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Isolation of Lipase from Candlenut Seeds and Its Immobilization in Polyacrylamide Gel

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Abstract: In general, lipase enzymes are used in the form of free lipase enzymes which can only be used once and are less effective, so that their use is costly. Therefore, to maximize the use of the lipase enzyme, immobilization of the enzyme that allows multiple use is a choice. The purpose of this study was to determine the activity of the candlenut (*Aleurites moluccana* (L.) Wild) lipase in its free form and in immobilization form. The stability of the immobilized lipase enzyme with repeated use was also investigated. The enzyme was immobilized by trapping method using polyacrylamide gel. The immobilized lipase enzyme was characterized to determine its stability after repeated use with various gel concentrations. The crude extract of the lipase of candlenut seed sprouts had an activity of 2.32 U/mL, whereas the activity of the immobilized lipase enzyme in polyacrylamide gel at concentrations of 6%, 8%, and 10%, were 2.133; 2,259; and 2,311 U/mL, respectively. The optimum temperature for the immobilized lipase enzyme was 30ºC and the optimum pH was 7.5. After five time us usage, the immobilized enzyme showed activities of 49.49%, 72.60% and 86.53%, for 6%, 8% and 10% polyacrylamide gel, respectively. These results indicate that the immobilized lipase enzyme from seed sprouts of candlenut (*Aleurites moluccana* (L.) Wild) by trapping method using 10% polyacrylamide gel can maintain its stability after five times of use.

Keywords: lipase; Candlenut; Activity; Immobilization; Polyacrylamide

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Introduction

Enzymes belong important commodity in industries, such as in food, chemical, and in pharmaceutical industry. One class enzyme that has a very broad role is the lipase. Lipase enzymes are used as biocatalysts to hydrolyze fats or oils into fatty acids and glycerol. Based on this activity, lipase has many applications in industry and biotechnology. Lipases are widely used in the industrial world such as the food and beverage industry, detergents, pharmaceuticals, agrochemicals, cosmetics, and the synthesis of organic materials.

Lipase enzymes can be isolated from microbes, animals, and plants. Plant lipases can be obtained from grains or seeds (Kawiński et al., 2021; Kumar et al., 2021). Plant based lipases are commonly obtained from germinated seeds, although fresh seed may also have significant lipase activity such as shown in castor bean seed (Tavares et al., 2018). During the germination process, the lipase enzyme activity in grains increases and high fat seeds are good sources of lipase (Kumar et al., 2021). Candlenut (*Aleurites moluccana* (L.) Wild), that has fat content of about 60%, is potential source of lipase (Tambun et al., 2020). The use of lipase enzymes from candlenut seeds in this study because it has high activity.

Candlenut trees are widely grown in Indonesia. Candlenut has been widely used such as in the household as a basic ingredient for cooking spices and in the pharmaceutical field. Candlenut seeds are

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classified as stone fruit because they have a hard shell resembling a shell with a rough and grooved outer surface. This seed shell is about 3-5 mm thick, and brown or black in color. Candlenuts from various regions have different levels of shell thickness.

Isolation of lipase enzymes has been widely carried out both from microorganisms, animals, and plants. In general, lipases in seeds have a high affinity for the dominant fatty acids in the seeds. Lipase from seeds also has an effective ability to hydrolyze triglycerides at the sn-1,3 position (Enujiugha et al., 2004). Such properties are needed by various industries. Therefore, exploration of lipases from seeds is growing, for example indigenous lipases of walnut seeds (Djarkasi et al., 2017), avocado seed sprouts (Sya'bani et al., 2017), durian seed sprouts (Dzulkarnain, 2018), cocoa beans (Permana et al., 2013).

