Available online at www.sciencedirect.com

ScienceDirect

Procedia Chemistry 18 (2016) 132 – 140

Procedia Chemistry

Molecular and Cellular Life Sciences: Infectious Diseases, Biochemistry and Structural Biology 2015 Conference, MCLS 2015

Isolation and Antibacterial Activity Test of Lauric Acid from Crude Coconut Oil (*Cocos nucifera* L.)

Febri Odel Nitbani[a]*, Jumina[a], Dwi Siswanta[a], Eti Nurwening Solikhah[b]

[a]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, Gadjah Mada University, Sekip Utara, Yogyakarta, Indonesia*
[b]*Pharmacology and Therapy, Medical Faculty, Gadjah Mada University, Sekip Utara, Yogyakarta, Indonesia*

Abstract

Isolation of lauric acid from crude coconut oil (CCO) has been done. Neutralization of CCO using 30% Na_2CO_3 solution could decrease its acid value from 1.69 to 0.48. Transesterification reactions of neutral coconut oil with methanol and K_2CO_3 at 55°C in 3 hours produced methyl laurate in 52% purity. Methyl laurate with 87% purity could be isolated by fractionatal distillation at 130-140°C. Hydrolysis of methyl laurate with NaOH produced solid lauric acidin 84% yield. Lauric acid at 5% concentration could inhibit the growth of all bacteria tested but it is still lower than Ciprofloxacin.

Peer-review under responsibility of the organizing committee of the Molecular and Cellular Life Sciences: Infectious Diseases, Biochemistry and Structural Biology 2015 (MCLS 2015)

Keywords: crude coconut oil; methyl laurate; lauric acid; antibacterial

* Corresponding author. Tel.: 082146219980; fax: -.
E-mail address: febri_nitbani@yahoo.com

Peer-review under responsibility of the organizing committee of the Molecular and Cellular Life Sciences: Infectious Diseases, Biochemistry and Structural Biology 2015 (MCLS 2015)
doi:10.1016/j.proche.2016.01.021

Nomenclature

CCO	Crude Coconut Oil
VCO	Virgin Coconut Oil
PKO	Palm Kernel Oil
GC-MS	Gas Chromatography-Mass Spectrometer
^{1}H NMR	Proton Nuclear Magnetic Resonance
NA	Nutrient Agar
w/v	Weight per volume expression of concentration of a solution
v/v	Volume per volume expression of concentration of a solution

1. Introduction

Indonesia has currently has the largest of palm plantations and thus is the largest coconut producer besides Malaysia and India[1]. All parts of coconut trees including fruits, stems and leaves can be used by human beings in a variety of applications. Due to its great benefits in a variety of applications, this tree is well known as the tree of life. However, the utilization of coconut products in Indonesia until recently was only 50% of the total production. The diversity of coconut tree based products in Indonesia is in fact only 22 in 2014, which is very small compared to 100 different products produced in the Philippines. This phenomenon therefore clearly explains the very low prices of coconuts in Indonesia. Thus, it is very important to increase the diversity of coconut tree based products, which in turn will lead to an increase of economic value of coconut trees and therefore contribute to the welfare of Indonesian coconut farmers.

Crude coconut oil (CCO) is one among all the products from coconut trees. Chemically, it consists of triglyceride compounds that contain large amount of saturated medium chain fatty acid. Lauric acid (C12:0) is the major fatty acid in CCO accounting for around 50% of the total fatty acids[2]. Due to its wide applications, there have been many research and investigations focusing on a variety of its applications, among which is its antimicrobial activity first shown by Prof. John Kabara in the 1970s. His research showed that lauric acid and the monoglyceride, monolaurin, had broad-spectrum antimicrobial activities including antibacterial, antiviral and anti fungal[3]. Despite their successful application as an antimicrobial agent, many researchers have reported on the challenging part, to synthesise and isolate lauric acid and monolaurin in high yield and purity from CCO and other oils that contains lauric acid.

