Research Article

Towards an alcohol-free process for the production of palm phytonutrients via enzymatic hydrolysis of crude palm oil using liquid lipases

Siti Hanifah Adiiba,[a,b] Eng-Seng Chan,[a,b] Yee Ying Lee,[b,c] Amelia,[a,b] Mun Yuen Chang[a,b] and Cher Pin Song[a,b*] [ID]

Abstract

BACKGROUND: Crude palm oil (CPO) is rich with phytonutrients such as carotenoids and tocols which possesses many health benefits. The aim of this research was to develop a methanol-free process to produce palm phytonutrients via enzymatic hydrolysis. In this work, triacylglycerol was hydrolyzed into free fatty acids (FFAs) using three different types of liquid lipases derived from *Aspergillus oryzae* (ET 2.0), *Aspergillus niger* (Habio) and *Candida antartica* (CALB).

RESULTS: ET 2.0 was found to be the best enzyme for hydrolysis. Under the optimum condition, the FFA content achievable was 790 g kg^{-1} after 24 h of reaction with 1:1 water-to-oil mass ratio at 50 °C and stirring speed of 9 × *g*. Furthermore, with the addition of 2 g kg^{-1} ascorbic acid, it was found that 98% of carotenoids and 96% of tocols could be retained after hydrolysis.

CONCLUSION: This work shows that enzymatic hydrolysis, which is inherently safer, cleaner and sustainable is feasible to replace the conventional methanolysis for the production of palm phytonutrients.
© 2022 The Authors. *Journal of The Science of Food and Agriculture* published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

Keywords: phytonutrients; enzymatic hydrolysis; lipases; crude palm oil; tocols; carotene

INTRODUCTION

Owing to the rise in heath awareness, there have been increasing interest and demand for functional food or nutraceuticals which can help to improve health or prevent diseases. Many plants contain phytonutrients which have been proven to confer health benefits. Interestingly, crude palm oil (CPO) is naturally rich with phytonutrients, specifically carotenoids and tocols, which have many benefits such as cardio-protection, anti-cancer and neuro-protection.[1] Various direct methods such as solvent extraction, molecular distillation and supercritical fluid extraction have been studied to extract phytonutrients from CPO.[2] However, an indirect method converts the triacylglycerides (TAGs) into a fatty acid derivative, followed by the separation of phytonutrients from the fatty acid derivative.

Methanolysis of palm-based feedstocks is the most preferred reaction route used in the indirect method. In this process, TAGs are converted into methyl esters, followed by the extraction of phytonutrients from methyl esters by distillation. However, undesired excess methanol could be present in the extracted phytonutrient concentrate. This is toxic to humans and long-term ingestion of a residual amount of methanol could lead to visual impairment or metabolic acidosis.[3] Besides its toxicity, methanol

is also highly flammable. Furthermore, methanol is primarily derived from fossil fuels, thus its production contributes to global warming. Owing to the increasingly stringent requirements for food safety, process safety, environmental protection and process sustainability, there is currently a strong industry interest to develop an alternative process which is inherently safer, cleaner, economical, and sustainable.

One option is to convert the TAG to free fatty acids (FFAs) via an enzymatic hydrolysis process. The phytonutrients concentrated in the unreacted CPO can then be separated from the FFA produced. Instead of methanol, this process uses water as co-reactant, thus

* Correspondence to: CP Song, Chemical Engineering Discipline, School of Engineering, Monash University Malaysia, Jalan Lagoon Selatan, 47500 Subang Jaya, Selangor, Malaysia. E-mail: song.cherpin@monash.edu*

a *Chemical Engineering Discipline, School of Engineering, Monash University Malaysia, Subang Jaya, Malaysia*

b *Monash-Industry Palm Oil Education and Research Platform (MIPO), Monash University Malaysia, Subang Jaya, Malaysia*

c *School of Science, Monash University Malaysia, Subang Jaya, Malaysia*

making the overall process inherently safer and more sustainable. Additionally, FFA is a by-product which has a high value and is in high demand. Moreover, the reaction can take place at low temperature (< 60 °C) and atmospheric pressure which will reduce energy consumption and protect the heat-labile phytonutrients, especially carotenes which begin to undergo thermal degradation at temperatures above 60 °C.[4,5] However, there are limited studies on the use of enzymatic hydrolysis in the production of phytonutrients from plant oils.

You *et al.*[6] hydrolyzed the CPO and crude palm olein to 950 g kg^{-1} fatty acids using *Candida rugosa* for 24 h at 50 °C, followed by carotene recovery through chromatography method. However, the carotenoid content was found to decrease by up to 18% during hydrolysis. However, Chu *et al.*[7] hydrolyzed the palm fatty acid distillate from 850 to 970 g kg^{-1} using Novozym 435 at 70 °C for 6 h. Subsequently, the fatty acid was neutralized and subjected to adsorption chromatography which concentrated the tocols from 3700 mg kg^{-1} to 43 200 mg kg^{-1}. Nevertheless, it was reported that 25% of the tocols were lost during the process. Moreover, there has been no further study carried out to improve the retention of the phytonutrient during hydrolysis.

Since phytonutrients are present in low concentration in vegetable oil and they are of high value, a small reduction in their concentration during hydrolysis would result in a substantial loss in potential profit. In this study, the enzymatic hydrolysis of CPO was carried out using different liquid lipases. We evaluated three commercial liquid lipases for their performance in hydrolyzing CPO and impacts on the retentions of tocols and carotene. In addition, we investigated the effects of ascorbic acid, which was added as a sacrificial antioxidant, on oil hydrolysis and phytonutrients retention.

MATERIALS AND METHODS

Materials
CPO was provided by Sime Darby Plantation Berhad (Selangor, Malaysia). The lipases derived from *Aspergillus oryzae*, also known as Eversa® Transform 2.0 (ET 2.0), and *Candida antartica* lipase B (CALB) were both supplied by Novozymes (Kuala Lumpur, Malaysia). Lipase from *Aspergillus niger* (Habio) was obtained from Mianyang Habio Bioengineering Co. Ltd (Sichuan, China). Isopropanol (IPA) with 99% purity and HPLC-grade methanol were purchased from JT Baker (Pulau Pinang, Malaysia) and Systerm Chemicals (Malaysia), respectively. The 99% *n*-hexane was purchased from Friendemann Schmidt (Kuala Lumpur, Malaysia). Sodium hydroxide (NaOH), potassium hydrogen phthalate and phenolphthalein were obtained from Sigma Aldrich (Selangor, Malaysia). All these lipases and chemicals were used without further purification.

Enzyme activity assay
The enzymatic activity of each liquid lipase was quantified by determining its hydrolytic activity.[8] In brief, 10 mL of refined palm oil was hydrolyzed with 10 mL of distilled water with the presence of 0.2 mL of liquid lipase in a 50-mL beaker. The process was carried out for 5 min at 50 °C and the stirring speed was set to 9 × *g*. The amount of FFA produced was then determined using an auto-titrator (Metrohm's Titrino Plus 848). The activity of the ET 2.0, CALB and Habio were found to be 7580, 1420, and 6700 IU mL^{-1}, respectively (1 IU represents the amount of enzyme that catalyzes the conversion of one μmole of titratable fatty acid per minute at 50 °C).

