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ISOLATING FREE FATTY ACIDS FROM VIRGIN COCONUT OIL USING LIPASES FROM DIFFERENT SOURCES

Van T. A. Nguyen[a,b*], Truong D. Le[a], Hoa N. Phan[b], Lam B. Tran[b]

[a]Industrial University of Ho Chi Minh City, Vietnam
[b]Ho Chi Minh City University of Technology, Vietnam

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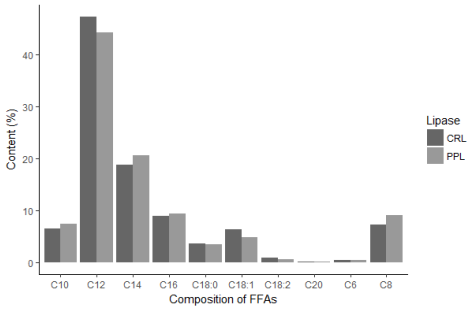
*Corresponding author
nguyenthiaivan@iuh.edu.vn

Graphical abstract



Abstract

To obtain free fatty acids (FFAs), virgin coconut oil (VCO) was hydrolyzed by two kinds of lipase: lipase from *Candida rugosa* (CRL) and lipase from porcine pancreas (PPL). The hydrolysis process was controlled under four parameters: VCO to buffer ratio, lipase concentration, pH condition and temperature. In term of CRL, the best conditions for hydrolysis reaction was 1:5 VCO to buffer ratio, 1.5% lipase (w/w oil), pH 7 and at 40°C. And for PPL was 1:4 VCO to buffer ratio, 2% lipase (w/w oil), pH 7.5 and at 40°C. Hydrolysis degree (HD) of VCO which was catalyzed by CRL reached 79.64%. Whereas, HD value as using PPL to hydrolyze VCO only achieved 27.94%, less than approximately three times compared to CRL. Morever, hydrolysis reaction for CRL also took less time than PPL. The length of hydrolysis time was 16 hours and 26 hours, respectively. FFAs were obtained from the hydrolyzed products and analyzed by GC – FID. It was obviously that lauric acid (C_{12}) took the biggest contribution to FFAs content (47.23% for CRL and 44.23% for PPL).

Keywords: Virgin coconut oil, lipase *candida rugosa*, lipase porcine pancreas, hydrolysis degree, free fatty acids

1.0 INTRODUCTION

Virgin Coconut Oil (VCO) contains a great amount of medium chain fatty acids (MCFAs) in which lauric acid takes the highest proportion (nearly 50%). MCFAs are easily absorbed and directly burned into energy in the liver. This creates the early satiety, so this may lead to the weight loss. Besides, MCFAs do not take part in the synthesis of cholesterol. Therefore, MCFAs is the best choice for obese people [1]. The research of Valente *et al.* [2] also showed that MCFAs in VCO had a effective contribution in controlling overweight condition for women. On the other side, antiviral, antifungal and antibacterial abilities of MCFAs in VCO also were concerned due to the presence of lauric acid in it. Parfene *et al.* [3] indicated that MCFAs in crude coconut oil could inhibit *Yarrowia lipolytica*. Shilling *et al.* [4] conducted experiments in antimicrobial effect of VCO and MCFAs against *Clostridium difficile*. The result showed that MCFAs in VCO were antibacterial agents, represented by capric acid, caprilic acid and lauric acid. In contrast, VCO had no effect on the growth of *Clostridium difficile*. With many benefits that MCFAs bring for the health, they can be applied in variety fields such as food, medicine and so forth. Particularly in food products, nowadays' tendency is organic food without chemcial additives. MCFAs can act as a natural preservative in food products. Thus, it is essential to extract MCFAs from VCO.

