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Investigation on enzymatic activity of rubber seed as source of plant lipase

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Abstract

Rubber seed is a non-edible seed that is abundantly available and considers agricultural wastes. A potential lipase from rubber seed was examined based on the enzymatic activity and its application in the hydrolysis reaction. The enzymatic activity characterization study was determined based on p-nitrophenol release in the hydrolysis reaction. The initial evaluation showed that temperature and pH significantly influence the reaction. The optimum condition based on enzymatic activity for rubber seed was found at 30 °C and pH 8. The rubber seed lipase extract was then used in enzymatic hydrolysis reactions of rubber seed oil, palm oil, and canola oil. The highest FFA percentage of 63% was found from the rubber seed oil. The results indicate that rubber seed extract has shown its potential enzymatic activity. However, further studies need to be done to apply this rubber seed in various lipase catalysed reactions.

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1.0 Introduction

Lipase is a hydrolase enzyme that provides an interface for substrates and it acts by hydrolysing the ester-carboxylate bonds that result in the production of organic alcohols and fatty acids (Wrolstad et al., 2005). Lipases can hydrolyse the long-chain TAGs as it can remain dissolved in oil water interface as most fats and oils of natural occurrence are triacylglycerols (TAGs) of long chain fatty acid that is known to have low solubility in water under natural conditions and (Rajendran et al., 2009). Seed lipases are generally more active with TAGs substrate with short-chain fatty acids (Barros et al., 2010).

The current major commercial lipase is microbial lipases are considered to be uneconomical as it has a high production cost (Hasan, et al., 2009). Plant lipases are being studied as an alternative lipase because plant lipase requires a lower cost to produce and higher specificity towards substrate during reactions example compared to other types of lipase. Studies by Seth et al. (2014) had shown that unpurified plant lipase can synthesizes and catalyse several reactions of lipids directly. In a different study by Barros et al. (2010) where castor bean lipase was used to hydrolyse pine seed oil and palm oil, a promising result was observed with fatty acids being produced (Barros et al., 2010). Although there were many studies conducted for the ability of plant lipase specifically the seed lipase, the study for rubber seeds lipase is still scarce. Rubber seeds have huge potential as alternative source of lipase and based on the study conducted by Njoku (1996) lipase activity was detected in the rubber seeds. Rubber seeds from the rubber tree (*Hevea brasiliensis*) are abundant in Malaysia. The abundant rubber seeds usually remain underused, as it is primarily used as rubber plantations planting material, while the unused rubber seeds ended up as agricultural waste. Hence, this increases the availability of rubber seeds to be used as a source of plant lipase.

The composition of the fatty acids in the oil can be observed from Table 1 (Ramadhas et al., 2013).There are two important parameters in the enzymatic activity, which are temperature and pH. The temperature has clear impact on the catalytic activity of plant lipases and is one of the elements that have to be considered to determine the enzymatic activity in the reaction that takes place (Avramiuc, 2016;Senrayan & Venkatachalam, 2018). Optimum temperature for various plant lipases used were in biosynthesis ranges between 30 and 55 °C. Reaction temperature higher than lipase physiological activity temperature will likely lead to a decrease in the synthesis yield

Table 1: Composition of fatty acids in the vegetable oil (Rajendran et al., 2009).

Fatty acid	Composition (wt. %)		
	Rubber seed	Canola	Palm
Palmitic C16:0	10.2	4.70	44
Palmitoleic C16:1	-	0.14	-
Stearic C18:0	8.7	1.65	4
Oleic C18:1	24.6	66.0	39
Linoleic C18:2	39.6	21.2	12
Linolenic C18:3	16.3	5.20	-
Arachidonic C20:0	-	0.90	-
Average molecular mass (g mol^{-1})	276.83	280.28	268.01

equilibrium and the inactivation of lipase. Wilfried et al. (2013) stated that the thermostability of seed lipase is high, due to the location of the lipase in the lipid bodies of the seeds as the lipid bodies contain a high level of TAGs and low moisture level (Wilfried et al., 2013). The low moisture level reduces the potential of thermal denaturation as Turner et al. (200) state that the quantity of water associated with the protein highly influenced the protein thermal denaturation temperature.