Enzymatic hydrolysis of CPO
The enzymatic hydrolysis reaction was carried out in a 1-L jacketed stirred-tank reactor which was covered with aluminum foil to minimize light exposure. Initially, 350 g of CPO was transferred into the reactor. Then, 350 g of water along with 7 g of dissolved enzyme (20 g kg^{-1} based on CPO weight) were also transferred into the reactor. For experimental runs involving the use of ascorbic acid, the ascorbic acid was first dissolved in the water before the solution was added into the reactor.

The reaction mixture was then stirred continuously at 50 °C and 9 × *g* for 24 h. Subsequently, the reaction mixture was subjected to phase-separation at 55 °C for 30 min. Following that, the top phase of the sample was extracted and subjected to centrifugation at 8500 × *g* for 3 min to enhance the separation of remaining water.

The reaction was repeated with varying temperatures (from 50 to 70 °C), water-to-oil mass ratio (from 0.6:1 to 1:1), ascorbic acid concentration (from 0–2 g kg^{-1} based on CPO weight) and stirring speed (from 9 × *g* to 18 × *g*). Other parameters such as pressure (1 atm), enzyme concentration (20 g kg^{-1}) and reaction time (24 h) were kept constant. Hydrolysis of CPO with the different set of process parameters were repeated with all three lipases (ET 2.0, Habio and CALB) unless stated otherwise.

Determination of FFA
FFA in the centrifuged top-phase sample was determined using an auto-titrator (Titrino Plus 848; Metrohm, USA) and a 0.1 M NaOH solution as the titrant. Approximately 0.3 g of the top-phase sample was extracted, heated, and then dissolved in 30 mL of IPA. A total of five drops of phenolphthalein was then added to the solution. Before the initiation of titration, the solution was stirred using the auto-titrator until the sample was dissolved in the IPA. The endpoint value which indicated the volume of NaOH required to titrate the solution was recorded. The FFA content in the top phase was calculated as shown in Eqn (1). The FFA molecular weight (MW) used was 256.4 mol g^{-1}, based on the MW of palmitic acid.

$$FFA\,(g\,kg^{-1}) = \frac{NaOH\ volume\,(L) \times NaOH\ molarity\,(mol/L) \times FFA\ MW\,(mol/g)}{Mass\ of\ sample\,(kg)}$$

(1)

Quantification of tocols
The preparation for tocols quantification started with the extraction of approximately 0.65 g of the centrifuged top-phase sample. This was followed by heating of the sample using a water bath at 55 °C to liquify the sample. The sample was then dissolved in 4 mL of IPA to avoid solidification, and 1 mL of the solution was then transferred into a glass vial. The tocols content was quantified using high-performance liquid chromatography (HPLC, 1260 Infinity II LC System; Agilent Technologies, Santa Clara, CA, USA) where 20 μL of the solution was injected into the system. The HPLC is equipped with an ultraviolet (UV) multiwavelength detector. The column used was C18 Reverse Phase Eclipse XDB, and mobile phase used was methanol–water (95:5 *v/v*). Flow rate of the mobile phase was maintained at 1.5 mL min^{-1} for 25 min during analysis. The peaks of *α*-tocopherol, *α*-tocotrienol, *γ*-tocotrienol and *δ*-tocotrienol peaks were identified by comparison with a standard curve previously generated using their respective standards. The peak areas were then converted to concentrations. The concentrations were expressed in mg kg^{-1}.

Quantification of carotenoids

Approximately 0.045 g of the centrifuged top-phase sample was melted by heating it in a water bath at 55 °C. Then, the sample was dissolved in 10 mL of *n*-hexane which is optically pure at 446 nm. Lastly, the sample was transferred to a quartz cuvette and the spectrophotometric absorbance at 446 nm was obtained using UV-visible spectroscopy (Genesys 10; Thermo Fischer Scientific, Waltham, MA, USA). The beta-carotene content was determined using Eqn (2) as shown:

$$\text{Carotene content}\,(\text{mg kg}^{-1}) = \frac{383 \times (\text{Absorbance of sample} - \text{Absorbance of pure } n-\text{hexane})}{100 \times \text{Sample mass}\,(\text{g})} \times \text{Volume of solvent}\,(\text{mL}) \tag{2}$$

where '383' and '100' in the equation were the simplification of the scaling factor and extinction factor of pure beta-carotene.

Statistical analysis

Each reaction run was carried out twice and the results shown are the mean values along with its calculated standard deviations. One-way analysis of variance (ANOVA) statistical analysis was carried out to analyze the statistical difference of the results, and a significant difference is identified when the P-value is below 0.05. For groups with a significant difference, a *post hoc* t-test analysis was carried out to identify significance between two variables in that specific group.

RESULTS AND DISCUSSION

Characterization of crude palm oil

The properties of the investigated CPO are presented in Table 1. Based on the results obtained, the initial FFA content found in the CPO from several batches were found to be ranging from 23 to 47 g kg^{-1}, which is well within the range of 30 to 50 g kg^{-1} FFA which is the agreement in other published reports.[9,10]

Likewise, the carotene and tocols content present in the CPO was found to be within the range reported by Nagendran *et al.*[11] and Mohd Top.[12] The carotene was found to range from 608 to 662 mg kg^{-1}, while the tocols content in CPO was found to be 650–754 mg kg^{-1}. Moreover, the composition of the α-tocopherol, α-tocotrienol, γ-tocotrienol and δ-tocotrienol in the investigated CPO were aligned with the known typical composition of tocols in CPO.[11]

Effect of lipase type

Three commercial liquid lipases (ET 2.0, Habio and CALB) were used as biocatalysts in the hydrolysis of CPO. Figure 1 shows the

Table 1. Characteristics of crude palm oil (CPO)

Properties	Values
Free fatty acid (g kg^{-1})	23–47
Phytonutrients	
Carotene content (mg kg^{-1})	608–662
Tocols content (mg kg^{-1})	650–754
$\quad\alpha$-Tocopherol (%)	18.3–20.5
$\quad\alpha$-Tocotrienol (%)	22.2–24.2
$\quad\gamma$-Tocotrienol (%)	44.9–45.2
$\quad\delta$-Tocotrienol (%)	12.2–12.5

FFA production, carotene and tocols retention after 24 h of enzymatic hydrolysis at 50 °C, 1:1 water-to-oil mass ratio and stirring speed of $9 \times g$. From the results, ET 2.0 was found to perform the best among the three lipases. High FFA content of 830 g kg^{-1} was achieved following 24 h of hydrolysis using ET 2.0 at 50 °C with a water-to-oil mass ratio of 1:1. Habio performed slightly less effectively for the hydrolysis of CPO, with its FFA content peaking at 780 g kg^{-1}. However, the results show that CALB produced poor results as the FFA content after 24 h of hydrolysis was only 120 g kg^{-1}.