In the past, there have been many reports and publications on the application of CCO as antibacterial agents, but most of them have reported it to have very low activity on microorganisms. For example, the research by Ugbobu *et al.*[4] used unpurified lauric acid from palm kernel oil (PKO) from the *Elaies guineensis* species to inhibit the growth of *Staphylococcus aureus*, *Streptococcus* sp. and *Candida albicans*. The composition of lauric acid was previously determined by the free fatty acid titration method. It was found that PKO was able to inhibit the growth of *S. aureus* and *Streptococcus* sp. but not *C. albicans.* In a recent paper, Loung *et al.*[5] explained the successful synthesis of lauric acid from virgin coconut oil and PKO through a hydrolysis reaction utilizing a lipase enzyme of *Thermomyces lanuginose* (TL IM). The antibacterial activity of the synthesized lauric acid was further tested on *Escherichia coli, Salmonella thypimurium* and *S. aureus* and its activity found to be very low. This may be due to the fact that the enzymatically-hydrolyzed lauric acid was not purified and hence it was still in its mixture with other compounds from VCO and PKO. A more interesting result was found by Silalahi *et al.*, who successfully conducted a partial hydrolysis of VCO using NaOH and lipase TL IM to produce lauric acid, which was further used as antibacterial agents[6]. They reported that enzymatic hydrolysis of VCO could be more effective in producing lauric acid, which was indicated by the increase of the acid number. This synthesized lauric acid was then tested for its antibacterial activity towards *Pseudomonas aeruginosa, S. aureus, Streptococcus epidermidis* and *P.acnes* and the result showed that this synthesized lauric acid was effective in inhibiting the growth of *P. aeruginosa.*

Until recently, the most commonly applied processused in industry for the synthesis of lauric acid is the Colgate-Emery steam hydrolysis of coconut oil, which can be done at high temperatures (250°C) and high pressure of 50 atm[7]. The advantage of this method is that it can yield fatty acid with a purity of higher than 97%. However, this method has been reported to be of relatively high cost and high energy consumption, which is due to the use of a special column for the breakdown off at sand oils. An alternative approach to replace the Colgate-Emery steam

hydrolysisis the use of non-specific lipase enzyme, which allows hydrolysis at 30 to 45°C and at atmospheric pressure to produce high purity fatty acid[8].

It is thus our interest to isolate and synthesise lauric acid from CCO and to test its antimicrobial activity on *S. aureus, Bacillus cereus, S. Thypimurium* and *E. coli.* The isolation of lauric acid from crude coconut oil was done through the neutralization of the free fatty acid, base transesterification using methanol, fractional distillation of the methyl ester mixture, and base hydrolysis of methyl laurate. The aim of this research was to find a new path of isolation and synthesis of lauric acid from CCO. Thus, this research will contribute to the diversification of coconut tree based products and therefore lead to the increase of Indonesian coconut farmers welfare. In the context of health, this research will potentially contribute in producing new antibacterial product from inexpensive and an abundant raw material such as coconut oil.

2. Methods

2.1 Materials

Materials used in this research were all analytical grade and used without further purification. They were purchased from Merck and include K_2CO_3, NaOH, Na_2CO_3, anhydrous Na_2SO_4, HCl, methanol, n-hexane. All the aqueous solutions were prepared using aquades. Crude coconut oil was obtained from Kupang, East Nusa Tenggara.

2.2 Experimental procedures

2.2.1 Neutralization of crude coconut oil

A standard method is used to determine the acidity number of crude coconut oil before the neutralization. 50 grams of crude coconut oil is dissolved in 50 mL of n-hexane and is then put into a separating funnel. 30 mL of 30% (w/v) Na_2CO_3 solution is added and the separating funnel is slowly shaken. Its yellow turbid bottom layer of gelatin-like consistency is separated, while 30 mL of 30% (w/v) Na_2CO_3 solution is added into its upper layer. The upper layer is then washed with distilled water until the pH is neutral. The clear yellow liquid obtained was dried with anhydrous Na_2SO_4 and evaporated to removen-hexane. The acid number of the neutralised product was redetermined. The neutralisation product could be further used for the synthesis of methyl ester if its acid number is below 1.

2.2.2 Transesterification of neutral coconut oil

0.25% (w/w) of K_2CO_3 calculated based on the total weight of the oil is dissolved in 0.21 mol of methanol (molecular weight = 32 g/mol; 6.72 g) in a 100 mL three-neck flask which is equipped with a thermometer and a condenser. 0.1 mol of neutral coconut oil (molecular weight = 638 g/mol; 63.8 g) was added into the mixture and then heated at 55°C for 3 h. The mixture is slowly cooled and left overnight in a separating funnel. The productis removed from the separating funnel, dissolved in n-hexane and washed with hot distilled water until reaching a neutral pH. The final product obtained is then dried with anhydrous sodium sulfate and is further characterized using gas chromatography-mass spectrometry (GC-MS).