Meanwhile, pH is a factor that has a marked impact on lipase activity in aqueous or organic media. It is responsible for the ionization state of a lipase. A study by Florkin & Stotz (1965) showed that lipase at pH 9 and temperature 37 °C with 1 mg of purified lipase and olive oil as the substrate has very high hydrolytic activity. The reaction can hydrolyse 12 moles of triolein per min. Meanwhile, the optimal range of lipase enzymatic activity is between pH of 6.0 and 8.0, and temperature between 30 °C and 40 °C (Wilfried et al., 2013).

There are few methods in determining the enzymatic activities, specifically lipase, among the common methods are titrimetric method and colorimetric method. In titrimetric method, the reaction of lipase can be detected by measuring the neutralized free fatty acids (FFAs) released, using a titration with triglyceride with sodium hydroxide solution. This quantitative enzyme activity method is defined as one μmol of FFAs release per min (Hasan et al., 2009). The colorimetric method involves analysing the solution containing substrate and lipase spectrophotometrically. In this method, p-nitrophenyl acyl ester can be used as the substrate to determine the lipase activity. The hydrolysis of p-nitrophenyl acyl ester will produce alcohol that can be monitor spectrophotometrically, from the colour change of the p-nitrophenol solutions at 405 nm or 410 nm. The enzyme activity for the hydrolysis of p-nitrophenyl can be expressed as μmoles of p-nitrophenol released per minute (Hasan et al., 2009).

In this paper, we reported study on enzymatic activity of rubber seed to assess its potential as source of lipase. The enzymatic activity of rubber seed was investigated at various pH and temperatures. The optimum pH and temperature were then used to study the rubber seed lipase ability to hydrolase various vegetable oil, such as rubber seed oil and canola oil. This study is expected to contribute to alternative sources of plant lipase, specifically from agricultural waste.

2.0 Methodology

2.1 Material

The rubber seeds were collected from Batu Anam Sime Darby Rubber Plantation, Segamat, Johor. The endosperm of the rubber seed was used for the study while the shell of the rubber seed was discarded. canola oil was obtained from local market. The rubber Seed oil used was extracted manually through solvent extraction using n-hexane as the solvent. Reagents used in the hydrolysis of the oil were 0.1 M Tris-HCl buffer, ethanol, and phenolphthalein.

2.2 Methods

2.2.1. Preparation and pre-treatment of rubber seed lipase

The endosperms were separated from the shell of the rubber seeds. The removed shells were discarded. 20 grams of the endosperms were grind using domestic food blender for 10 minutes until fine powder is obtained as shown in Fig. 1.

Acetone was added to the powder with solvent ratio of 1:5 (w/v) for the defatting process to pre-treat the rubber seed powder. The defatting process removed the rubber seed oil at atmospheric pressure and room temperature (Isbilir et al., 2008). The mixture was kept under gentle mechanical stirring at 150 rpm at 4 °C for 15 minutes. Buchner funnel was used to vacuum filter the mixture with excess acetone (Santos et al., 2013). The filtered product was sieved to obtain product with particle size of 750 μm to gain rubber seed powder with higher surface area. The defatted seed powder was stirred with eight times its weight of phosphate buffer (0.1 M and pH 7) at 4 °C for 30 minutes. The mixture

Fig 1: Fine rubber seed endosperm

Fig 2: Fine rubber seed endosperm after mixing with 0.1 M Phosphate Buffer.

was centrifuged for 30 minutes at 5,000 rpm at 4 °C (Yesologlu & Baskurt, 2008). The supernatant was collected as shown in Fig. 2, and used as rubber seed lipase extract for enzymatic activity and hydrolysis.

2.2.2. Analysis of rubber seed enzymatic activity

Standard curve of p-nitrophenol was plotted with p-nitrophenol volume from 0.05 mL to 0.50 mL. Each of the varied volumes was diluted with 5 mL of 0.1M Tris-HCl buffer, pH 8.2. The absorbance value of the mixtures was observed at 410 nm with 0.1M Tris-HCl buffer, pH 8.2 as the buffer. The standard curve produced was used to convert rubber seed lipase samples absorbance values to enzymatic activity, (Wrolstad et al., 2005).

The analysis of enzymatic activity of rubber seed lipase was conducted by mixing 2.5 mL of 0.1 M Tris-HCl buffer pH 8.2 with 2.5 mL of 420 µM p-nitrophenyl acetate solution in test tubes. 1 mL was added to one of the test tubes as blank and 1 mL of rubber seed lipase was added to the test tube to initiate the enzymatic reaction. The test tubes were placed in water bath at temperature 30 °C, 150 rpm for 15 minutes. 6 mL of acetone (v/v) was added to the test tube after 15 minutes to terminate the reaction (Santos et al., 2013; Wrolstad et al., 2005). The absorbance value of the sample at 410nm was obtained using UV-Vis. Standard curve of p-nitrophenol was used to convert the absorbance value to the enzymatic activity value of rubber seed lipase as suggested by Wrolstad et al.