Enzyme production requires a fairly high cost and is generally used only once. Therefore, to carry out further reactions, a new lipase enzyme is needed so that the resulting product is more optimal. The use of lipase enzymes that can only be used once is less effective and costs a lot (Mulko et al., 2019). Therefore, to maximize the use of the lipase enzyme, enzyme immobilization can be carried out. Enzyme immobilization is a method of binding enzymes to a solid material or matrix that is not soluble in water. It aims to separate the enzymes at the end of the reaction, without contaminating the reaction products so that they can be used sustainably.

Enzyme immobilization can be done by several methods, one of which is the adsorption method because it is easy to operate. However, this method produces immobilized enzymes with weak immobilization bonds, immobilized enzymes are very sensitive to changes in temperature and pH, and the amount of immobilized enzymes is very small (Zhao et al., 2015). Therefore, the method used in this research is the trapping method. When compared with the adsorption method, this method is relatively stable. Besides being easy to do, this method is inexpensive, produces stable immobilized enzymes and is able to trap enzymes in large quantities and with high enzyme activity (Bilal et al., 2019). The support matrix commonly used in this method is polyacrylamide.

Polyacrylamide as a support matrix will trap the enzyme in a free state and not bound to the supporting material so that relatively the catalytic function and natural structure of the enzyme molecule do not change so that the enzyme can still function. In addition, the pore size of the polyacrylamide can be adjusted as needed. Since candlenut has a great potential to be use for various purposes, it is of interest to provide an immobilization platform to facilitate it activity. And since polyacrylamide is well-known to effectively bind enzyme for repetitive uses, the present research aims to optimize the immobilization of candlenut lipase in different polyacrylamide gel concentrations. The enzyme stability and usability after several cycle of uses is described.

Method

Materials

Candlenut seeds were obtained from a traditional market in Mataram and from a local candlenut plantation in Lingsar, Lombok. Chemical for enzyme isolation was 5 mM phosphate buffer pH 7. Biuret reagents for protein determination consisted of Casein (Sigma), $CuSO_4$ (Merck), Na_2KHPO_4 and NaK_2HPO_4 (Merck), and 7.5% NaOH. The materials for enzyme assay were Lombok VCO (Virgin Coconut Oil) Al-Amiin, crude extract of lipase, immobilized lipase, technical n-hexane (Merck), 0.1 M phosphate buffer pH 7.5, 96% alcohol (Merck), technical acetone (Merck), 0.01 M NaOH, and indicator phenol phthalein (Merck).

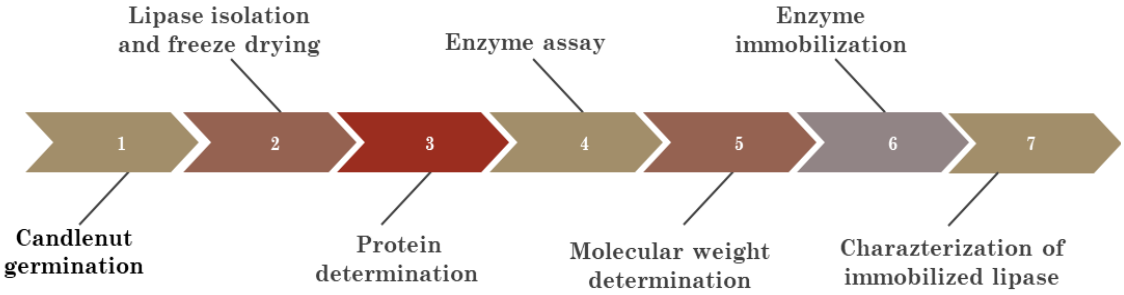
Materials for Sodium Dodecyl Sulfate Polaycrylamide Gel Electrophoresis (SDS-PAGE) and Native-PAGE electrophoresis were aquadest, Tris-HCl buffer, Na_2HPO_4 (Merck), NaH_2PO_4 (Merck), Tris-base (bio-WORLD), acrylamide (Bio Basic Canada), *bis*-acrylamide (Bio Basic Canada), SDS (Bio Basic Canada), ammonium persulfate (Merck), SDS (Bio Basic Canada), Tetramethylethylenediamine (TEMED, Sigma), glycine (Bio Basic Canada), glycerol, beta-mercaptoethanol (Sigma), bromophenol blue, Coomassie Brilliant Blue R-250 (Sigma), glacial acetic acid pa (Merck), methanol p.a. (Merck), electrophoresis running buffer, and protein marker (Thermoscientific).