2.2.3 Isolation of methyl laurate

Methyl laurate is subsequently isolated from the mixture of methyl ester obtained from transesterification step. The isolation is done by using the reduced pressure distillation technique generally known as fractional distillation. The distillation fraction is taken at a variety of ranges including 80-110, 110-120, 120-130, 130-140 and140-150°C. Each fraction is further analyzed by using gas chromatography. Redistillation can also be done to increase the amount of methyl laurate. The structure of methyl laurate obtained is further verfied by analysis with IR spectrometer, gas chromatography and mass spectrometer. The gas chromatograph (operated at 260°C, stationary phase CP-FFA, and carrier H_2 at 40 mL/min) is equipped with a flame ionization detector (FID).

2.2.4 Hydrolysis of methyl laurate

A total of 0.01mol of methyl laurate (molecular weight = 214.3 g/mol; 2.143 g) and 0.08 mol of NaOH (molecular weight = 40 g/mol; 3.4 g) are mixed in a 100 mL three-neck flask and stirred for 30 min. 30 mL of distilled water is then added to this mixture and stirring is continued until all solids dissolved. The product is removed from the flask, put in a separating funnel and then neutralized with 30 ml of 10% HCl solution until reaching pH 1. The product is subsequently extracted with 3×15 mL n-hexane. The organic layer is washed with distilled water to get a neutral pH and dried with anhydrous Na_2SO_4. Evaporation is done to remove the remaining n-hexane solvent and the residue is further analyzed by IR spectrometry and ^{1}H NMR (Delta 2-NMR, ECA 500MHz).

2.2.5 Antibacterial test using "well diffusion" method

Antibacterial activity test is conducted to find out the potential inhibition ability of isolated lauric acids on the growth of several bacterial pathogens including *S. aureus, B. cereus, S. Thypimurium* and *E. coli*. Sterilization of materials and tools used in actibacterial activity test is done prior to the antibacterial experiment. The sterilisation is done for 30 min in an autoclave at 121°C. Seeding medium of nutrient agar (NA) is prepared by dissolving 20 g of NA into 1 L of aquades and then heated until all the NA completely dissolved. This medium is then resterilized in autoclave. Antibacterial activity test is done by incubating NA medium which has been streaked by one loop of test bacteria at 37°C for 24 h. One loop of bacteria which has been kept for 24 h is put into a test tube containing 9 ml of sterile physiological sodium chloride and synchronized with the 0.5 MacFarland. A sterile swab is further inserted into a test tube, pressed at the walls and scratched homogenously on NA medium. A pitting with a diameter of 6 mm is made and kept for several minutes. 100 µL lauric acid with the concentration of 5%, 10%, 15% and 20% are prepared and added into each pitting. Similar procedures are also done for commercial ciprofloxacin with the concentration of 0.5% as comparison. After the incubation at 37°C for 24 h, the inhibition zone diameter is determined by measuring the clear zone formed around the well. This is further taken as inhibitory diameter.

3. Results and Discussion

3.1 Neutralization of crude coconut oil (CCO)

CCO generally contains triglycerides, free fatty acids and other impurities such as sterols. The amount of free fatty acids in coconut oil is indicated by the acid number in which the higher the acid number, the higher the amount of free fatty acids. It is assumed that the free fatty acids can also react with base catalyst used in the transesterifications of coconut oil, therefore leading to lower quality of the transesterification product. It is thus necessary to remove free fatty acids from CCO through a reaction with a weak base such as Na_2CO_3. This reaction will result in the production of removable water-soluble salts. In this research, the neutralization of free fatty acids from coconut oil was done by using Na_2CO_3 and the result indicates that it can reduce the acid number of crude coconut oil from 1.69 to 0.48. Indicated by its final acid number which is lower than 1, this neutralized crude coconut oil can be categorized as a good reagent for further transesterification reactions.