There are several methods to acquire FFAs such as, acidic hydrolysis, alkaline hydrolysis or enzymatic

hydrolysis. In which, using enzymatic hydrolysis is a friendly way to protect the environment and receive hydrolyzed products as desired. It means that we can control the hydrolysis reaction by using the positional enzyme or partial hydrolysis. The enzyme reaction is conducted under milder condition of temperature and pH when compared to acidic or alkaline hydrolysis. Thus, it can avoid some danger from undesirable side-reactions, have no effect to color of products or limit oxidized products [5]. Morever, enzymatic hydrolysis requires no co-factor or any chemical additive, and the enzyme also can be reusable [6]. So in this study, the hydrolysis of VCO to obtain FFAs was conducted using two type of enzymes from different sources: *Candida rugosa* lipase (CRL) and porcine pancreas lipase (PPL). The aim of this study was to determine the best hydrolysis conditions for these two lipases and compare the hydrolysis ability of them.

2.0 METHODOLOGY

2.1 Materials

VCO was funded by Luong Quoi Coconut Co., Ltd (Bentre Province, Vietnam). CRL (Type VII, ≥700 unit/mg solid) and PPL (Type II, 100-500 units/mg protein) were purchased from Sigma-Aldrich Co. (Canada). Chemicals used in this research were KOH, ethanol, iso-octane and the others were purchased from Merck (Germany) with purification more than 95%.

Devices used for experiments were high speed homogenizer (IKA T 25 digital ULTRA-TURRAX, Germany), overhead stirrer (OS20, USA), orbital shaker incubator (LM-2575RD) from Yihder Technology Co. (Taiwan), evaporator (IKA RV digital V) from Germany and GC – FID SHIMADZU 2010 Plus.

2.2 Hydrolysis Process

The hydrolysis process was conducted under four parameters that affect significantly to hydrolysis degree of VCO, such as: VCO to buffer ratio, lipase concentration, pH and temperature.

The initial mixture included VCO and buffer which is suitable for pH condition of the two lipases. For CRL, the experiment was conducted by using phosphate buffer, whereas borate buffer was used for PPL. Iso-octane as solvent to dissolve VCO was added to the mixture with 1:1 VCO to solvent ratio [7]. Then the mixture was emulsified in 15 minutes by a stirrer at speed of 10000 rpm before adding the appropriate amount of lipase. The mixture was continuously stirred in 5 minutes to dissolve completely lipase powder. The hydrolysis reaction was incubated in orbital shaker incubator device. The reaction was stopped after 2 hours reaction and determined the hydrolysis degree (HD)

The hydrolysis degree of VCO was calculated as the following equation:

$$HD = \frac{V_{KOH} * M_{KOH} * M_{FFAs}}{10 * m} (\%) \quad [8]$$

According to the equation, V_{KOH} is the volume of Potassium hydroxide titrated (mL), M_{KOH} is the molarity of KOH solution (mol/L), M_{FFAs} is the average molecular weight of FFAs and m is mass of the VCO (g).

2.3 FFAs Isolating

The process of FFAs isolation was carried out according to the method of Shimada *et al.* [9]. First of all, the mixture after finishing the hydrolysis process was added KOH to neutralize the released FFAs. Then the mixture was added to the separatory funnel, the residual glycerides which was not hydrolyzed by lipase was extracted out of the funnel by adding n-hexane. The upper phase containing n-hexane and residual glycerides was withdrawed. The lower phase containing FFAs was continued to extract after returning to free form by adding 4N HCl in the funnel. FFAs was isolated by using rotary evaporator to remove n-hexane.

2.4 Analysis Composition of FFAs

FFAs compositon was detemined by using Gas Chromatograph – flame ionization detection. FFAs were converted into fatty acid metyl ester (FAME) before injecting into the column. 2µl of sample was injected using split injection mode with the split ratio 1:25. The column DB-FFAP (0.25 mm internal diameter, 30 m length and 0.25 µm film thickness) was used at a column of head pressure of 12 psi. Helium played a role as carrier gas, FID and injector temperature were both at 250°C during the process. The initial column temperature was 100°C, temperature program was set up to 230°C at 7°C /min and was maintained this temperature until the end of analysis process [10].

All the experiments were conducted in triplicate and data output were statistically analyzed by R software.

