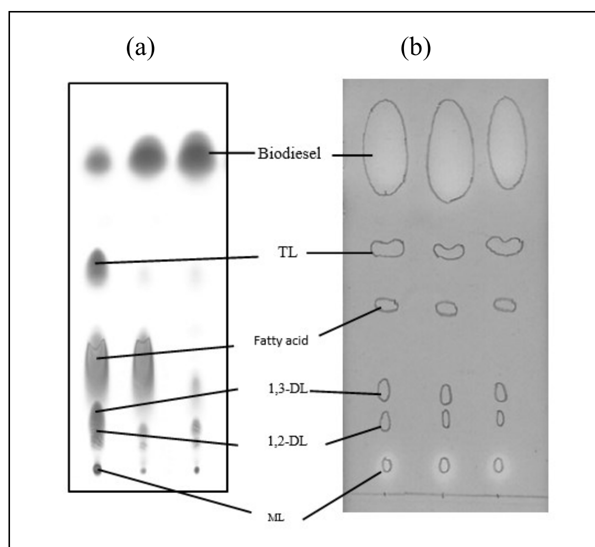

Figure 5. TLC Plate of glycerolysis reaction (a) reference TLC on lipid separation [20] and (b) TLC of sample

Gas chromatography analysis
Synthesized ML was directly compared with the standard mixtures of ML, DL and TL chromatogram (Figure 6). The percentage of ML composition was determined based on the total area of the chromatogram [21]. A relevant idea from a previous study mentioned that fatty acids of methyl esters with similar carbon chain have the same response factor and volatility. Therefore, the sample can be directly compared with the peak area to determine the composition [18]. While, Figure 7 shows a single peak GC chromatogram of purified ML after solid phase extraction (SPE) was performed. Thus, the synthesized ML has been successfully separated and purified from the side products (dilaurin and trilaurin).

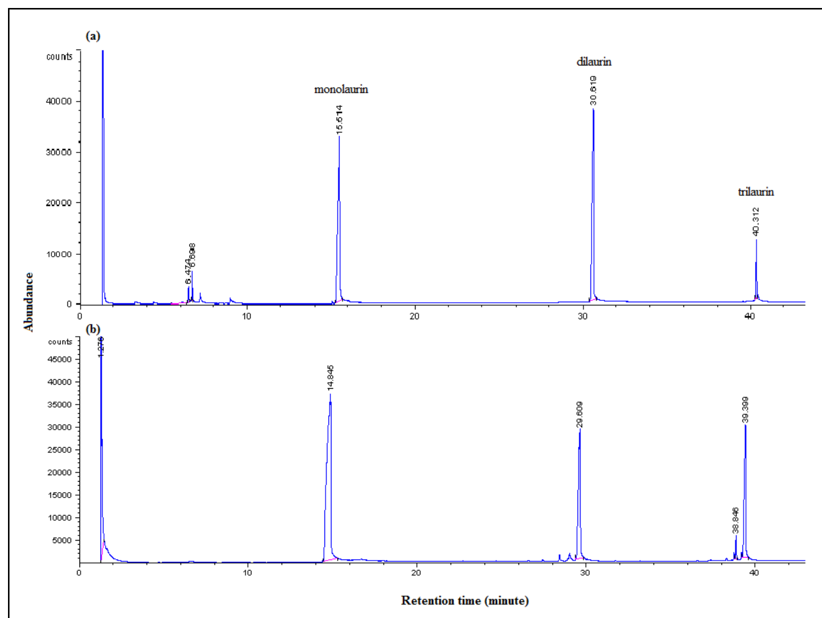

Figure 6. Comparison of GC-FID chromatogram (a) synthesized ML (b) standard mixtures of ML, DL, TL