3.2 Transesterifications of neutral crude coconut oil

Transesterifications of neutral CCO is done by reacting 1 mol of neutral coconut oil and 2.1 mol of methanol with 0.25% of the total weight of the oil of K_2CO_3 as the catalyst. The reaction was initially conducted by mixing methanol and K_2CO_3 under constant stirring until the mixture forms a solution containing potassium methoxide base which can react with triglycerides of the coconut oil. The transesterification reaction is kept at a constant temperature of 55°C for 3 h. The result shows that this reaction results in the formation of two immiscible layers where the top layer is a mixture of methyl esters and the bottom layer is glycerol. The top layer, the methyl ester of fatty acids, is then removed, dissolved in the non-polar n-hexane solvent and washed with distilled water. This is done to remove the remaining alkaline K_2CO_3, which is indicated by the neutral pH of the washing water. A certain amount of anhydrous sodium sulfate is then added into the solution to absorb the remaining water in the solvent and

the solvent is further removed by evaporation. The final product of this transesterification reaction is a yellow viscous liquid (Fig. 1a) which is mixture of methyl esters of fatty acids produced with 97% yield.

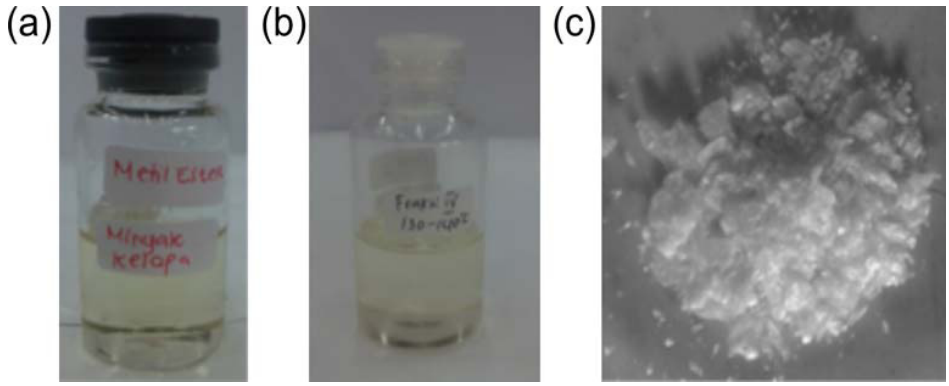

Fig. 1. (a) Mixture of methyl ester of fatty acids product; (b) Distillate of fraction IV (130-140°C); (c) The product of base hydrolysis of methyl laurate

Furthermore, the product of the transesterification reaction is analyzed using GC-MS and its chromatogram is presented in Fig. 2a. This chromatogram clearly shows that there are nine peaks with retention times ranging from 8.7 to 40.5 min. The peak with the greatest abundance is shown by the highest peak with retention time of 32.5 min having relative percentage of 52%. Further analysis by mass spectrometry shown in Fig. 2b indicates that this peak is methyl laurate, characterized by a molecular ion at m/z = 214, which is in very good agreement with the molecular weight of methyl laurate. Mass spectra of other peaks showed good evidence that the final product of transesterification is purely a mixture of fatty acid methyl esters.