Despite being a lipase which generally catalyzes the hydrolysis of lipids, CALB in its immobilized form (Novozym 435) has been reported to work better in reaction medium where its water content is at minimum.[13] As a result, the FFA conversion obtained using the CALB-catalyzed reaction was the lowest among the investigated lipases since a large amount of water was used to hydrolyze CPO. This is in good agreement with a previous study where a low conversion of buriti oil to FFA (i.e. approximately 190 g kg^{-1}) was reported using CALB.[14] This finding is also supported by Bresolin *et al.*[15] who found that immobilized CALB had ten-fold lower enzyme hydrolytic activity compared to the immobilized ET 2.0.

For the phytonutrients retention, the results show that carotene and tocols retentions were both higher for CALB reactions in comparison to ET 2.0 and Habio. Since the experiments were carried out at a similar condition, the results show that the retention of phytonutrients could be correlated to the extent of hydrolysis in the reaction mixture. Higher FFA contents in the reaction mixture catalyzed by ET 2.0 and Habio were found to give lower retention of both carotene and tocols, whilst lower FFA content in the reaction mixture catalyzed by CALB gave higher retention. This finding indicates that FFA plays a major role in degrading the phytonutrients since the FFA is prone to auto-oxidation.

The unsaturated fatty acids produced during hydrolysis are prone to auto-oxidation due to their double bonds. As a result of the low bond dissociation energy of double bonds, the hydrogen is more easily removed.[16] These unsaturated fatty acids would react with oxygen in an autocatalytic auto-oxidation process which involves three stages: initiation, propagation, and termination.[17]

When the concentration of radicals is high enough, two radicals can join together to form non-radical products in the termination stage.[18] Before the termination stage is reached, the phytonutrients such as carotenes and tocols present in the CPO would act as antioxidants to break the propagation cycle and prevent the formation of non-radical products. Naturally, the phytonutrients (AH) would donate its hydrogen to the free radicals, R$^{\bullet}$ and ROO$^{\bullet}$, and would then turn into antioxidant radicals (A$^{\bullet}$) which are stable radical products which do not have any antioxidant activity.[16,18] The mechanism of chain-breaking antioxidants is shown in Eqns (3) and (4):

$$\text{AH} + \text{R}^{\bullet} \rightarrow \text{RH} + \text{A}^{\bullet} \tag{3}$$

$$\text{AH} + \text{ROO}^{\bullet} \rightarrow \text{ROOH} + \text{A}^{\bullet} \tag{4}$$

Effect of ascorbic acid

In order to minimize the degradation of phytonutrients due to the formation of fatty acids, the incorporation of a sacrificial antioxidant was investigated in this study. Ascorbic acid, a type of antioxidant soluble in water, was selected as a sacrificial antioxidant for
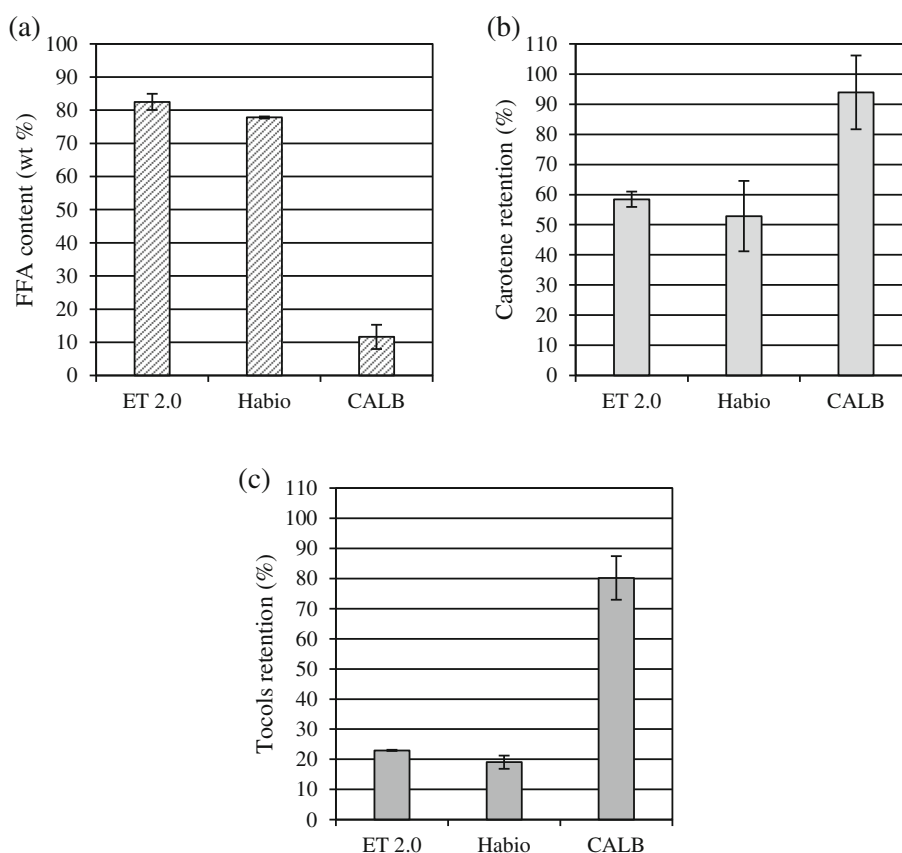
Figure 1. Effects of lipase type on (a) FFA production, (b) carotene retention and (c) tocols retention. Error bar in the graph represents standard deviation of the mean value.

the hydrolysis of CPO. Its concentration was varied from 0 to 2 g kg^{-1} to determine its effects on hydrolysis and phytonutrients retention.

Figure 2 shows the effects of ascorbic acid concentration on the FFA production, carotene and tocols retention after 24 h of enzymatic hydrolysis at 50 °C, 1:1 water-to-oil mass ratio and stirring speed of 9 × *g*. As shown in Fig. 2(b,c), the phytonutrients retention for all types of lipases were found to increase dramatically with an increasing concentration of ascorbic acid. For ET 2.0-catalyzed hydrolysis, the addition of 2 g kg^{-1} ascorbic acid was found to increase the retention of carotene and tocols to 98% and 96%, respectively. Meanwhile, the retention of carotene and tocols for Habio-catalyzed hydrolysis was increased to 94% and 99%, respectively. However, the addition of ascorbic acid, either at 1 or 2 g kg^{-1}, did not have significant effect on the retention of carotene for CALB-catalyzed hydrolysis; but it increased the retention of tocols from 80% to 100%.

