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The Activity of Lipase From Jatropha Seed (*Jatropha Curcas* L.) And Its Application On Hydrolysis of Castor Oil In Organic Solvent

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Abstract

Jatropha curcas L. is a seed plant which has lipase activity and widely used in hydrolysis processes and chemical synthesis. This study aims to determine the lipase activity that reacts with castor oil in organic solvents. In this research, organic solvents, especially hydrophobic solvents, were used in the hydrolysis reaction to increase the enzyme catalytic activity. The organic solvent used has a hydrophobicity level between 2 and 4, namely hexane. The research stages consisted of lipase isolation, lipase assay, the effect of adding metal ions assay, and analysis of castor oil composition. The results showed that jatropha seeds had lipase activity in crude of 0.603 U/mL and increased to 0.911 U/mL after addition of K^+. The result of hydrolysis and trans-esterification of castor oil by lipase are ricinoleic acid (4.58%) and methyl ricinoleic (11.67%), and the concentration of ricinoleic acid (17.09%) and methyl ricinoleic (60.83%) were increased after addition of K^+. The esterification reaction produces alkyl ester compounds such as methyl palmitate, ethyl pentadecanoate, methyl linoleate, methyl oleate, and methyl stearate. The lipase isolated from jatropha seeds are not only catalyzes the hydrolysis reaction but also catalyzes chemical synthesis reactions such as esterification and trans-esterification.

Keywords: *lipase enzyme, Jatropha seeds, isolation, biocatalyst, hexane.*

INTRODUCTION

Lipase is an enzyme that has an essential role in modern biotechnology as a biocatalyst and has high activity in hydrolysis reactions and chemical syntheses such as esterification, inter-esterification, alcoholysis, and acidolysis [1]. Lipases are classified as hydrolase and esterase enzymes that can break down a triglyceride in hydrophobic solvents. The utilization of organic solvents, especially hydrophobic solvents, can increase the catalytic activity of enzymes so that the resulting product has high efficiency [2]. According to Masyittah and Thahjono (2017), many reactions catalyzed by lipase enzymes can take place well in organic solvents, especially those involving water-insoluble substrates or reaction products such as triglycerides [3]. Organic solvents open the lipase enzyme 'lid' due to the activation of the interface with different efficiencies between solvent types. Moreover, solvent utilization needs to be evaluated case-by-case, especially when different lipase sources or substrates are used [4].

Recently, the process of developing enzyme production utilizes seed plants. Jatropha seed, which has the Latin name *Jatropha curcas* L., is a plant with many benefits, one of which is a source of lipase enzymes. The seed kernel is the part of the jatropha seed that can potentially have lipase activity. The kernel has a high-fat content, which can be hydrolyzed by lipase. The advantages of lipase enzymes from jatropha seeds are it has high specificity, low production costs, abundant availability in nature, easy purification, and environmentally friendly. In addition, lipases in grains have a high affinity for the dominant fatty acids in these seeds [5]. The hydrolytic activity of lipase enzymes from *Jatropha curcas* L. has been reported by Abigor et al. (2002) on palm oil substrate, de Sousa et al. (2010) on soybean oil substrate, and Avelar et al. (2013) on canola oil substrate [2]. The type of substrate is very influential on lipase activity. Lipases are enzymes that work specifically on specific substrates. According to the induced-fit theory, the lipase enzyme model has an active site that is flexible to work specifically on suitable substrates. It means that the enzyme and substrate have similarities to form an enzyme-substrate combination [6]. In the following study, castor oil from the plant *Ricinus communis* L. was used as a substrate, which is closely related to the *Jatropha curcas* L. plant seed. It is expected that good interaction between the lipase enzyme and the substrate to obtain the desired product are occurred.