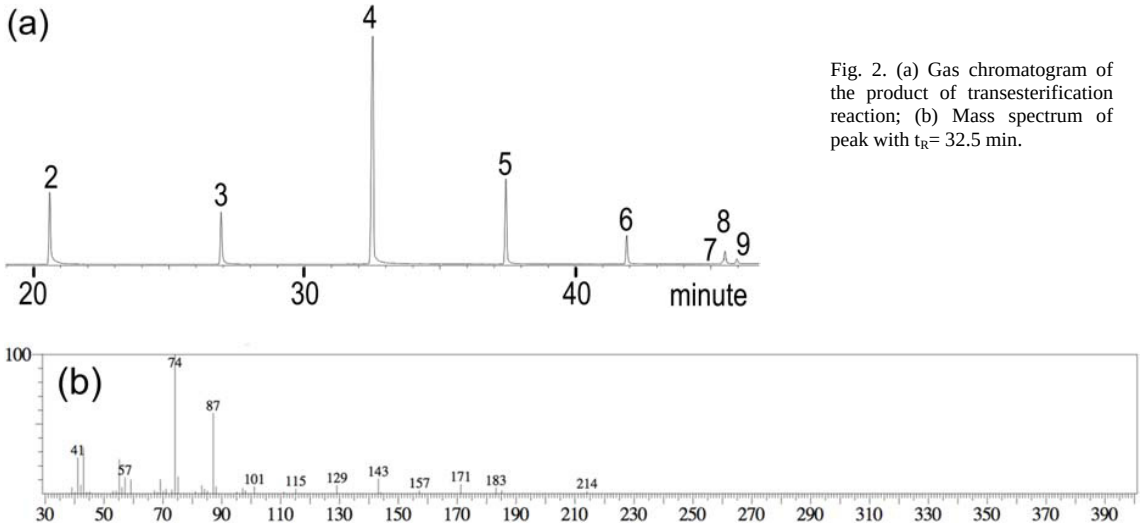

Fig. 2. (a) Gas chromatogram of the product of transesterification reaction; (b) Mass spectrum of peak with t_R= 32.5 min.

3.3 Isolation of methyl laurate

The isolation of methyl laurate, the component with the greatest abundance in the transesterification product is done through distillation techniques. This is theoretically based on the difference in boiling point among all compounds in the mixture of methyl esters. The transesterification product is a mixture of saturated fatty acid methyl esters such as methyl caprylate, methyl caprate, methyl laurate, methyl myristate, methyl palmitate and

methyl stearate, and unsaturated fatty acid methyl esters such as methyl oleate and methyl linoleate. Distillation with reduced pressure is further chosen for the separation methyl laurate from the mixture of these methyl esters as they have very high boiling points above 200°C. 100 mL of methyl ester mixture is distilled and the vapor is collected in different fractions as shown in Table 1.With this technique, it is estimated that methyl laurate evaporates at boiling point between 130-140°C.

Table 1. Data of distillate fraction temperature range and the yield of each fraction

Fractions	Temperature range (°C)	Volume (mL)
I	80-110	12.8
II	110-120	9.8
III	120-130	17.8
IV	130-140	30.6
V	140-150	10.6

Table 1 presents the yield of each fraction, with fraction IV having the largest quantity indicated by its largest volume among the five fractions. The collected fraction IV appears as a yellow viscous liquid (Fig. 1b). It was further analyzed by GC and its chromatogram is presented in Fig. 3a.