Ascorbic acid is a type of secondary antioxidant which scavenge free radicals and oxygen-deprived species, i.e. hydroxyl radicals, hydroperoxides and singlet oxygen.[19] To stabilize free radicals and oxygen-deprived species, ascorbic acid donate its electrons/hydrogen and form relatively ascorbyl-free radicals.[20] The small-sized ascorbic acid molecules enhances its ability to donate hydrogens, making it an effective sacrificial antioxidant for this hydrolysis process.[21]

In addition, the secondary antioxidant ascorbic acid (AsH) could replenish the hydrogen atoms of the primary antioxidants such as tocols which belong to the vitamin E group by donating its hydrogen atom to the tocols radical (T·).[20,22] This process would regenerate the natural phytonutrients such as tocols (TH) and its corresponding antioxidant activity.[16] The process of tocols regeneration through the reduction of tocol radicals is as shown in Eqn (5):

$$T^{\cdot} + AsH \rightarrow TH + As^{\cdot} \quad (5)$$

In this study, ascorbic acid was found to improve the retention of carotenes and tocols during hydrolysis of CPO. However, ascorbic acid with high concentration could promote oxidation by catalyzing the reduction of free transition metal ions that could promote free radical reactions.[23] Therefore, it is essential to determine the optimum concentration of ascorbic acid for the process.

Inhibitory effect of ascorbic acid
Although the addition of ascorbic acid was found to improve the retention of carotene and tocols during hydrolysis, the addition of ascorbic acid could also affect the hydrolysis performance of lipases. This is evident when the hydrolysis reaction was performed using Habio (see Fig. 2(a)). The FFA content was found to decrease significantly from 780 to 500 g kg^{-1} when ascorbic acid was added at 1 g kg^{-1}. The FFA content dropped further to 400 g kg^{-1} when the ascorbic acid concentration was increased to 2 g kg^{-1} ($P < 0.05$). However, the absence and presence of ascorbic acid (up to 2 g kg^{-1}) in the hydrolysis reactions involving ET 2.0 and CALB lipases did not affect the FFA contents because the differences in the results were found to be statistically insignificant ($P > 0.05$).
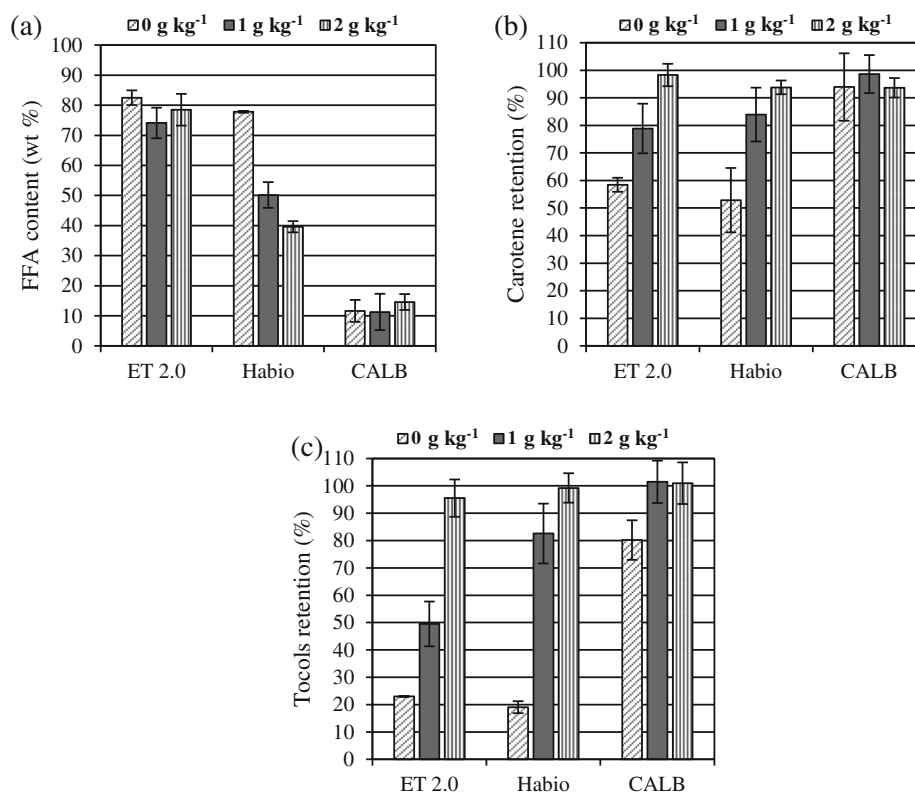
Figure 2. Effects of ascorbic acid concentration on (a) FFA production, (b) carotene retention and (c) tocols retention. Error bar in the graph represents standard deviation of the mean value.

The addition of ascorbic acid was found to result in a change in the pH of water which could affect the lipase activities. With no ascorbic acid, the pH of water was 7.5. Upon the addition of 1 g kg^{-1} and 2 g kg^{-1} of ascorbic acid, the pH dropped to 3.3 and 3.1, respectively. This could affect the reaction because each lipase has a different optimum pH range where their enzyme activity would be at its maximum. Specifically, lipase derived from *Aspergillus oryzae* (i.e. ET 2.0 in this study) has been found to be able to perform optimally at pH lower than *Aspergillus niger* (i.e. Habio in this study).[24,25] At low pH, acid-induced protein denaturation of the lipase could take place, thus affecting the enzymatic activity.[26]

Degradation rate of palm phytonutrients

The results illustrated through Fig. 3(a) gives insight to the difference in carotenoids and tocols retention, using the ET 2.0 reactions as an example. This is because ET 2.0 is the lipase which is able to perform well without being disrupted by the presence of ascorbic acid. A distinct difference in the amount of carotene and tocols retention was observed when no ascorbic acid was added. With no ascorbic acid, the carotene retained was 58% as shown in Fig. 3(a). However, the tocols retention was as low as 23% after 24 h of reaction.

This study has found that during hydrolysis, the tocols which belong to the vitamin E group were degraded before the pro-vitamin A carotenoids when no ascorbic acid was present. These observations show that tocols are more sensitive to auto-oxidation during hydrolysis. Similar findings were observed in the CALB-catalyzed reactions where tocols were found to degrade but not carotene in the absence of ascorbic acid even at low FFA production (see Fig. 2(c)). This result is in good agreement with previous studies which have mentioned that α-tocopherol can shield β-carotene from auto-oxidation.[27]

Aside from the difference in the carotene and tocols degradation, a detailed study on the degradation rate of each tocol isomer was also carried out. This was done by performing a kinetic study on the reaction using ET 2.0 lipase, 50 °C, 1:1 water-to-oil mass ratio and no ascorbic acid as this was the run which produced the highest FFA content. As shown in Fig. 3(b), α-tocopherol, α-tocotrienol and γ-tocotrienol degraded rapidly in the first 4 h, particularly in the first 2 h. The fast degradation was due to the rapid production of FFA at the beginning of the hydrolysis reaction.

By the eighth hour, the α-tocopherol isomer was completely degraded first before the other isomers. These results agree with Schauss et al.[28] who found that α-tocotrienol was the least stable tocotrienol isomer and most prone to degradation. Following that, the α-tocotrienol isomer was found to degrade completely between 12th and 16th hour (see Fig. 3(b)). However, γ-tocotrienol was found to degrade gradually over the 24 h of reaction. Interestingly, the retention δ-tocotrienol remained practically unchanged throughout the whole 24 h of reaction.

