Lipase Production of *Aspergillus aculeatus* MS. 11 Using Solid State Fermentation on Rubber Seed Press Cake

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Abstract. Lipase is an enzyme that has various use in industrial sector. However, the enzyme production cost is considerably high mainly due to expensive fermentation substrate. Therefore, it is necessary to develop effective media formulation to increase production of lipase enzyme without reducing the amount of production. This research aims to know the effectiveness of rubber seed press cake as a fermentation substrate for *Aspergillus aculeatus* Ms. 11. We observed the effect of glucose and olive oil as a carbon source addition on the substrate, determined the growth profile and lipase production on the substrate in lipase production. Isolates of Ms.11 was inoculated in 14,28 mL MGM medium with substrate variation rubber seed press cake, rubber seed press cake + glucose, rubber seed press cake + olive oil, and combination substrate (rubber seed press cake + glucose + olive oil) during 0, 2, 4, 7 days. The highest lipase activity and lipase productivity in this study shown at 19.23 U/mL and 186.51 Yield/day, there were obtained from rubber seed press cake + olive oil on day 2, whereas the highest specific activity of lipase enzyme (1.16 U/mg) was obtained in substrate variation rubber seed press cake + olive oil on day 7. We concluded that maximum lipase production of Ms. 11 can be obtained using rubber seed press cake with addition of olive oil.

Keywords: Glucose · Rubber seed · SSF

1 Introduction

Microorganism have an various role in life and applied in various sector such as food, beverages, chemicals, and pharmaceutical product [1]. Microorganism were the preferred source in enzyme industry because they were easy to obtain, have a fast growth rate, and high-quality product. A large macromolecule that was composed with some amino acid that linked by amide bonds with molecular weights range from kilodaltons to megadaltons were defined as enzyme. Enzymes have a level of specificity in substrates. The specificity of enzymes used for catalyzing reactions between one type of chemical compound as a basic for classification [2]. One of the most enzymes studied was lipase enzyme.

Lipase (triacylglycerol hydrolases) EC3.1.1.3 was enzyme that has the ability to hydrolyze ester bonds from water-insoluble substrates and belong into hydrolases

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enzyme [3]. Lipase was also acts to support esterification, trans esterification, and hydrolysis reaction [4]. The study is also supported by the research that reported by Durr-E-Nayab et al. [5] that lipase was responsible for the reactions of lipolytic metabolism there are acidolysis, alcoholysis, hydrolysis, aminolysis, esterification and interesterification. Naturally serine in nature will be hydrolyze ester into fatty acid and glycerol [6]. Lipase enzyme activity was influenced by pH, temperature, ionic strength, and chemical substance. Temperature has a significant effect on kinetic energy of enzyme and substrate molecules. pH affect in enzyme stability by changing the electrostatic interaction of protein structure that cause amino acids changes [3]. Biodiesel is an application of lipase. Biodiesel production can be carried out using chemical or enzymatic catalysts. Enzymatic catalysts have a several advantages, one of them was to improve the product quality, easy reaction, and included to catalyst group that can reused so it has not negative impact on the environment. However, the used of this catalyst has a lower conversion and reaction rate than chemical catalyst [7].

Lipase enzyme will be catalysed two reactions to produce methyl ester. The reaction were esterification and trans esterification [7]. Lipase will be catalyze the hydrolysis reaction when under of high water availability conditions on the substrate, while lipase will be catalyzed the esterification reaction when under of low water availability conditions on substrate [8]. The esterification reaction was conversion reaction of fat into esters using fatty acid and ethanol as a catalysts [9].

Biodiesel can also produce by transesterification reaction. The principle of this reaction was alcoholysis which is a reaction between lipids and alcohols to produce esters and glycerol as byproducts. Transesterification has 3 reaction mechanisms. This reaction is reversible. The first step was conversion of triglycerides into diglycerides. The second step was conversion of diglycerides into monoglycerides. The last step was conversion of monoglycerides into glycerol. This reaction will be produce one one ester molecule in each step [10, 11]. Lipase enzymes are used in the process of transesterification and esterification due to economic and environmental reasons. This is because lipases have the ability to use monoglycerides, diglycerides and triglycerides as well as free fatty acids [12].

The most biodiesel is produced using methanol and alkaline catalysts. This reaction requires two step esterification reaction. This is because of some oils and fats still contain with free fatty acids (FFA) which cannot be converted into biodiesel if we use an alkaline catalyst. The reaction begins with acid catalyzed pretreatment to convert FFA into fatty acid methyl esters. To complete the reaction, the reaction was terminated by alkaline catalyst [11]. However, the enzyme production cost is considerably high mainly due to expensive fermentation substrate. Therefore, it is necessary to develop effective media formulation to increase production of lipase enzyme without reducing the amount of production.

Hevea brasiliensis (Willd. ex A. Juss.) Müll. Arg.) [13] which we know as rubber tree was export commodity for plantations. Indonesia, Thailand, Malaysia, Vietnam, and India were the largest natural rubber producer. The total area of Indonesian rubber plantations in 2016 reached 3.64 million hectares with a productivity (1,080 kg/ha). The 85 percent of rubber production in Indonesia is exported abroad. The five countries that most import from Indonesia are the United States, China (RRC), Japan, Singapore, and

Brazil [14]. Rubber production was also contributed to Indonesia GDP and Indonesia has produce around 2.88 million metric tons of rubber in 2020 [15]. Rubber trees produce rubber seeds which have more oil content than castor seeds and karanja seeds. The extracted oil has inedible properties, so it is widely used as biodiesel [16].