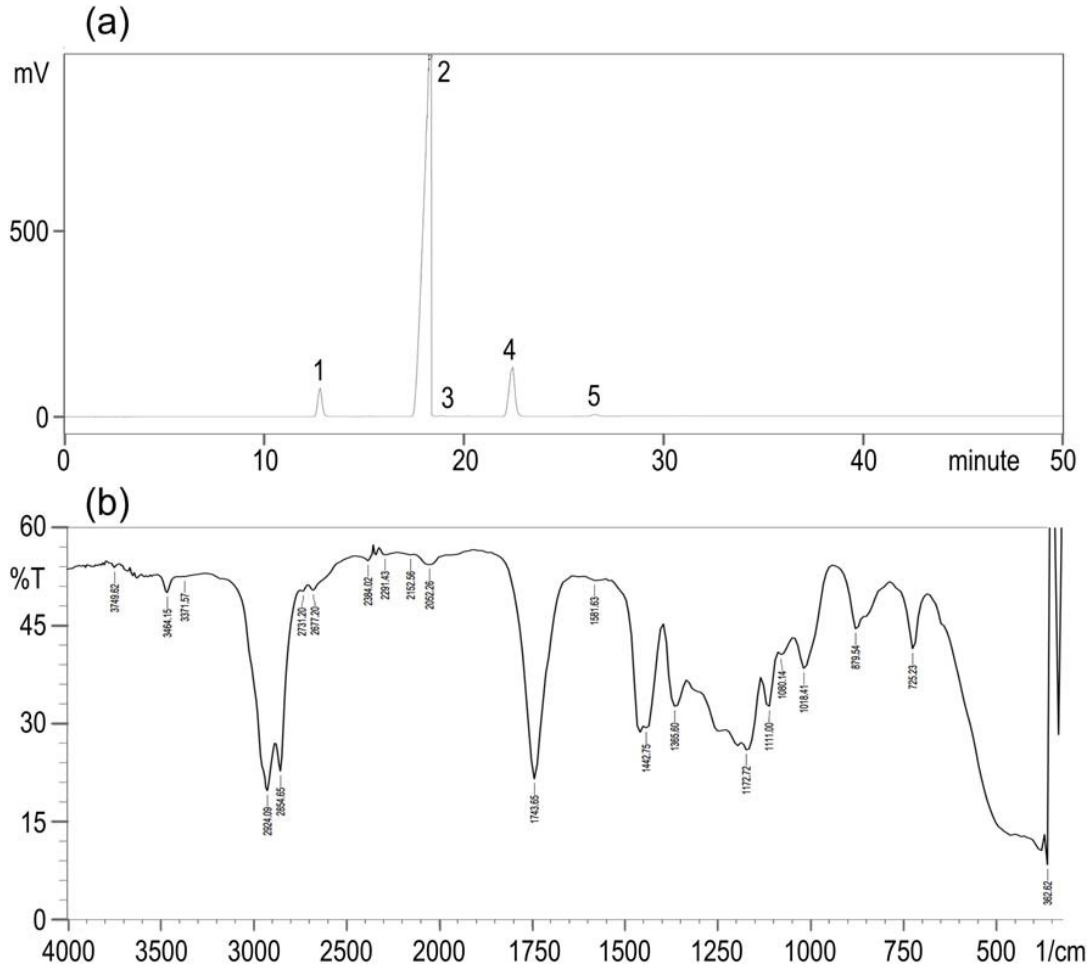

Fig. 3. (a) Chromatogram of fraction IV; (b) IR Spectra of fraction IV

It is indicated by Fig. 3a that there is one highest peak among all the peaks shown. This peak has a retention time of 18.4 min with a purity of 87%. This peak is in a very good agreement with the standard chromatogram of methyl laurate, thus it can be certainly concluded that this peak represents methyl laurate, which collected as fraction IV of reduced pressure distillationat 130-140°C.

Subsequent analysis of fraction IV used an IR spectrometer and the result is presented in Fig. 3b. It can be seen from the IR spectrum that there appear some typical absorptions of an ester compound. These include the absorption of ester group C=O at 1700 cm^{-1} region and the absorption of ester C-O-C stretch at 1117 cm^{-1}. Typical absorption of C-H vibration due to the presence of sp^3 can be found at 2900 and 2800 cm^{-1}. There also appear an absorption of -CH_3 groups in the area of 1357 cm^{-1} and a typical absorption of -CH_2- groups around 1400 cm^{-1}. Thus, it can be inferred that majority of the fraction IV content is of methyl laurate.

It was also found that further MS analysis of the compound which has the highest peak in the chromatogram of Fig. 3a resulted in the same MS spectra as shown by Fig. 2b. This result clearly showed the successful isolation of methyl laurate from its mixture as it is indicated by comparable molecular fragmentation at m/z = 214 (molecular ion), m/z=183, m/z=87 and the base peaks at m/z = 74. These peaks are in a very good agreement with the fragmentation of methyl laurate. Thus it can be concluded that the highest peak with a relative level of 87% in the chromatogram is methyl laurate, which is indicated by the molecular ion of m/z = 214.

3.4 Hydrolysis of methyl laurate

Methyl laurate was hydrolyzed to lauric acid using NaOH solution with the mole ratio of methyl laurate to NaOH of 1:8. The reaction scheme for base hydrolysis of methyl laurate to lauric acid is presented in Fig. 4a. The product of this reaction was a white solid (Fig. 1c) having a yield of 84%. This product was analysed by using IR spectrophotometer and the result is presented in Fig. 4b.

It can be seen from Fig. 4b that there has been a conversion of ester compound to the carboxylic acid compound, which is shown by shifting absorption of C=O group to a shorter wave number of 1600cm^{-1}. The figure also shows a disappearance of C-O-C absorption around 1117cm^{-1}, which strongly indicates that methyl laurate has all been converted into lauric acid.

In order to support the IR data, the hydrolyzed product was also analysed by using ^{1}H NMR spectroscopy and the result is presented in Fig. 4c. The result of ^{1}H NMR clearly indicates the full conversion of methyl laurate into lauric acid. This is clearly illustrated by the chemical shift at δH = 0.88 ppm (3H, t) which is the chemical shifts of the three protons bonded to the C_{12} (-CH_3) atom. Due to the influence of the neighboring protons bound to C_{11}, this appears to be a triplet of peaks. There is also a chemical shift δH = 1.28 ppm (16H, s) which is due to resonance of methylene protons attached to the C_4-C_{11} atoms. This is also supported by the appearance of pentuplet of peaks at δH = 1.61 ppm with 2 protons, which is due to the of the protons bound to the C_3 atom. A resonance from protons bound to C_2 on the other hand results in a more downfield chemical shift at δH = 2.34 ppm. This indication can be strongly attributed to the less protected protons bound to C_2, which is due to the resonance effect of the carboxyl group (-COOH) of lauric acid.