The reason to the difference in the stability and antioxidant activity of the tocol isomers can be explained by their structural difference.[29] Between the tocotrienol isomers, it is the methyl groups on the chromanol heads that sets them apart. α-tocotrienol is a trimethylated isomer with three methyl substitutes in C-5, C-7 and C-8. However, γ-tocotrienol has two methyl substitutes in C-7 and C-8, whist δ-tocotrienol is a monomethylated isomer with only one methyl substitute in C-8. A trimethylated tocol such as the α-tocopherol and α-tocotrienol are reported to be the most active among the various E-vitamins.[30] Due to that, the more active α-tocols are degraded first, and the monomethylated δ-tocotrienol is the most stable and least prone to degradation.
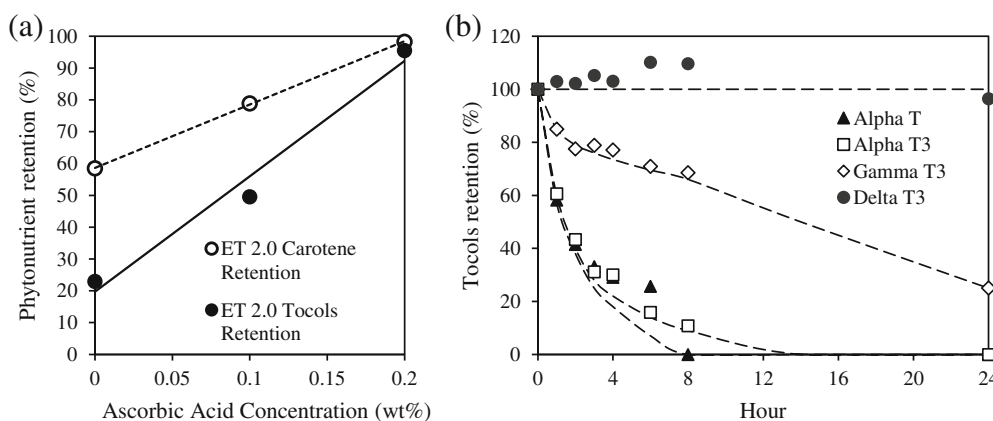
Figure 3. (a) Effect of ascorbic acid concentration on the retention of carotene and tocols for hydrolysis performed using ET 2.0. (b) Changes in tocol isomers concentration (in ppm) during hydrolysis using ET 2.0 without the addition of ascorbic acid. Alpha T, α-tocopherol; Alpha T3, α-tocotrienol; Gamma T3, γ-tocotrienol; Delta T3, δ-tocotrienol.

Overall, it can be concluded that ascorbic acid plays an important role in maintaining the phytonutrient level of the product during hydrolysis. Lietz and Henry[31] performed hydrolysis of red palm oil using *Candida cylindracea* lipase (100 g kg^{-1}) under nitrogen blanketing in the presence of a very small amount of ascorbic acid (0.05 g kg^{-1}). They reported 100% retention of carotenes after a short duration (4 h) of hydrolysis. However, the retention of tocols and the retention of carotenes without ascorbic acid addition were not reported in the study. In this work, it was found that both carotene and tocols could be effectively retained after 24 h of reaction without the need for nitrogen blanketing but with the use of higher ascorbic acid content (2 g kg^{-1}).

Effect of temperature
Enzymes are heat sensitive and each type of lipase have different heat tolerance and optimum temperature, typically ranging between 20 and 70 °C.[32] Additionally, phytonutrients are also heat-labile which could degrade due to exposure to high temperature. Thus, this study investigated the effect of temperature towards the enzymatic hydrolysis of CPO, as well as the retention of the phytonutrients. Figure 4(a) shows the FFA content in the oil phase after 24 h of enzymatic hydrolysis (using ET 2.0 and Habio) with a water-to-oil mass ratio of 1:1, 2 g kg^{-1} ascorbic acid and stirring speed of $9 \times g$. CALB was excluded for further investigation because the lipase did not perform well in hydrolysis.

As shown in Fig. 4(a) and as verified by the statistical *t*-test analysis, the FFA productions were approximately the same for the ET 2.0-catalyzed reactions conducted at 50 °C and 60 °C ($P = 8.6 \times 10^{-1}$). However, the FFA final content dropped significantly to 550 g kg^{-1} when the temperature was increased from 60 °C to 70 °C ($P = 2.7 \times 10^{-2}$), indicating that ET 2.0 could have denatured at this temperature. This shows that temperature of 70 °C and above reduces the lipase's activity, and this agrees with the lipase specification which reports that ET 2.0 begins to lose its effectivity at temperatures above 45 °C.[33] The optimum temperature for ET 2.0 is actually 40 °C, but temperatures lower than 50 °C was not investigated because the reaction mixture would start to solidify at a temperature below 47 °C, thus hindering the homogenization of the oil and water mixture.

However, the FFA production of reactions using Habio was found to decrease when the temperature was increased from 50 °C to 60 °C and then 70 °C ($P = 5.2 \times 10^{-3}$). These results show

that Habio lipase which was derived from *Aspergillus niger* was more heat sensitive compared to ET 2.0. In fact, previous studies have shown that the optimum temperature for oil hydrolysis using *Aspergillus niger* lipases was between 35 and 45 °C.[34,35]

With regards to carotene, its retention was found to decrease with increasing temperature for ET 2.0-catalyzed reactions (see Fig. 4(b)). The carotene content was found to drop from 98% to 72% when the reaction temperature was increased from 50 °C to 70 °C ($P < 0.05$). The results are consistent with the findings reported in the literature where higher temperature was found to cause higher degradation of carotene.[36] Specifically, Kha *et al.*[37] reported that carotene retained at 50 °C was twice the amount retained at 60 °C during the pre-treatment of Gac oil. However, in this study, higher temperature was not the sole cause for higher degradation of carotene. The combined effects of high FFA content and high temperature could have contributed to the higher degradation of carotene.

The degradation of carotene in the Habio-catalyzed reactions was significantly less than that of the ET 2.0-catalyzed reactions, even at a reaction temperature of 70 °C. At this temperature, it is worth noting that the FFA content in the reaction catalyzed by Habio was only 130 g kg^{-1}. This finding lends further support to our hypothesis that the presence of FFA played a key role in degrading the carotene.

Contrarily, based on one-way ANOVA statistical analysis, the increase in temperature resulted in no significant change in the tocols retention for both ET 2.0 and Habio-catalyzed reactions ($P > 0.05$). This result shows that the degradation of tocols was less affected by the temperature than that of the carotene. Therefore, the optimum temperature for the enzymatic hydrolysis of CPO with both lipases is 50 °C by taking into consideration both FFA production and the phytonutrients retention.