3.0 RESULTS AND DISCUSSION

3.1 Effect of VCO to Buffer Ratio

According to Figure 1, the hydrolysis degree for each lipase depend on a different VCO to buffer ratio. The highest hydrolysis degree was obtained at 1:4 VCO to buffer ratio for PPL. Whereas CRL needed an amount of buffer more than PPL, the best result obtained at 1:5 of VCO to buffer ratio.

Catalytic activity of lipase is conducted on elmusion of oil and water. Therefore, increasing the amount of buffer makes lipase have more substrates to catalyze because there are more links between oil and buffer which are established in the mixture of

reaction. This leads to the rise of hydrolysis degree but the excess of buffer amount will decline the catalytic ability of lipase remarkably because it occurs the competition of substrate (water will bind to lipase and prevent it bond to substrate). As the result, the hydrolysis degree is decreased [7].

In general, oil to buffer ratio was not equivalent to other studies due to substrate specificity of lipase and different hydrolysis methods. With different substrates or hydrolysis methods, solubility's lipase and linking to ester bond of triglycerides also were different, leading to difference in adding the amount of buffer. Such as the study of Sharma *et al.* [7], using CRL to hydrolyze cod liver oil was conducted with 1:4 of oil to buffer ratio and iso-octane also was used as solvent to dissolve cod liver oil. Meanwhile, Freitas *et al.* [11] used CRL and PPL to hydrolyze soybean oil with 1:4 of oil to buffer ratio without iso-octane in the mixture. Instead, the study was added 2.5% of Arabic gum to the mixture.

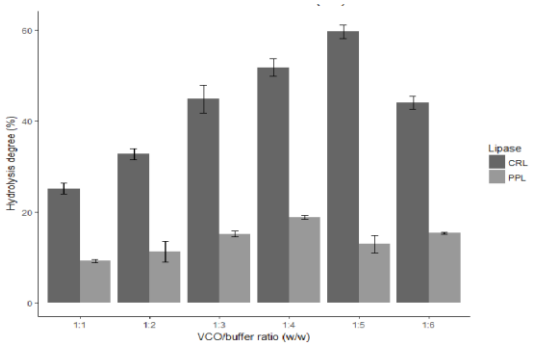


Figure 1 Effect of VCO to buffer ratio on HD

3.2 Effect of Lipase Concentration

In Figure 2, the result showed that at 2% of PPL concentration got the highest hydrolysis degree. Meanwhile, CRL needed only concentration of 1.5% to get the highest hydrolysis percentage. This showed that catalytic ability of CRL was stronger than PPL. The percentage of FFAs released by CRL was more than about three times by PPL.

The more lipase concentration was added the more hydrolysis degree was obtained. However, the hydrolysis degree will increase insinigficantly if lipase is saturated with subtrates. In this case, adding more lipase will not make the growth of hydrolysis degree [12].

This result also was not equivalent to other researches, the same to the result of oil to buffer ratio, either high or low lipase concentration depends on the substrate which was catalyzed and hydrolysis method. Therefore, solubility or linking to ester bond of triglycerides of each lipase is different. This lead to the difference in the amount of lipase added to achieve the subtrate saturation. As the result, each study gave different results in the amount of lipase. Such as, the study of Sharma *et al*. [7] using CRL with substrate was cod liver oil, the best condition for enzymatic hydrolysis was at 2% of lipase concentration. And another research of Freitas *et al.* [11], the amount of CRL and PPL added to the mixture to hydrolyze soybean oil was 1% of lipase concentration.

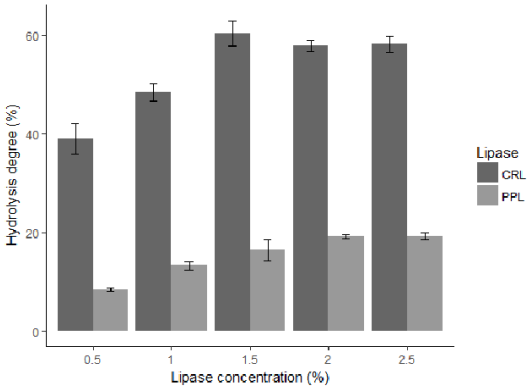


Figure 2 Effect of lipase concentration on HD

3.3 Effect of pH

Figure 3 showed that the highest hydrolysis degree was obtained at pH 7 for CRL (Figure 3 a)) and 7.5 for PPL (Figure 3 b)). Overall, PPL prefers to catalyze in slightly alkaline medium than neutral, meanwhile CRL was suitable at neutral pH.