Lipase activity in a seed proves the ability of an enzyme to catalyze the decomposition of fat triglycerides. Lipase activity that acts on a substrate can be affected by adding metal ions. Metal ions are one of the cofactors needed by enzymes in catalytic processes. These cofactors can act as activators that can increase enzyme activity and as inhibitors that can inhibit enzyme work. Previous research has been reported by Khairunnisa et al. (2021) on camped seed sprouts, in which K^+ and Mg^{2+} were act as activators that can increase lipase activity to 101%, 680%, and 131.09% [7]. Sui et al. (2012)

research on coconut endosperm using addition of Na^+ as an inhibitor work to 64% [8]. Another study by Yesiloglu and Baskurt (2013) explained that the Mg^{2+} could strongly inhibit the lipase activity of almond seeds [9].

Based on the description above, previous studies only reported that a seed plant has the potential as a source of lipase, characterized by the presence of lipase hydrolysis activity and several optimum conditions that can affect lipase enzyme activity. These conditions include the effect of pH, temperature, incubation time, type of substrate, the addition of activator-inhibitor ions, and addition of solvents. However, in previous studies, the analysis of the products produced by lipases when reacted with a substrate has not been carried out nor analyzed. Therefore, this current research decided to conduct research to determine the lipase activity isolated from Jatropha seed (*Jatropha curcas* L.) in decomposing triglycerides from a substrate that is closely related to the enzyme source, namely castor oil, in n-hexane solvent, and analysis of the compound using the Gas Chromatography-Mass Spectroscopy (GC-MS) (Shimadzu QP-2010S) instrument.

MATERIALS AND METHODS

Materials and Instrumentation

Material used were Jatropha seeds *(Jatropha curcas* L.), *castor oil* (*Ricinus communis* L.) (Darjeeling), ethanol 96% (technical), acetone (technical), sodium hydroxide (Sigma Aldrich), di-sodium hydrogen phosphate (Merck), sodium di-hydrogen phosphate (Merck), phenolphthalein indicator (Merck), sodium sulfate anhydrous (Merck), n-Hexane (technical), n-Hexane (Emsure®), sulfuric acid (Merck), sodium chloride (Merck), potassium chloride (Merck), magnesium chloride (Merck), diethyl ether (Merck), tri-chloro acetate (Merck).

Laboratory instrumentations used were cooling centrifugator (Tommy MX-307), waterbath shaker (Memmert), freezer –20 °C (Thermo Scientific), pH-meter (Walk LAB), gas chromatography - mass spectroscopy (GC-MS) (Shimadzu QP-2010S).

Method

Isolation of Crude Lipase

Isolation of crude extract of Lipase was done using a modified method developed by Abigor et al. (2002) [2]. A 25 g of jatropha seeds were added with 100 mL of 5 mM phosphate buffer pH 7, then the mixture was homogenized and centrifuged at 10,000 rpm at 4˚C for 30 minutes. The results of homogenization and centrifugation were extracted again. The supernatant that was obtained is a crude extract of the lipase enzyme.

Lipase assay

The assay was done using a modified method developed by Monnet et al. (2012) [10]. The activity assay was carried out by mixing 5 g *castor oil,* 2.5 mL n-hexane, 5 mL phosphate buffer 100 mM pH 7.5-, and 1-mL crude extract. Next, the mixture was incubated at 37° C for 45 minutes in a water bath shaker. After the incubation, the mixture was added with 25 mL of acetone-ethanol (1:1, v/v) and four drops of phenolphthalein indicator and then titrated with 0.01 M NaOH. Lipase activity was calculated using the following equation:

$$\text{Activity (U mL}^{-1}) = \frac{(A-B)[NaOH]1000}{45}$$

Note: A = sample volume, B = blank volume, 1000 = mmol to mol conversion value, and 45 is reaction time (minutes).

The Effect of Metal Ions-Addition

Determination on the effect of metal ions addition was carried out the same way as the lipase assay method, as developed by Monnet et al. (2012) [10]. The difference between the processing steps and the activity test is addition of chloride salt solutions, such as KCl, NaCl, and $MgCl_2$ with a concentration of 10 mM each of 1 mL. The lipase activity after the addition of metal ions was calculated using the following equation:

$$A = \frac{A_o}{A_c}\ x\ 100\%$$

Note: A_o = Sample activity, A_c = Control activity.