Effect of stirring speed
Oil and water are immiscible by nature, therefore efficient mixing could result in higher production of FFA during hydrolysis. Higher stirring speed could improve the hydrolysis of CPO as it would enhance the homogenization of the oil and water, increasing the interfacial area for reaction. However, the increased shear stress induced by high stirring speed could result in the unfolding of lipase and denaturation.[38]

The final FFA content, carotene retention and tocols retention after 24 h of enzymatic hydrolysis at 50 °C, water-to-oil mass ratio of 1:1 and ascorbic acid concentration of 2 g kg^{-1} with varying
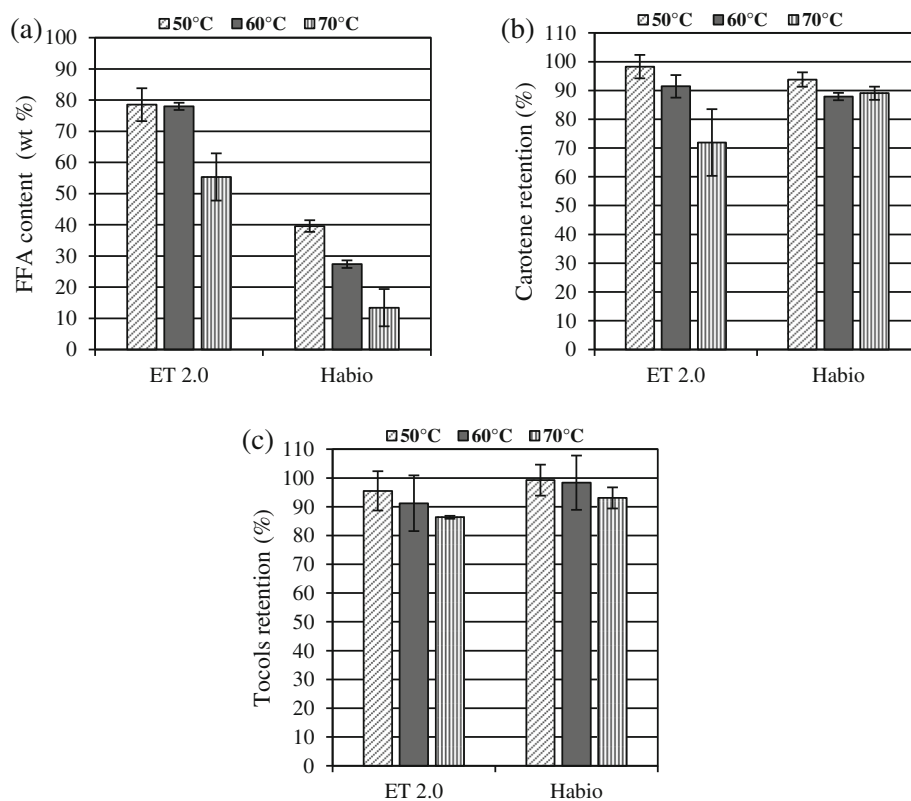
Figure 4. Effects of temperature on (a) FFA production, (b) carotene retention and (c) tocols retention. Error bar in the graph represents standard deviation of the mean value.
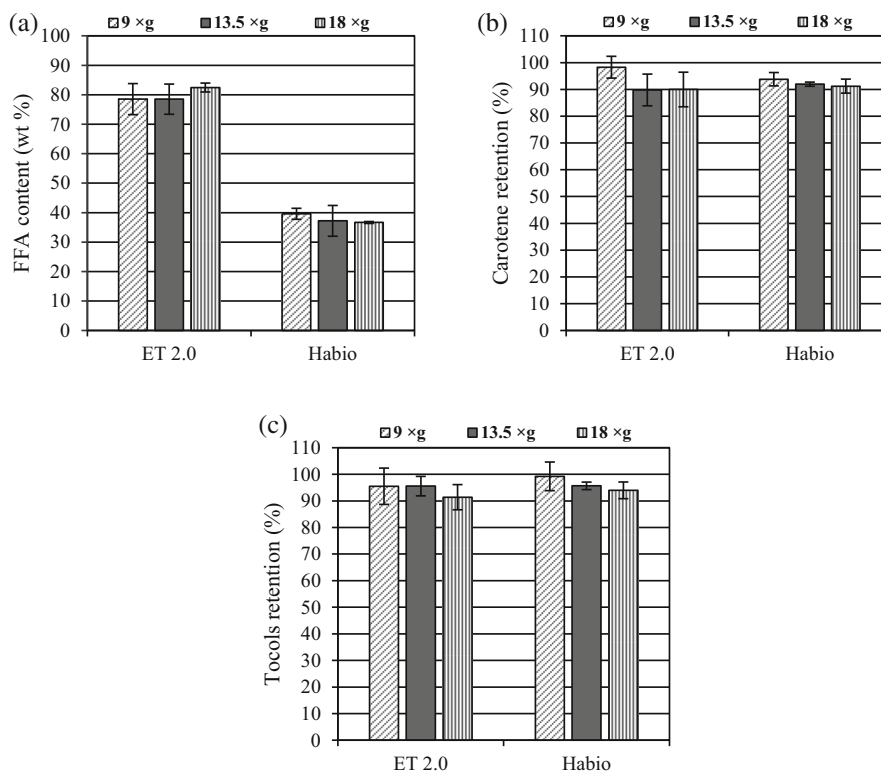

Figure 5. Effects of agitation speed (in × g) on (a) FFA production, (b) carotene retention and (c) tocols retention. Error bar in the graph represents standard deviation of the mean value.
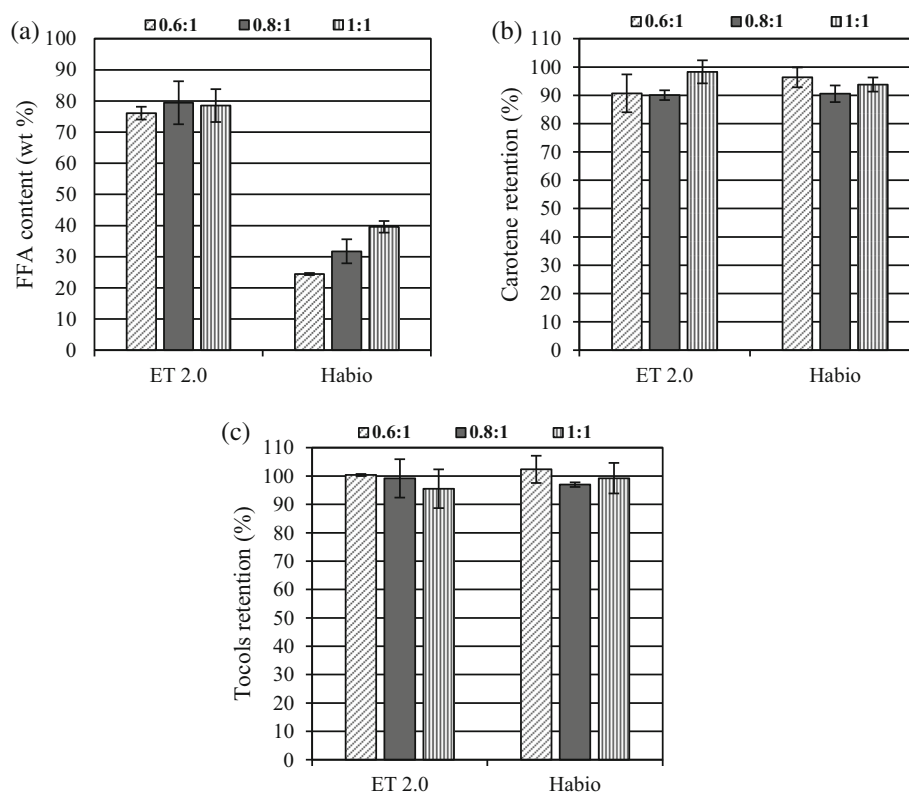
Figure 6. Effects of water-to-oil mass ratio on (a) FFA production, (b) carotene retention and (c) tocols retention. Error bar in the graph represents standard deviation of the mean value.