2.2.3 Hydrolysis of various vegetable oil using rubber seed lipase extract

The hydrolysis reactions of vegetable oil (rubber seed oil, canola oil and cooking palm oil) were carried out in 250 mL conical flasks containing 8 g of vegetable oils mix with 0.1 M Tris-HCl buffer with pH 8.2, based on the stoichiometric ratio and the addition of 1.5% of rubber seed lipase from the total mass of the mixture. The mixture was agitated in water bath at 30 °C for 6 hours at 150 rpm. The reaction was conducted for 6 hours in order to find the optimum time for the enzymatic hydrolysis reaction. One gram of sample was collected with 1hour interval. The sample was mix with 10 mL 2-propanol to denature the enzyme hence stopping the enzymatic reaction. Few drops of phenolphthalein indicator were added to sample mixture and the samples were titrated against 0.1 M sodium hydroxide solution. The titration was conducted to analyse the percentage of free fatty acids (FFA), which is expressed as percentage of oleic acid. Eq. (1) was used to determine the percentage of FFA (Santos et al., 2013):

$$FFA(\%) = \frac{V_{NaOH} \times 10^{-3} M_{NaOH} \times MM_{Oleic\ Acid}}{W_t} \times 100 \quad (1)$$

where V is the volume of 0.1 M sodium hydroxide solution (NaOH) used during titration, M is the molar concentration of NaOH used, MM is the molecular weight of oleic acid and W_t is the weight of sample taken.

3.0 Results and discussion

3.1 The effect of different reaction temperature on rubber seed enzymatic activity

The enzymatic activity of the rubber seed lipase was studied and determined from the method suggested by

Wroldstad et al. (2005) with slight modification by using UV-Vis (Agilent Technologies Cary 60 UV-Vis). The amount of p-nitrophenol released from the enzymatic reaction was analyzed from the absorbance value at 410 nm. The different colours of p-nitrophenyl acetate solution can be observed based on Fig. 3. The colour difference indicates that p-nitrophenol has been released after the reaction.

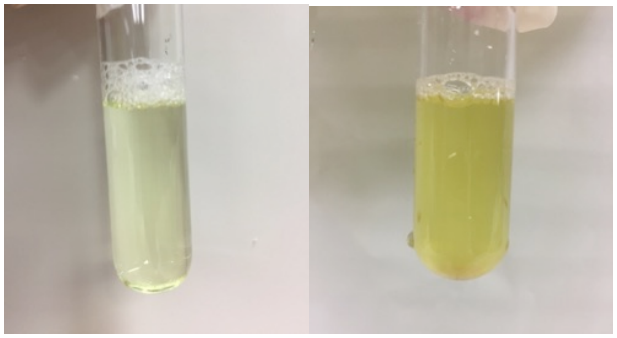

Fig. 3 Left: Initial colour of p-nitrophenyl acetate. Right: Final colour of p-nitrophenyl acetate solution reaction with rubber seed lipase.

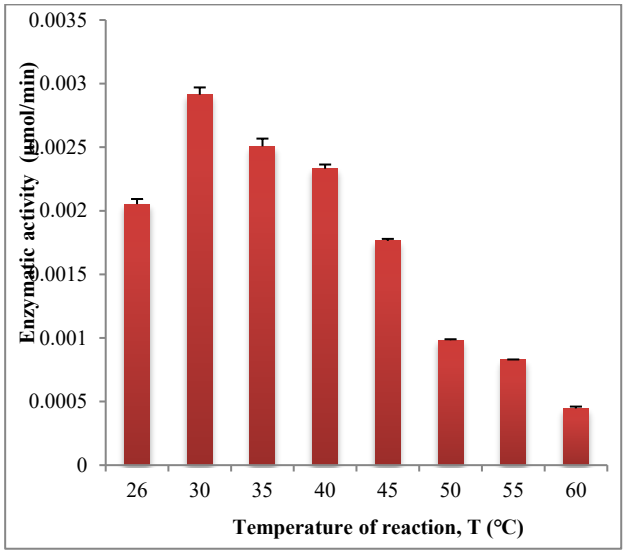

Fig. 4: Enzymatic activity at various temperatures based on the amount of p nitrophenol released