Ingredients for immobilized enzymes were crude extract of lipase enzyme, aquadest, Tris-HCl buffer, acrylamide (Bio Basic Canada), bis-acrylamide (Bio Basic Canada), TEMED, and ammonium persulfate (Merck).

Equipment

The equipment employed in this study were centrifuge (Tommy MX-307), water bath shaker, hot plate stirrer (Scy-supply), micro centrifuge (IECMicro–MB centrifuge), pH-meter (HANNA pH 211), freeze dryer (Alpha 1-2 LD) plus), freezer –20 °C, UV-1601 PC Shimadzu spectrophotometer, SDS-PAGE and Native-PAGE (GE Healthcare) apparatus.

Supporting equipment: 96-well microplate, 50 mL and 15 mL conical tubes, analytical balance (Kern ABJ), micropipette (Eppendorf 0.5-1000 L), micro tip (0.5-1000 L), measuring cup 50 mL (Pyrex® Iwaki Glass), 250 mL Erlenmeyer flask (Pyrex® Iwaki Glass), 500 mL and 1000 mL measuring flasks (Pyrex® Iwaki Glass), blender (Phillips), 50 mL burette (Pyrex® Iwaki Glass), pole stand, plastic filter, funnel (Pyrex® Iwaki Glass), spatula, magnetic stirrer.



Scheme 1. The experiment performed in this study are summarized in the above scheme

Candlenut seeds were washed and rinsed with 0.02% KNO_3 for 24 hours. The germination was carried out in a container filled with soil that was kept moist by regular watering. After 15 to 30 days, germination of candlenut seeds was observed by the appearance of sprout. Candlenut seeds that have germinated were collected for use as source of lipase. Candlenut shells were broken to get candlenut seeds flesh. Candlenut seeds were crushed and prepared in the form of suspension in phosphate buffer pH 7.0. The suspension was transferred into a 50 mL conical tube and centrifuged at 6000 rpm, 4 °C for 10 minutes. The clear brown candlenut supernatant was separated from debris and skim fractions. Supernatants (crude extracts) were stored in aliquots for use in advanced testing.

Protein concentration was determined by Biuret method. Casein solutions with concentration from 0 to 2500 µg/mL were used as standard solutions. Optimum absorbance was first determined prior to standard solutions and samples measurement (Taurina & Andrie, 2021).

Candlenut lipase activity was measured from its ability to hydrolyze VCO substrate. Lipase activity was determined by titrating the fatty acids released in the hydrolysis reaction (Monnet et al., 2012) which has been modified. The mixture consisting of 5 grams of VCO, 2.5 mL of n-hexane, 5 mL of 0.1 M phosphate buffer pH 7.5 and 1 mL of lipase enzyme was incubated in a water bath shaker at 30 °C for 45 minutes. After incubation, 25 mL of acetone-ethanol (1:1, v/v) and 4 drops of pp indicator were added, and then titrated with 0.01 M NaOH until a clear pink color.

The treatment of the blank was the same as that of the sample but without enzymes. Lipase enzyme activity can be calculated by the formula:

$$A = ((V.s-V.b) \times [NaOH] \times 1000)/(V.enz \times t) \quad (1)$$

Where:
V.s : Volume of sample titrant (mL)
V.b : Volume of blank titrant (mL)
[NaOH]: Concentration of NaOH (M)
V.enz : Enzyme volume (mL)
t : Incubation time (minutes)
1000 : Conversion factor from mmol to mol

The determination of the molecular weight of candlenut seed lipase was undertaken by method according to Laemmli (Laemmli, 1970). SDS PAGE electrophoregram was compared to that of electrophoresis under undenaturing condition (native-PAGE).