Analysis Compound of Castor Oil Substrate

The hydrolysis reaction of castor oil refers to a modified study conducted by Mulyani et al. (2018) [11]. In this study, a variation of enzyme: substrate (v/w) was used. Furthermore, each sample was added with 40 mL of n-hexane solvent, 0.1 M phosphate buffer pH 7.5, and then the mixture was incubated for 48 hours. Each sample was centrifuged to separate the enzyme phase and the oil phase. The formed oil phase was inactivated with 3 mL of 5% TCA solution at high temperature and rapid heating. The product was then analyzed with the GC-MS instrument (Shimadzu QP-2010S). The instrument GC-MS condition for analysis were: stationary phase: 100% dimethyl polysiloxane; column type: TG-WaxMS; column length: 30 m; column temperature: 40-250 °C; column flow: 0.7 mL/min; injection temperature: 250 °C; pressure: 24.9 kPa; linear speed: 30.2 cm/sec.

RESULTS AND DISCUSSION

Lipase Activity

Lipase activity was measure by titrimetric method with triple repetition. In this study, crude Lipase was reacted with castor oil as a substrate. Castor oil is hydrolyzed in the form of fatty acids, then the fatty acids were calculated based on the titration volume. The more free-fatty acids produced, the greater the volume of NaOH required for the titration. The more free-fatty acids indicate that the enzyme activity will be higher [1].

Lipase activity is expressed in units (U). One enzyme unit can be defined as the amount of enzyme needed to produce 1 mol of free fatty acids per minute (U = µmol/min) at its optimum condition. In this study, the lipase activity acting on the castor oil substrate was 0.603 U/mL. The results obtained are more significant than the research results conducted by Abigor et al. (2002), which is 0.376 U/mL [2].

Lipase Activity After Metal Ions Addition

Metal ions can increase or decrease enzyme's activity. According to Bijay et al. (2016), metal ions can stimulate lipases that catalyze hydrolysis reactions in oil by removing fatty acids from the oil-water interface and allowing lipases to act freely on oil molecules [12]. It means that the ability of the enzyme to bind to the substrate can be changed by metal ions, thus the activity of an enzyme can decrease (as an inhibitor) or increase (as an activator). Metal ions tested include Na^+, K^+, and Mg^{2+}, the metal ions used in this study are in the form of chloride salts. Table 1 shows the activity of the lipase enzyme after the addition of metal ions.

Table 1. Effect of Metal Ions for Lipase Activity

Metal Ion	Activity (U/mL)	Relative Activity (%)
Lipase	0.866	100
Lipase + K^+	0.911	105.196
Lipase + Na^+	0.578	66.743
Lipase + Mg^{2+}	0.546	63.048

The table shows that K^+ at a concentration of 10 mM can act as an activator which can increase lipase activity up to 105.195%. This condition is the same as Lipase that produced from camped seed sprouts in the study of Khairunnisa et al. (2021), in which the metal ion K^+ increases lipase activity to 101.68% [7]. The increasing lipase activity happens because a metal ion can bind to the enzyme protein and change the conformation of the enzyme to achieve better stability. In addition, metal ion binds to enzyme proteins directly to stabilize the conformation of the active site or induce the formation of the enzyme's active site, therefore it can form one or more coordination bonds with the substrate [13].

Different from the case of metal ions, Na^+ and Mg^{2+} can act as inhibitors at a concentration of 10 mM, reducing lipase activity to 63.048% and 66.743%. This condition is close to research by Sui et al. (2012) on coconut-endosperm , in which the lipase enzyme activity increased to 64% after adding 5 mM of Na^+ [8]. Another study by Yesiloglu and Baskurt (2013) explained that the Mg^{2+} metal ion could very strongly inhibit the lipase activity of almond seeds [12]. According to Vitolo (2021), enzyme inhibitors are compounds that can change the active site of an enzyme due to the interaction between metals and enzymes, thus it will reduce the enzyme activity [6]. In contrast to research by Bijay et al. (2016) and Khairunnisa et al. (2021), addition of Mg^{2+} can increase lipase activity [8][9]. This difference is because, at specific concentrations, metal ions can act either as activators or as inhibitors [10].