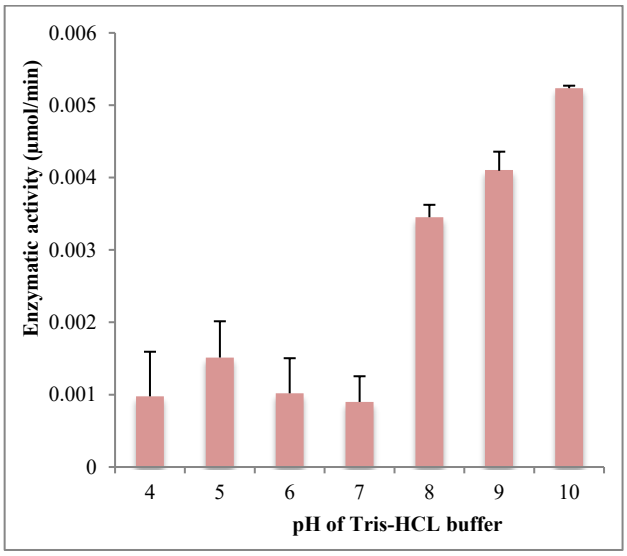

Fig. 5: The amount of p-nitrophenol released for various pH

The enzymatic activity at different temperatures were performed at temperature from 10 °C to 60 °C , with 5 °C intervals for 15 minutes each. Rubber seed lipase exhibits optimum activity at temperature 30 °C and it starts to inactivate drastically from temperature 45 °C upwards. The much lower amount of p-nitrophenol released at high temperatures can cause the denaturation of the enzyme as it is above the typical lipase physiological activity temperature (Florkin & Stotz, 1965).

Based on Fig. 4, it can be observed that temperature of the reaction influences rubber seed enzymatic activity. At the optimum temperature that is 30 °C , the enzymatic activity will be at the highest point meanwhile as temperature starts to increase, the enzymatic activity decreases due to the enzyme in the system starts to become dormant or denatured.

3.2 The effect of reaction pH on rubber seed enzymatic activity

The effect of pH on the enzymatic activity of rubber seed lipase was also studied by ranging the pH of the 0.1 M Tris-HCl buffer from pH 4 to pH 10 at the optimum temperature of 30 °C. The pH was varied by adding 0.1 M sodium hydroxide (NaOH) solution to the buffer to produce alkaline buffer and 0.1 M hydrochloric acid (HCl) to produce acidic buffer (Wrolstad et al., 2005). The pH of the buffer was tested using Mettler Toledo pH meter.

Based on Fig. 5, it can be observed that the optimum pH for rubber seed lipase is pH 8 as the enzymatic activity of rubber seed lipase increase starting from pH 8 onwards. Study conducted Yesologlu et al. (2008) also verified that at pH 8, optimum enzymatic activity was detected for almond seed lipase. High pH for the enzymatic reaction indicates that the rubber seed lipase is an alkaline lipase. At low pH, relatively low enzymatic activity was observed. Low pH may lead to the denaturation of the enzyme proteins making it unable for the enzymes to convert p-nitrophenyl acetate to p-nitrophenol.

3.3 Hydrolysis various vegetable oils using rubber seed lipase

Generally, the effectiveness of an enzyme is dependent both on the fat-splitting action and the ability to promote emulsion between the two phases,

which is the oil aqueous phase in the hydrolysis reaction. Based on stoichiometric ratio, one mole of triglyceride requires three moles of water (oil to water molar ratio = 1:3) to produce three moles of (free) fatty acids and one mole of glycerol as shown in Eq. 3.

$$Triglycerides + 3H_2O \leftrightarrow 3Fatty\ Acids + Glycerol \quad (3)$$

The ratio will influence the ability and effectiveness of an enzyme to hydrolyse the reaction. Increasing or decreasing the ratio will either reduce or increase the hydrolysis rate. Furthermore, excess water would drive the equilibrium towards the end products – free fatty acids and glycerol. In this reaction, rubber seed lipases will hydrolyse the ester bonds of storage triacylglycerols based on the stoichiometric ratio (Mazaheri et al., 2019). Hydrolysis was performed in a stirred-tank reactor for 6 hours using rubber seed oil in order to evaluate the optimum time for maximum hydrolysis of the oil. The hydrolysis reaction was conducted at optimum of temperature of 30 °C and pH 8.2. For this study, oleic acid was chosen as the indicator for fatty acid formation as it is the major fatty acid in most vegetable oil as observed on Table 1. Based on Fig. 6, it is found that the percentage of fatty acid is decreasing with increasing of reaction time. However, the sharp declining was until 2 hours of reaction with maximum of 58.24% of fatty acid (oleic acid) released. After 2 hours, the hydrolysis of rubber seed oil reaction remains constant showing neither significant increment nor reduction for the remaining 5 hours of the reaction as observed on Fig. 6.