Each lipase has an optimum pH condition which gives the best hydrolysis degree. Determining suitable pH value for each lipase is very important for the experiments because pH has a significant effect on hydrolytic ability of lipase. With dramatic changes in pH (very high or very low) may affect the ionization of substrate, lipase or lipase-substrate complex. This will make lipase denature, breakdown of substrate, leading to a decrease of hydrolysis degree [13].

Some studies had equivalent results at these pH values. Hydrolysis of cod liver oil using CRL was conducted at pH 7.0 [7]. Hydrolysis of soybean oil using CRL and PPL at pH 7 and 7.5 respectively was done by Freitas *et al.* [11]. Hermansyah *et al.* [14] also used CRL to hydrolyze triolein at pH 7.0. Zou *et al.* [15] had a comparison between free PPL and PPL immobilized on ionic liquid modified mesoporous silica SBA-15 about their hydrolysis activity, the optimum pH for free PPL was 7.5.

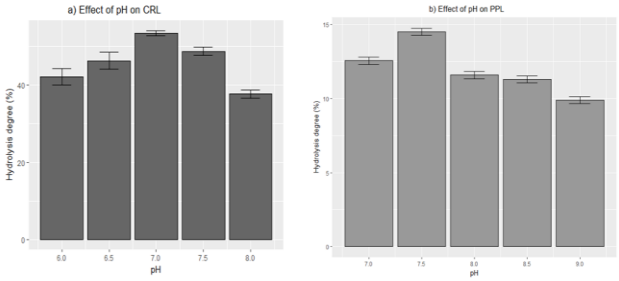


Figure 3 Effect of pH on HD of a) CRL and b) PPL

3.4 Effect of Temperature

As well as pH, temperature has a strong effect to hydrolysis reaction of lipase. Increasing temperature will decrease viscosity of mixture reaction and molecular of lipase becomes flexible, leading to easy linking between lipase and subtrate. However, lipase will be deactivated if temperature is too high because the nature of enzyme is protein, guiding to a fall of hydrolysis degree of lipase.

As shown in Figure 4, the hydrolysis degree got the highest rate of both two lipases at temperature of 40°C. This temperature was also equivalent to the study of Freitas *et al.* [11] in using CRL and PPL to hydrolyze soybean oil at 40°C. Zhou *et al.* [16] hydrolyzed Jatropha oil for biodiesel production using CRL at 40°C. In research about improving catalytic hydrolysis reaction of sol-gel-encapsulated CRL was conducted by Ozyilmaz *et al.* [17], the optimum temperature for encapsulated CRL was 35°C but for free CRL was nearly 40°C. For the determination of positional distribution of fatty acids in *Mimusops elengi* and *Parkinsonia aculeata* seed oils, Sharma *et al.* [18] set up the experiments using PPL to hydrolyze them at 40°C.