3.5 Antibacterial activity test using well diffusion method

Antibacterial activity of lauric acid is conducted to determine its ability to inhibit the growth of two Gram-positive bacteria, *S. aureus* and *B. cereus*, and two Gram-negative bacteria, *S. thyphimurium* and *E. coli*. Lauric acid was previously dissolved in n-hexane as a solvent and as shown in Table 2, its concentration was varied from 5% to 10%, 15% and 20%. Based on the diffusion pitting method, the ability of lauric acid to inhibit the growth of bacteria was determined by measuring the diameter of the clear zone formed around the wells that contain lauric acid solutions with various concentrations. The concentration of both lauric acid and ciprofloxacin were varied to determine the effect of the concentration of both compounds on inhibition zone diameter. The results of this antibacterial test are presented in Table 2.

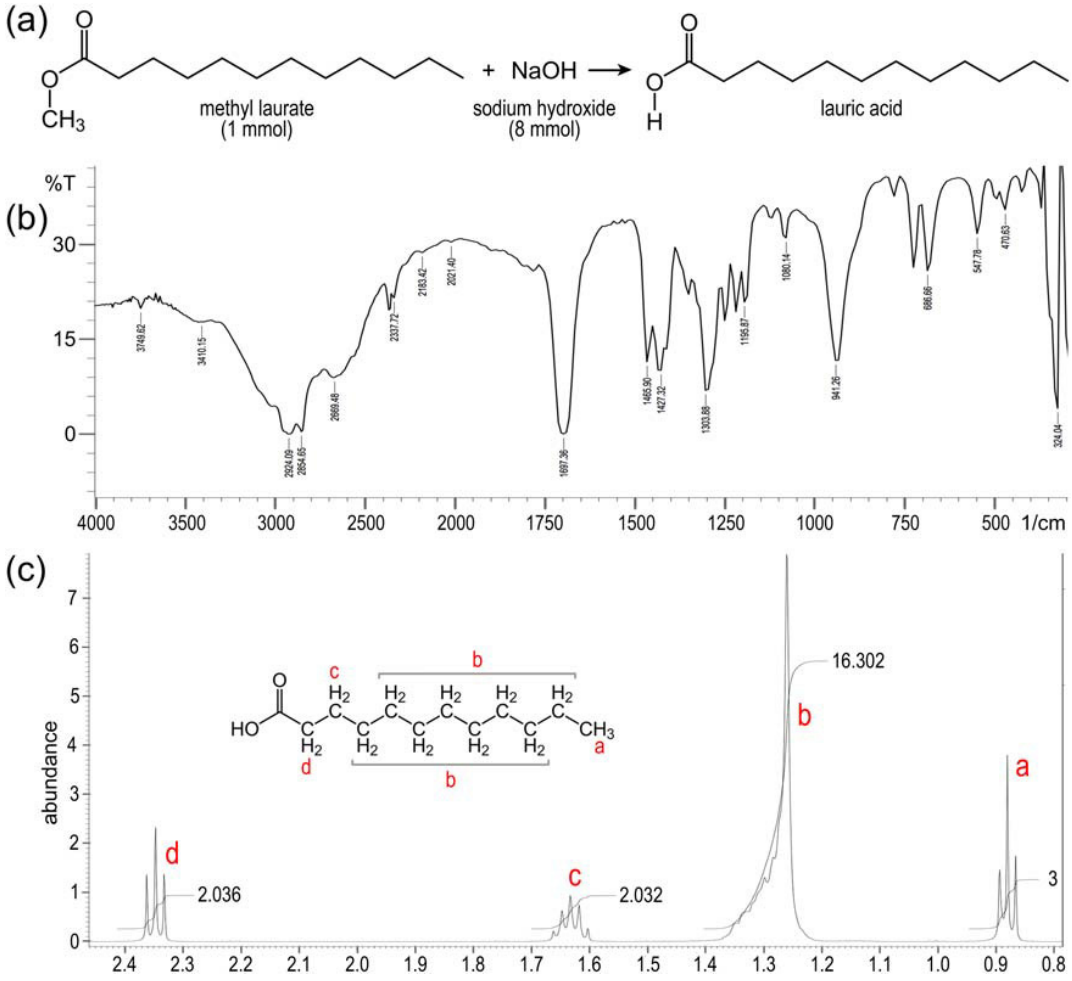

Fig. 4. (a) Reaction scheme for base hydrolysis of methyl laurate; (b) IR spectrom of lauric acid; (c) ^{1}H NMR spectrum of lauric acid