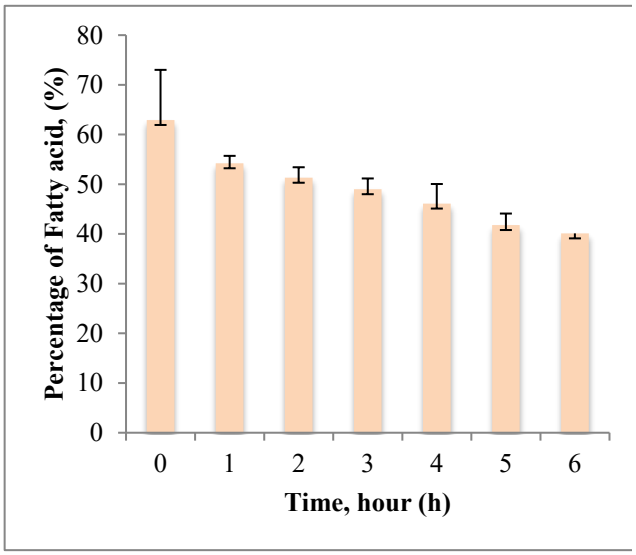

Fig. 6: The percentage of oleic acid (%) released from the hydrolysis of rubber seed oil.

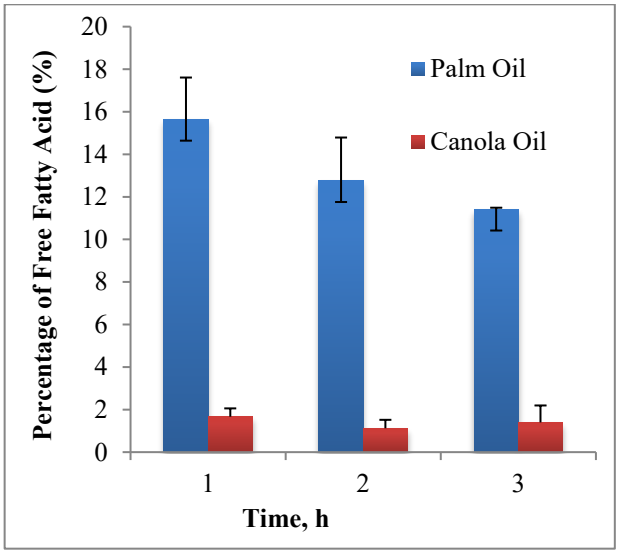

Fig. 7: The percentage of oleic acid (%) released from the hydrolysis

As comparison, hydrolysis reactions were also carried out with canola and palm oil using rubber seed extract as lipase. Similar trends were also observed, where free fatty acid was high initially and reduced with the time. As hydrolysis is reversible reaction, this is most probably the cause. The fatty acid was reversed when the reaction time is extended, and the reaction was carried out at its stoichiometric reaction. The palm oil and canola oil have relatively low free fatty acid which are 15% and 1.6 %, respectively, compared to rubber seed oil This is because each oil is commercial cooking vegetable oil

Fig. 7 shows that reducing free fatty acid was more significant in palm oil compare to canola oil, This may be due to the substrate specificity between rubber seed lipase and palm oil (Santos et al., 2013)that is used for domestic.

4.0 Conclusions

Enzymatic activity of rubber seed has been investigated in the hydrolysis of p-nitrophenyl. The result indicates that the optimum temperature and pH for rubber seed lipase activity are relatively moderate Further application of rubber seed lipase extract in hydrolysis of various vegetable oil such as rubber seed oil, palm oil, and canola oil, showing a possible reverse of fatty acid when conducted at its stoichiometric reaction. It is found that the highest free fatty acid was from rubber seed oil. The results from these studies showing rubber seed enzymatic activity. However, further studies and application of the rubber seed extract in various lipase catalysed reactions are suggested.

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