The results of this study indicate that K^+ act as activators while Mg^{2+} and Na^+ act as inhibitors. According to Sharma (2013), a metal may acts as an activator if the metal binds to the allosteric site of the enzyme causes a change in the shape of the active site of the enzyme to match the substrate [14]. According to Sholeha (2021), metal ions will act as inhibitors if the metal ion structure resembles the substrate, thus there will be competition with the substrate to bind to the enzyme, which then lead to formation of an enzyme-inhibitor complex [15].

Catalytic Activity of Lipase in Castor Oil

Determination of catalytic activity of Lipase in castor oil was carried out with an incubation time of 48 hours at 37˚C with enzymes:substrate concentration of (1:6) (v/w), with and without addition of K^+. The product was then analyzed by GC-MS. The chromatogram is shown in **Figure 1,** while its data interpretation is presented in **Table 2**. **Figure 1** shows the chromatogram from the GC-MS analysis on the hydrolysis product of castor oil substrate with lipase enzyme that isolated from *Jatropha curcas* L. in n-hexane. Chromatograms are presented in the order of the reaction of castor oil with a lipase catalyst **(a)**; castor oil with a lipase catalyst with the addition of metal ions K^+ **(b)**; castor oil without the addition of lipase **(c)**; taking into account the identified peaks formed as a product of the reaction of the substrate with enzyme lipase.

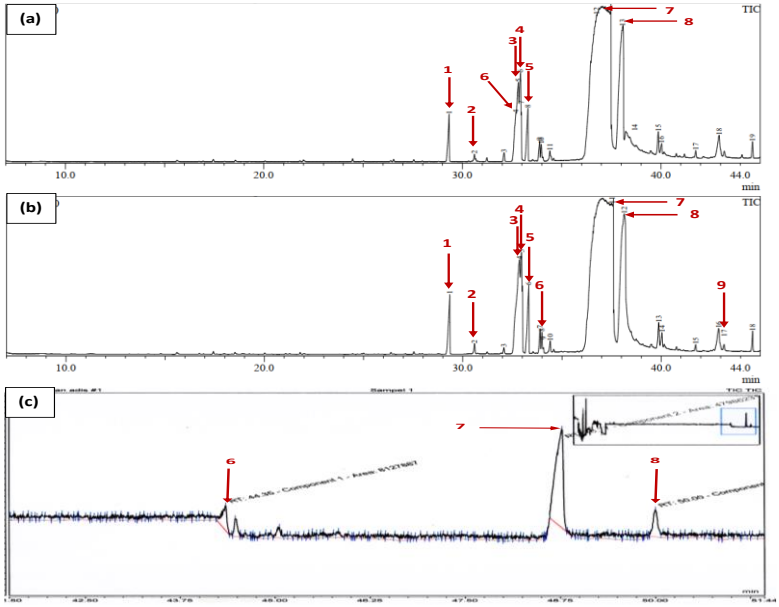

Figure 1. Chromatogram of the Reaction with Lipase **(a),** with Lipase and K^+ **(b),** and without Lipase **(c)**

Table 2. Product of Enzymatic Reaction of Lipase in Castor Oil

Product Compound	Peak	Result					
		Castor Oil with Lipase **(a)**		Castor Oil with Lipase and K^+**(b)**		Castor Oil **(c)**	
		RT (min)	% Area	RT (min)	% Area	RT (min)	% Area
Methyl Palmitate	1	29.311	4.49	29.343	2.09	-	-
Ethyl Pentadecanoate	2	30.606	0.80	30.596	0.18	-	-
Methyl Linoleate	3	32.814	10.12	32.857	7.84	-	-
Methyl Oleate	4	32.922	8.44	32.963	4.19	-	-
Methyl Stearate	5	33.293	4.87	33.322	2.36	-	-
Methyl-9,12-Octadecadienoic	6	32.692	6.31	33.894	0.62	44.350	21.30
Methyl Ricinoleic	7	36.758	11.67	37.074	60.83	48.770	83.70
Ricinoleic Acid	8	38.228	4.58	38.147	17.09	50.000	35.90
2-Hexadecanoyl Glycerol	9	-	-	43.174	0.19	-	-