The data in Table 2 clearly indicate that lauric acid with a concentration of 15% and 20% has larger diameter of inhibition zone than that of standard antibiotic compound of ciprofloxacin which is only 30 mm. At a concentration of 5% and 10%, the inhibition zone diameter of lauric acid was, on the other hand, smaller than that of the standard antibacterial compound except for *Bacillus cereus*, which has a similar inhibition zone diameter to that of the ciprofloxacin at the concentration of 10%. Overall, it can be concluded that in the concentration of 5%, lauric acid is able to inhibit the growth of both Gram positive and Gram negative bacteria but the antibacterial activity of lauric acid is still lower than Ciprofloxacin. The higher the concentration of lauric acid, the greater the antibacterial activity which is obviously indicated by its steep increase in the diameter of the inhibiting zone obtained from the test on all four bacterial species.

Table 2. Inhibiting zone diameter of lauric acid compound

	Diameter of inhibiting zone (mm)				
Bacterial test	20%	15%	10%	5%	Ciprofloxacin (0.5%)
Staphylococcus aureus	40	37	28	25	30
Bacillus cereus	40	35	30	25	30
Salmonella thypimurium	38	30	28	25	30
Escherichia coli	41	36	28	26	30

4. Conclusions

Neutralization reaction of crude coconut oil using 30% (w/v) Na_2CO_3 solution could decrease its acid value from 1.69 to 0.48. The product of the transesterifications reaction of triglycerides from coconutoil (neutral coconut oil) with methanol using K_2CO_3 as the base catalyst is a mixture of methyl ester with a methyl laurate composition of 53%. Methyl laurate was successfully separated from its mixture by fractional distillation at a temperature of 130-140°C with a purity of 87%. Lauric acid has been successfully produced through the hydrolysis of methyl laurate in 84% yield. It is found that lauric acid is able to inhibit the growth of *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhimurium* and *Escherichia coli* at a concentration of 5% but it is still lower than Ciprofloxacin. The higher the concentration of lauric acid, the greater the diameter of inhibition zone against *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhimurium* and *Escherichia coli*.

References

1. Menteri Pertanian RI. Mengembalikan Kejayaan Kelapa Indonesia. *Tabloid Sinar Tani Maret 2014*; diakses 22 Juni 2014.
2. Bouaid A, Martínez M, Aracil J. Biorefinery approach for coconut oil valorisation: A statistical study. *Bioresour. Technol* 2010; 101 : 4006-4012
3. Widiyarti G, Hanafi M, Soewarso WP. Study on synthesis of monolaurin as antibacterial agent against Staphylococus aureus. *Indo. J. Chem.* 2009, 9 (1) : 99-106.
4. Ugbogu OC, Onyeagba RA, Chigbu OA. Lauric acid content and inhibitory effect of palm kernel oil on two bacterial isolates and *Candida albicans. Afr. J. Biotechnol.* 2006, 5 (11) : 1045-1047
5. Loung FS, Silalahi J, Suryanto D. Antibacterial activity of Enzymatic hydrolyzed of Virgin Coconut oil and Palm Kernel oil against *Staphylococcus aureus, Salmonella thypi and Escherichia coli, Int.J.Pharm.Tech. Res.* 2014. 6(2) : 628-633.
6. Silalahi J, Yademetripermata, Putra EDL, Antibacterial Activity of Hydrolyzed Virgin Coconut Oil. *Asian J. Pharm. Clin. Res.* 2014.7(2) : 90-94
7. Bornscheuer UT. Lipase-catalyzed syntheses of monoacylglycerols. *Enzyme Microb. Technol* 1995; 17 : 578-586.
8. Brady C, Metcalve L, Slabozewski D, Frank D, Lipase Immobilized on a Hydrophobic, Microporous Support for the Hydrolisis of Fats. *J. Am. Oil Chem. Soc*. 1998; 65(6) : 917-921.