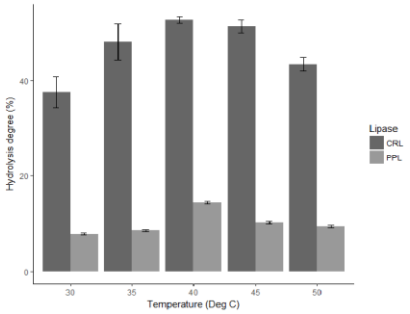


Figure 4 Effect of temperature on HD

3.5 Time of Hydrolysis Reaction

Figure 5 illustrated the hydrolysis degree of two lipase during 30 hours. Hydrolysis degree of VCO by using PPL reached 27.64% after 26 hours hydrolysis. Whereas VCO hydrolyzed by CRL not only got the higher hydrolysis degree but also took less time than PPL (HD achieved about 80% after 16 hours reaction). When compare the result of hydrolysis time of CRL in this study with other researches, the hydrolysis time was saved. In study of Freitas *et al.* [11], after 24 hours reaction, the maximum percentage about 70% when using CRL to hydrolyze soybean oil and 23% of hydrolysis degree was obtained for PPL. Iso-octane is a solvent to dissolve VCO. It made viscosity of VCO go down as well as increasing the interface of VCO and water. Because of this, lipase had more substrate to catalyze the hydrolysis reaction. As the result, the hydrolysis time was shortened and had a increasing in hydrolysis degree [19].

CRL gave a higher catalytic activity. The percentage of FFAs released from hydrolysis reaction using CRL was more than trippled compared to PPL. Morever, time course of VCO hydrolysis using CRL was also shorter than PPL. Because of positional specificity of PPL, PPL hydrolyzes triglycerides in sn-1,3 position and remains sn-2 position. But CRL is a non-specific lipase, it can hydrolyze completely all three ester bonds in triglycerides. Hence, hydrolysis degree of VCO by CRL was more than PPL [18].

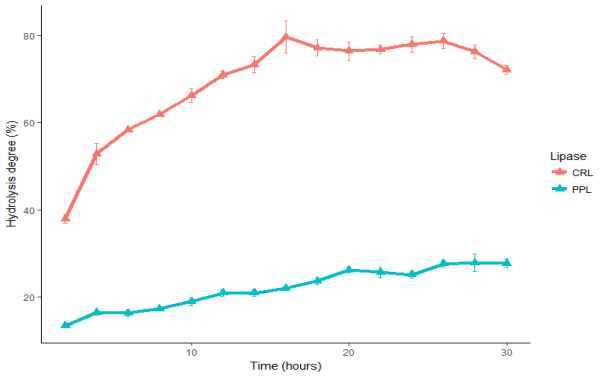


Figure 5 Hydrolysis time

3.6 Composition of FFAs

The composition of FFAs was shown in Figure 6. In general, the main composition that contributes to FFAs content was MCFAs with lauric acid (C_{12}) took the highest percentage. Enzymatic hydrolysis VCO by CRL and PPL released 47.23% and 44.23% of lauric acid content, respectively. Although PPL is specific lipase at sn-1,3 position, the component of FFAs released by PPL was nearly equivalent to CRL (non-specific lipase). So this can be explained that all fatty acids of the tested VCO have about equal distribution between sn-2 and sn-1,3.

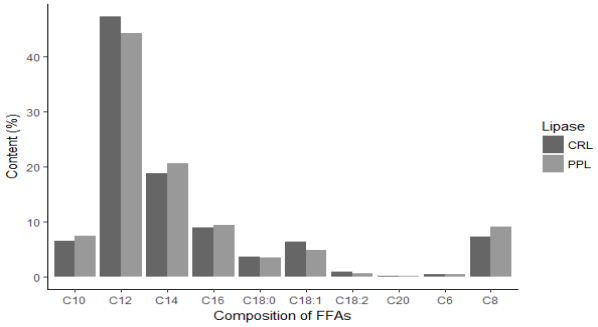


Figure 6 Compositon of FFAs obtained after hydrolysis reaction

4.0 CONCLUSION

The best conditions for hydrolysis VCO by CRL was determined at 1:5 of VCO to buffer ratio, 1.5% of CRL concentration, pH 7 and temperature at 40°C. For

PPL, the best result was obtained at 1:4 of VCO to buffer ratio, 2% of PPL concentration, pH 7.5 and temperature at 40°C. The results of pH and temperature were suitable with natural properties of each lipase. Morever, it also was equivalent to previous hydrolysis process conducted at these pH and temperature. VCO to buffer ratio and lipase concentration had differences because of substrate specificity of lipase and hydrolysis method.

Overall, in these hydrolysis reactions, catalytic ability of CRL was higher than PPL because of it's non-positional specificity, so it could hydrolyze three ester bonds of triglycerides, leading to a great amount of released FFAs.

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