Immobilization of the lipase enzyme with polyacrylamide gel referred to Kumar and co-worker (Kumar et al., 2014), with modification. The mixture to make polyacrylamide gel with 10% crude extract was put into a 50 mL conical tube. Then 200 µL of the mixture was put into each well of a U-bottom model 96 Well microplate (diameter 6.94 mm). The well plate was stored in the refrigerator at 4 °C until the gel hardened. The immobilized enzyme was washed using 10 mL of 5 mM pH 7 phosphate buffer. The immobilized lipase enzyme was then tested for changes in activity during repeated use. The protein bound to the gel was also determined until the fifth use.

Results and Discussion

Germination of candlenut seeds

Candlenut lipase enzymes prepared from candlenut seeds which were conditioned so that they were in the germination phase. This condition was chosen because lipase activity generally increased in this phase, where nutrients and energy were mobilized rom fat reserves in the seeds (Tavares et al., 2018).

Figure 1. Germination process of candlenut seeds. Candlenut seeds were soaked in KNO_3 (left) prior to germination (middle). Right picture shows germinating candlenut seed after 15-30 days.

Isolation of lipases from candlenut seeds

The nut meat from the pecan seed sprouts separated from the shell was mashed with a blender. The resulted in suspension was subjected to centrifugation to allow separation of the skim fraction (Figure 2). This fraction was stored in a freezer for the purpose of further analysis.

Figure 2. isolation of lipase from candlenut seeds

Lipase activity

The resulted in crude extract was used in the lipase activity assay as described in the method. The lipolytic activity if lipase hydrolyze the ester bonds between glycerol and fatty acids. The liberated fatty acids were measured by base titration. It was revealed that the obtained crude extract has lipolytic activity of 2.12 U/mL

Protein determination

The protein determination was undertaken by the biuret method. This method utilizes the reaction between Cu^{2+} metal with protein peptide bonds. Protein content was measured based on color intensity which was measured using visible spectrophotometry. The maximum wavelength of the casein standard protein solution was first determined in the wavelength range of 450 - 800 nm and the highest absorbance was at a wavelength of 551 nm (not shown). A calibration curve was then created by using casein absorbance at 551 nm after coloring solution of Biuret reaction was developed (Fig. 3).

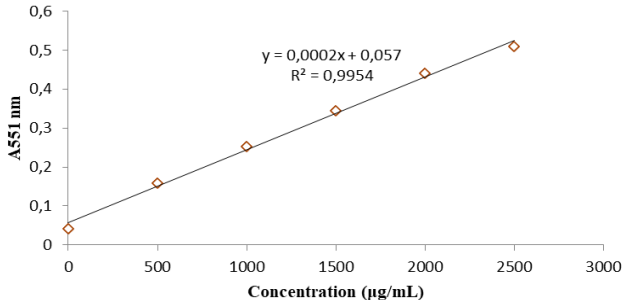


Figure 3. Calibration curve for protein determination

The protein content of the sample was obtained by substituting the absorbance value of the lipase into the equation y = 0.0002x + 0.057 (Fig. 3). Solving the equation resulted in a protein concentration of 1.030 mg/mL. From this protein concentration data, it can be determined that the specific activity of the crude extract of the lipase enzyme is 2.07 U/mg.

Molecular weight determination

To find out the protein profile of candlenut seeds (including lipases from candlenut seeds), SDS-PAGE and native-PAGE electrophoresis were performed on the sample. The obtained lipase is active and in native-PAGE electrophoregram appears as a single band with a molecular weight of about 120 kDa. Since electrophoresis in denatured conditions (SDS-PAGE) shows that the lipase consists of several protein subunits (Fig 4), it is likely that candlenut lipase is complex protein consisting of different subunits. Whether the individual subunits active like a recent report for coconut lipase (Savalas et al., 2021) is yet to be investigated.