stirring speed (from $9 \times g$ to $18 \times g$) are shown in Fig. 5. From Fig. 5(a), it can be seen that there was no significant difference in the FFA production for both ET 2.0 and Habio as the stirring speed was increased from $9 \times g$ to $18 \times g$ ($P > 0.05$).

The results indicate that the reaction mixture was well mixed even at a low mixing speed of $9 \times g$ and there was no mass transfer limitation observed. However, increasing the mixing speed to $18 \times g$ did not reduce nor increase the production of FFA either. Similarly, varying the stirring speed did not cause significant difference in the retention of carotene and tocols for both lipases.

Effect of water-to-oil mass ratio

In enzymatic hydrolysis, a high excess amount of water is necessary to shift the equilibrium towards hydrolysis. An increase in water-to-oil mass ratio should theoretically drive the reaction equilibrium forward. However, excessive amount of water may also lead to excessive dilution of lipase and generation of a large amount of wastewater. The effects of water-to-oil mass ratio to the final FFA content, carotene retention and tocols retention after 24 h of reaction at 50 °C, 2 g kg^{-1} ascorbic acid and stirring speed of $9 \times g$ is shown in Fig. 6.

Varying the water-to-oil mass ratio from 0.6:1 to 1:1 for hydrolysis using ET 2.0 lipase did not affect the FFA production over the 24 h of reaction. It was discovered that this difference is not statistically significant, and the one-way ANOVA analysis resulted in a P-value of 6.8×10^{-1} ($P > 0.05$). As seen in Fig. 6(a), the values of final FFA content for hydrolysis reactions with water-to-oil mass ratio of 0.6:1 to 1:1 could vary between 760 and 790 g kg^{-1}. The trends observed shows that a water-to-oil mass ratio of 0.6:1 (equivalent to 28:1 molar ratio, 9.5-fold of the stoichiometric requirement) is already enough to shift the equilibrium for the processes using ET 2.0. Thus, the

use of this 0.6:1 water-to-oil mass ratio would be preferable for the minimization of materials (i.e. water) during the process.

Contrarily, the final FFA content in the oil phase increased significantly with increasing water-to-oil mass ratio for hydrolysis using Habio lipase ($P = 8.7 \times 10^{-3}$). Increasing the water-to-oil mass ratio further may potentially result in a higher production of FFA using Habio lipase. A similar trend to that of Habio in the presence of ascorbic acid is observed for enzymatic hydrolysis using lipase from *Candida rugosa*.[39] The study by You and Baharin[39] observed an increase in FFA production as the water-to-oil mass ratio was increased from 0.2:1 to 1:1 and an eventual decrease when the water-to-oil mass ratio is higher than 1:1.

With regards to phytonutrient retention, no significant changes were observed in both carotene and tocols retentions by varying the water-to-oil mass ratio for both lipases (see Fig. 6(b,c)). In this study, the ascorbic acid which was added to help preserve the phytonutrients is soluble in water.[40] The lack of change in phytonutrients preservation indicates that the amount of ascorbic acid added into the system (i.e. 2 g kg^{-1} of the CPO) is sufficient to protect the phytonutrients for water-to-oil mass ratio up to 1:1.

CONCLUSIONS

This study investigated the retention of carotene and tocols during enzymatic hydrolysis of CPO to FFAs. Lipase derived from *Aspergillus oryzae* (ET 2.0) was found to perform the best in catalyzing the hydrolysis of CPO, followed by the lipase derived from *Aspergillus niger* (Habio). Lipase derived from *Candida antartica* lipase B (CALB) was found to be inefficient in performing oil hydrolysis. The addition of a sacrificial antioxidant (i.e. ascorbic acid) significantly improved the retention of tocols and

carotenoids. Using ET 2.0, the highest FFA content attainable was 790 g kg^{-1} with 98% of the carotenoids and 96% of the tocols retained after 24 h of reaction using an ascorbic acid concentration of 2 g kg^{-1}, 1:1 water-to-oil mass ratio at 50 °C and 9 × g. This finding shows that it is feasible to develop a methanol-free process to produce palm phytonutrients via enzymatic hydrolysis, thus improving the overall process sustainability and safety.

ACKNOWLEDGEMENTS

CONFLICT OF INTEREST

The authors declare no competing interests.

REFERENCES

1 Loganathan R, Selvaduray KR, Radhakrishnan AK and Nesaretnam K, Palm oil: rich in health promoting phytonutrients. *Palm Oil Dev* **50**:16–25 (2009).

2 Malekbala MR, Soltani SM, Hosseini S, Babadi FE and Malekbala R, Current technologies in the extraction, enrichment and analytical detection of tocopherols and tocotrienols: a review. *Crit Rev Food Sci Nutr* **57**:2935–2942 (2017).

3 Ernstgård L, Shibata E and Johanson G, Uptake and disposition of inhaled methanol vapor in humans. *Toxicol Sci* **88**:30–38 (2005).

4 Stefanovich AF and Karel M, Kinetics of beta-carotene degradation at temperatures typical of air drying of foods. *J Food Process Preserv* **6**:227–242 (1982).

5 Leng CCS, Baharin BS, Che Man Y and Tan CP, Optimisation of enzymatic hydrolysis for concentration of squalene in palm fatty acid distillate. *J Sci Food Agric* **88**:1512–1517 (2008).

6 You LL, Baharin BS, Quek SY, Abdullah MA and Takagi S, Recovery of palm carotene from palm oil and hydrolyzed palm oil by adsorption column chromatography. *J Food Lipids* **9**:87–93 (2002).

7 Chu BS, Baharin BS, Quek SY and Man YBC, Separation of tocopherols and tocotrienols from palm fatty acid distillate using hydrolysis-neutralization-adsorption chromatography method. *J Food Lipids* **10**:141–152 (2003).

8 Chang MY, Chan E-S and Song CP, Biodiesel production catalysed by low-cost liquid enzyme Eversa® transform 2.0: effect of free fatty acid content on lipase methanol tolerance and kinetic model. *Fuel* **283**:119266 (2021).