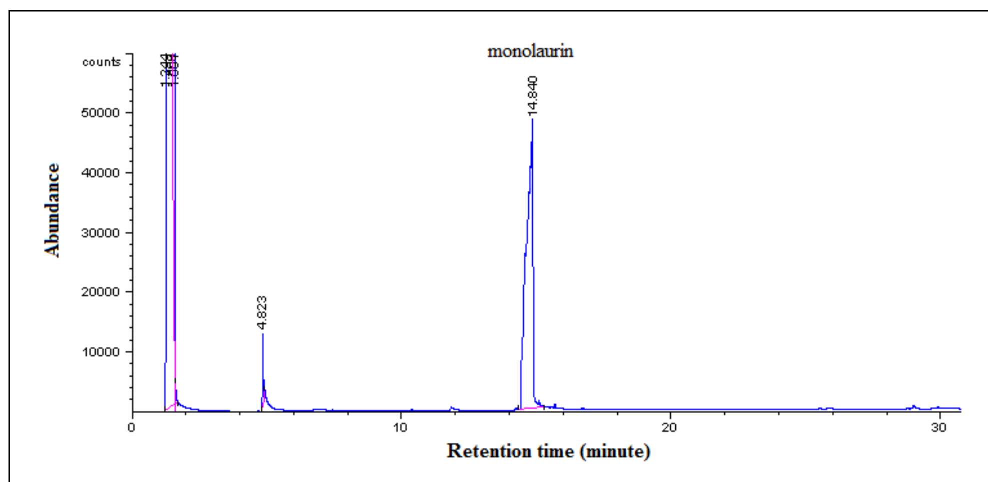

Figure 7. GC chromatogram of purified monolaurin

Infrared spectra

Figure 8 shows comparison of methyl laurate and purified monolaurin infrared spectra. Based on the figure, the present of hydroxyl group can be confirmed by looking at the O-H stretching broad band spectrum at wavenumber 3205 cm^{-1}. It proves the glycerolysis reaction has been successfully performed.

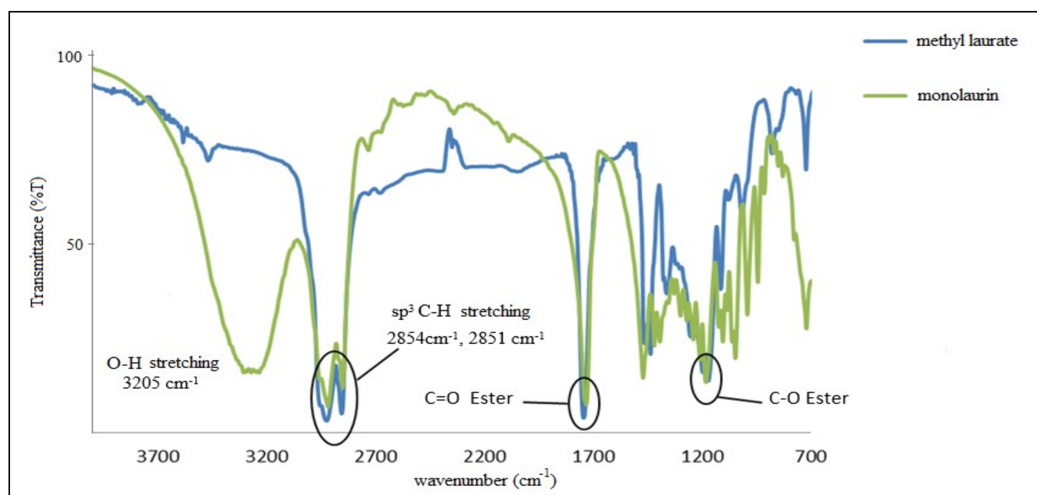

Figure 8. Comparison of infrared spectra between monolaurin and methyl laurate

Conclusion

Monolaurin was successfully synthesized (47.6% yield) and purified by enzymatic glycerolysis of methyl laurate which catalyzed by *Candida antarctica* lipase b. The optimum glycerolysis reaction conditions was using CALB (N-435) 5%wt at 24 hours of reaction time. Monolaurin was successfully characterized using TLC, GC-FID and FTIR analysis techniques. Acrylic resin that bonded onto CALB (N-435) was a robust support material because it restrained the enzyme from getting into the organic media.

Acknowledgement

The authors would like to acknowledge the Universiti Kebangsaan Malaysia for the research facilities and financial support through research grant no. GGPM-2014-033. Also, thanks to the Ministry of Higher Education for the funding through fundamental research grant, FRGS/2/2014/ST01/UKM/02/2 and MyBrain15 scholarship.

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