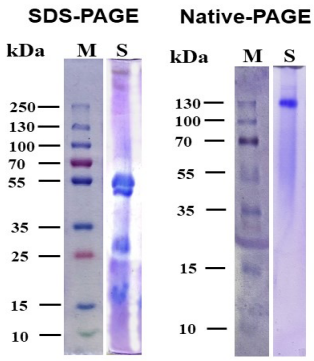


Figure 4. Electrophoregram. SDS-PAGE shows candlenut lipase has several subunits, i.e., 55 and 50 kDa, 28 kDa and 20 kDa subunits (left). Native-PAGE shows that candlenut lipase appears a single band of c.a. 120 kDa (right).

Lipase immobilization

Immobilization of the lipase enzyme by the entrapment method using polyacrylamide gel was carried out because of the ease of the gel preparation, inexpensive and lead to stable (Zhao et al., 2015; Zou et al., 2020) The polyacrylamide support is known for its stability and capacity to trap a large number of enzymes while keeping high enzyme activity (Bilal et al., 2019). The PAG that employed to entrap candlenut lipase is shown in Fig 5.

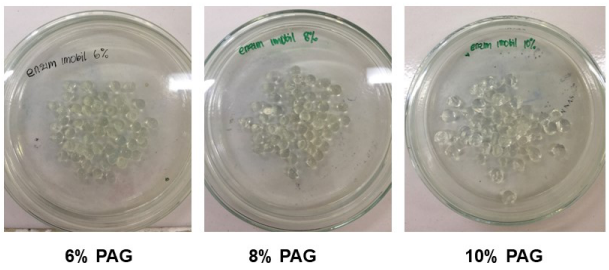


Figure 5. Candlenut lipase immobilization with polyacrylamide gel

Immobilized lipase stability in repeated use

The activity of the immobilized lipase enzyme was determined in the same procedure as for free lipase determination described in method section, using VCO as substrate. The test results of immobilized lipase enzyme activity after repeated use for different gel concentration is shown in Table 1 and schematically depicted in Fig 6.

Table 1. Immobilized candlenut lipase activity after repeated use for various polyacrylamide gel concentration

Lipase activity (U/mL)			
Gel conc.	6%	8%	10%
1st use	2.13	2.26	2.31
2nd use	1.61	1.90	2.26
3rd use	1.24	1.64	2.07
4th use	0.65	1.32	1.80
5th use	0.11	1.30	1.60
Av. activity	1.15	1.68	2.01

Fig 6 shows that the activity of the immobilized lipase enzyme increases along with the increase in the concentration of the polyacrylamide gel. The initial activity of immobilized lipase enzymes at concentrations of 6, 8, and 10%, was 2.13 U/mL; 2.26 U/mL; and 2.31 U/mL, respectively. The difference in lipase activity is determined by the density of the polyacrylamide gel pores. The higher the polyacrylamide gel concentration, the higher the polyacrylamide gel density or the smaller the polyacrylamide gel pores so that the number of enzymes trapped in the polyacrylamide gel increases. Other enzymes also show increased retention and reusability when immobilized in higher polyacrylamide gel concentration, such as protease (Sattar et al., 2018) and amylase (Mulko et al., 2019).

The stability of immobilized enzyme upon repeated use was tested for each gel concentration. The result is depicted in Fig 6. It shown that after 5 time of use, the enzyme immobilized in 10% gel is the most stable when compared to the same enzyme immobilized in 6 and 8% polyacrylamide.

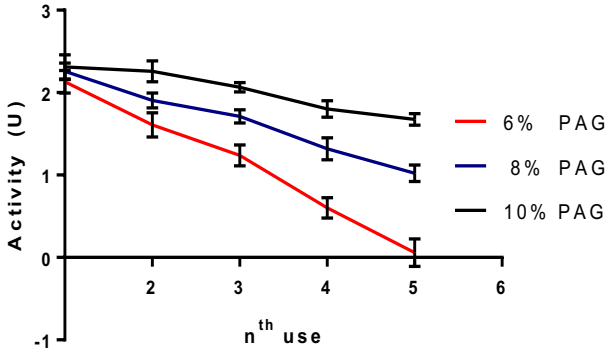


Figure 6. Lipase activity after repetitive use. Each measurement was made triplicate.