9 Azeman NH, Yusof NA and Othman AI, Detection of free fatty acid in crude palm oil. *Asian J Chem* **27**:1569–1573 (2015).

10 Bahadi MA, Japir A-W, Salih N and Salimon J, Free fatty acids separation from Malaysian high free fatty acid crude palm oil using molecular distillation. *Malaysian J Anal Sci* **20**:1042–1051 (2016).

11 Nagendran B, Unnithan UR, Choo YM and Sundram K, Characteristics of red palm oil, a carotene- and vitamin E–rich refined oil for food uses. *Food Nutr Bull* **21**:189–194 (2000).

12 Mohd Top AG, Content of vitamin E in palm oil and its antioxidant activity. *Palm Oil Dev* **12**:25–27 (1990).

13 Novozymes, *Immobilized Lipases for Biocatalysis*. Novozymes A/S, Denmark (2016).

14 Ribeiro BD, Coelho MAZ and Barreto DW, Production of concentrated natural beta-carotene from Buriti (*Mauritia vinifera*) oil by enzymatic hydrolysis. *Food Bioprod Process* **90**:141–147 (2012).

15 Bresolin D, Hawerroth B, de Oliveira RC, Sayer C, de Araújo PHH and de Oliveira D, Immobilization of lipase Eversa Transform 2.0 on poly(-urea–urethane) nanoparticles obtained using a biopolyol from enzymatic glycerolysis. *Bioprocess Biosyst Eng* **43**:1279–1286 (2020).

16 Choe E and Min DB, Mechanisms of antioxidants in the oxidation of foods. *Compr Rev Food Sci Food Saf* **8**:345–358 (2009).

17 Taghvaei M and Jafari SM, Application and stability of natural antioxidants in edible oils in order to substitute synthetic additives. *J Food Sci Technol* **52**:1272–1282 (2015).

18 Rizwanul Fattah IM, Masjuki HH, Kalam MA, Hazrat MA, Masum BM, Imtenan S *et al.*, Effect of antioxidants on oxidation stability of biodiesel derived from vegetable and animal based feedstocks. *Renewable Sustainable Energy Rev* **30**:356–370 (2014).

19 Al-Malaika S, Reactive antioxidants for polymers, in *Reactive Modifier for Polymers*, ed. by Al-Malaika S. Springer, Dordrecht, pp. 266–302 (1997).

20 Pehlivan FE, Vitamin C, An antioxidant agent, in *Vitamin C*, ed. by Hamza AH. IntechOpen, Rijeka, pp. 23–36 (2017).

21 Noon J, Mills TB and Norton IT, The use of natural antioxidants to combat lipid oxidation in O/W emulsions. *J Food Eng* **281**:110006 (2020).

22 Varatharajan K and Pushparani DS, Screening of antioxidant additives for biodiesel fuels. *Renewable Sustainable Energy Rev* **82**:2017–2028 (2018).

23 Kaźmierczak-Barańska J, Boguszewska K, Adamus-Grabicka A and Karwowski BT, Two faces of vitamin C—antioxidative and pro-oxidative agent. *Nutrients* **12**:1501 (2020).

24 Martínez-Sanchez JA, Arana-Peña S, Carballares D, Yates M, Otero C and Fernandez-Lafuente R, Immobilized biocatalysts of Eversa® transform 2.0 and lipase from *Thermomyces lanuginosus*: comparison of some properties and performance in biodiesel production. *Catalysts* **10**:738 (2020).

25 Mosavi-Movahedi AA, Wilkinson AE and Jones MN, Characterization of *Aspergillus niger* catalase. *Int J Biol Macromol* **9**:327–332 (1987).

26 Konermann L, Protein unfolding and denaturants, in *eLS*. John Wiley & Sons Ltd, Chichester (2012).

27 Liu C, Russell RM and Wang X-D, α-Tocopherol and ascorbic acid decrease the production of β-Apo-carotenals and increase the formation of retinoids from β-carotene in the lung tissues of cigarette smoke–exposed ferrets in vitro. *J Nutr* **134**:426–430 (2004).

28 Schauss AG, Endres JR and Clewell A, Safety of unsaturated vitamin E tocotrienols and their isomers, in *Tocotrienols*, 2nd edn, ed. by Tan B, Watson RR and Preedy VR. CRC Press, Boca Raton, pp. 17–36 (2012).

29 Shahidi F and de Camargo AC, Tocopherols and tocotrienols in common and emerging dietary sources: occurrence, applications, and health benefits. *Int J Mol Sci* **17**:1745 (2016).

30 Woollard DC and Indyk HE, TOCOPHEROLS | Properties and determination, in *Encyclopedia of Food Sciences and Nutrition*, 2nd edn, ed. by Caballero B. Academic Press, Oxford, pp. 5789–5796 (2003).

31 Lietz G and Henry CJK, A modified method to minimise losses of carotenoids and tocopherols during HPLC analysis of red palm oil. *Food Chem* **60**:109–117 (1997).

32 Loh JM, Amelia, Gourich W, Chew CL, Song CP and Chan E-S, Improved biodiesel production from sludge palm oil catalyzed by a low-cost liquid lipase under low-input process conditions. *Renewable Energy* **177**:348–358 (2021).

33 Novozymes, *The Novozymes Enzymatic Biodiesel Handbook*. Novozymes A/S, Denmark (2016).

34 Carvalho PO, Campos PRB, Noffs MD, Fregolente PBL and Fregolente LV, Enzymatic hydrolysis of salmon oil by native lipases: optimization of process parameters. *J Braz Chem Soc* **20**:117–124 (2009).

35 Qiao H, Zhang F, Guan W, Zuo J and Feng D, Optimisation of combi-lipases from *aspergillus Niger* for the synergistic and efficient hydrolysis of soybean oil. *Anim Sci J* **88**:772–780 (2017).

36 Shehu UE, Chow TQ, Hafid HS, Mokhtar MN, Baharuddin AS and Nawi NM, Kinetics of thermal hydrolysis of crude palm oil with mass and heat transfer in a closed system. *Food Bioprod Process* **118**:187–197 (2019).

37 Kha TC, Phan-Tai H and Nguyen MH, Effects of pre-treatments on the yield and carotenoid content of Gac oil using supercritical carbon dioxide extraction. *J Food Eng* **120**:44–49 (2014).

38 Goswami D, Basu JK and De S, Optimization of process variables in castor oil hydrolysis by *Candida rugosa* lipase with buffer as dispersion medium. *Biotechnol Bioprocess Eng* **14**:220–224 (2005).

39 You LL and Baharin BS, Effects of enzymatic hydrolysis on crude palm olein by lipase from Candida rugosa. *J Food Lipids* **13**:73–87 (2006).

40 Yuan J-P and Chen F, Degradation of ascorbic acid in aqueous solution. *J Agric Food Chem* **46**:5078–5082 (1998).