Based on **Figure 1**, the major product of reaction of castor oil substrate with lipase is methyl-ricinoleic, as evidenced by the formation of compound with the widest % area. The percentage area shows the number of components of the compound in the sample or mixture in comparison to the relative peak areas. In addition, several compounds were formed, which is detailed in **Table 2.**

Based on **Table 2**, fatty acids and some methyl esters were formed. This shows that the reaction was not only related to hydrolysis process but also esterification and transesterification processess. In sample **a**, the enzymatic reaction of castor oil was occurred without the addition of K^+ ions. Under these conditions, the lipase catalytic activity in the hydrolysis, esterification, and transesterification processes was balanced. The fatty acids indicated this, which is supported that the formed alkyl ester concentrations were not much different. The transesterification product produces methyl ricinoleate (11.67%) and methyl-912 octadecadienoic (6.31%), while the hydrolysis process produces ricinoleic acid (4.58%). In addition, lipase also catalyze esterification reactions, and this is supported by the formation of alkyl esters such as methyl palmitate (4.49%), ethyl pentadecanoate (0.80%), methyl linoleate

(10.12%), methyl oleate (8.44 %), and methyl stearate (4.87%).

In the sample **b**, there is an enzymatic reaction of castor oil with addition of K^+ ions. In this condition, the transesterification process occurs optimally. The highest methyl ricinoleate compound content indicates this among other compounds, which is 60.83%. In addition, a hydrolysis process was characterized by the formation of ricinoleic acid compounds of 17.09% and 2-hexadecanoyl glycerol of 0.19%. Therefore, the lipase catalytic activity in the hydrolysis and transesterification reactions is high, and this is because the K^+ can increase the activity of lipase. However, in this condition, the concentration of the products resulting from the esterification reaction decreased, namely methyl palmitate (2.09%), ethyl pentadecanoate (0.18%), methyl linoleate (7.84%), methyl oleate (4.19%), and methyl stearate (2.36%). The results of the esterification reaction are decreasing due to the esterification tend to be occurred in the condition where lipase actively performs without any K^+.

Sample **c** is the result of the initial analysis of the castor oil substrate. In this condition, castor oil was firstly undergo a transesterification reaction, aiming to determine the components of fatty acid compounds contained in the substrate. Based on **Table 2**, the highest content in castor oil is methyl ricinoleate (83.70%). These results are in accordance to research conducted by Mulyani et al. (2018), in which methyl ricinoleic was the main compound of castor oil (88.666%) [11]. Before carrying out the transesterification reaction, the triglycerides of castor oil made the hydrolysis reaction occurs earlier. This was indicated by the presence of ricinoleic acid (35.90%) and the remaining transesterification product, namely methyl-9,12-octadecadienoic (21.30%).

According to **Table 2**, transesterification reaction by lipase without K^+ ions **(a)** resulting in 11.67% of methyl ricinoleate, which is increased to 60.83% after the addition of K^+ ions **(b)**. This was continuously increase to 83.70% in castor oil samples **(c)**. Similarly, it is also happen in the hydrolysis process by lipase. First, 4.58% ricinoleic acid were formed, which then increased to 17.09% and to 35.90%. This increases are due to K^+ ions are activators that can increase the catalytic activity of lipases that act on a substrate, thus the lipase catalyzes multiple reactions. A drastic increase occurred in the castor oil component, and this happened because only transesterification and hydrolysis reactions were took place, thus the resulting products were less.

CONCLUSIONS

Lipase that isolated from jatropha seeds, and then reacted with castor oil in hexane solvent had a catalytic activity of 0.603 U/mL. The catalytic activity was increased after the addition of K^+ (0.911 U/mL). The products produced by lipase after breaking down triglycerides are fatty acids, alkyl esters, and glycerol by-products. The products include ricinoleic acid, methyl ricinoleate, methyl-9,12-octadecadienoic-ricinoleic acid, methyl-palmitate, ethyl-pentadecanoate, methyl-linoleate, methyl-oleate, methyl-stearate, 2-hexadecanoyl glycerol.

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