The lipase immobilized in 6% polyacrylamide experienced a significant decrease in activity from the 2nd use, whereas the lipase activity in the 10% polyacrylamide gel did not decrease significantly until the 5th use. At the 5th use, the immobilized lipase enzyme activity was still high (1.60 U/mL). In general, the activity of lipase decreases following repetitive usage, due to the loss of bound enzyme from supporting matrix. In other word, the more the immobilized enzyme used, the less enzymes trapped in the polyacrylamide gel. The damage in the protein structure integrity after repetitive use also accounts for the reduced activity.

Similar observation was also recorded for rice bran lipase immobilized on activated carbon (Firdaus et al., 2017) and yan lipase immobilized in powdered chitosan (Wardoyo et al., 2015), where the lipases showed stability up to 5th and 6th use, respectively. This indicates that the immobilized lipase enzyme can be reused despite the decrease in its activity. The use of polyacrylamide as supporting matrix for enzyme may also be generalized, as immobilized protease using polyacrylamide showed stability up to 4 cycles of use, and only showed significant decrease after the 5th use (Mubarik, 2001).

Lipase enzyme trapping stability in polyacrylamide gel

The stability of the lipase entrapment in the polyacrylamide gel is shown in the following figure:

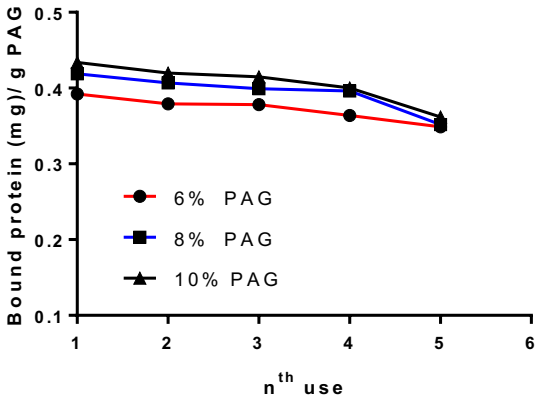


Figure 7. Protein load after repetitive use

The number of lipase enzymes trapped in the polyacrylamide is indicated by the value of protein loading (Minovska et al., 2005). The protein content obtained from the washing filtrate of the immobilized lipase enzyme was used to obtain the value of protein loading in the polyacrylamide gel. The protein loading values were 0.392 mg/g, 0.419 mg/g; and 0.434 mg/g in 6, 8 and 10% gel, respectively. Although the 6% gel retains less enzyme after 5th use, the decrease in its hydrolytic activity is more dramatically (more than 50% loss of activity, Table 2). At the same condition, the 10%

gel only slightly has more enzyme (Fig 7) and after 5th use only loses 14% of its initial activity (Table 2).

Immobilization Efficiency

The immobilization efficiency was obtained from the comparison of the value of the immobilized lipase enzyme activity with the crude extract activity of the lipase enzyme. The value of this immobilization efficiency indicates the level of success of the immobilization method used. The higher the immobilization efficiency value, the better the results obtained (Thangaraj & Solomon, 2019).

Table 2. Average activity of immobilized lipase

Gel concentration (%)	Average Activity (U/mL)	Av. Immobilization efficiency (%)
6	1.148	49.49
8	1.684	72.60
10	2.007	86.53

Conclusion

From this study, it is concluded that the lipase enzyme from candlenut could be immobilized by trapping method using polyacrylamide gel. The highest stability was found when the enzyme was trapped in 10% polyacrylamide, a condition that maintains the lipase activity above 86% after five time of uses. This research can be completed by observing the effect of immobilized enzyme storage time on repeated use.

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