The utilization of rubber seeds as a substrate for cultivation or the growth of microorganisms has been ignored. Every year many rubber seeds are only treated as an agricultural waste, but when we see from the nutritional content, rubber seeds have the potential as an efficient substrate for growth microorganisms. Besides of being a cheap substrate, rubber seeds are also easy to obtain and economical in terms of cost [17]. Rubber seeds have the characteristics of a hard outer shell, so there is a need for a rubber seed splitting technique. Rubber seed breaking or pressing is done mechanically using a hydraulic press machine to get the rubber seed press-cake and rubber seed oil. This rubber seed press cake is used for the formulation of the medium in fermentation [18].

Fermentation is the main technique for producing various enzymes from fungi and bacteria when fermented on the right substrate. The Solid-State Fermentation (SSF) method is preferred when enzymes have to be extracted from fungi, which require lower water potential. Enzymes can be produced if the right substrate is used, such as rubber seed press cake. This is because the rubber seed press-cake has a high nutrient content and acts as a carbon source for microbial growth [17], therefore this study was conducted lipase production of *Aspergillus aculeatus* Ms.11 using solid state fermentation on rubber seeds press cake medium.

The mold that we used in this study was *Aspergillus aculeatus* Ms. 11. From Faculty of Biology UGM. *A. aculeatus* Ms. 11 was isolated from non-dairy creamer industrial waste PT. Kievit Indonesia, Salatiga, Central Java. These isolates have characteristics in producing high lipase. It can be shown by the medium containing olive oil and glucose at 96 h and 120 h incubation, which has lipase activity of 5.13 ± 0.30 U/mL and 5.22 ± 0.59 U/mL. The study was also showed the results of optimal lipase activity in used of methanol and ethanol at pH 7.0, 30 °C [19].

Based on the previous research, *Aspergillus aculeatus* Ms. 11 had an ability to produce lipase using SmF method (Submerged Fermentation) with variations pH, temperature, and incubation time. However, the ability of *A. aculeatus* Ms. 11 to producing lipase using Solid State Fermentation (SSF) in rubber seeds press-cake medium is not yet known. Rubber seed press cake is a good and efficient substrate in lipase production because it has high protein content. This research observed about the effect of carbon source addition on substrate to lipase production, and determined the growth profile and lipase production. The research aims to know the effectiveness of rubber seed press cake as a fermentation substrate for *Aspergillus aculeatus* Ms. 11. We observe the effect of glucose and olive oil as a carbon source addition on the substrate, determined the growth profile and lipase production of *Aspergillus aculeatus* Ms. 11.

2 Materials and Methods

2.1 Materials

Aspergillus aculeatus strain Ms. 11 was provided by Faculty of Biology in department of Microbiology Laboratory, UGM. The isolate was isolated from solid waste (non-dairy

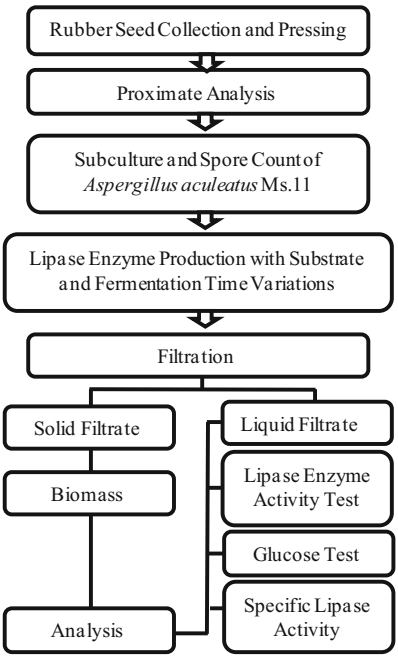

Fig. 1. Research design flowchart.

creamer) PT. Kievit Indonesia, Salatiga, Central Java. Most of non-dairy creamer was made from coconut oil and palm oil that commonly used to reduce the color (coffee and tea) and as flavor enhancer in drinks [19, 20].

The growth media were rubber seeds press-cake obtained from the Keling, Jepara, Central Java, PDA (Potato Dextrose Agar) (HiMedia), glucose (Merck), olive oil (Bertolli), ammonium sulphate [$(NH_4)_2SO_4$] (Chemix), Urea [NH_2CONH_2] (Chemix), sodium dihydrogen phosphate [NaH_2PO_4] (Chemix), potassium dihydrogen phosphate [KH_2PO_4] (Merck), magnesium sulphate heptahydrate [$MgSO_4.7H_2O$] (BDH), and calcium chloride [$CaCl_2$] (Merck). Other chemical materials used were Triton X-100 (Merck), isooctane (Merck), ethanol (Merck), Cu-acetate reagent (Merck), pyridine (Merck), Lowry reagent (Chemix), DNS reagent (Aldrich), HCl (Merck), NaOH (Merck), and oleic acid (Subur Kimia Jaya).

2.2 Methods

(See Fig. 1)

2.2.1 Rubber Seeds Collection and Subculture

Rubber seeds are collected from rubber seeds plantations in the Keling, Jepara, Central Java. The rubber seeds are broken down and pressing in the PAU Postgraduate Engineering Lab with a pressure of 100 KN to produce oil and rubber seeds press-cake.

Table 1. Substrate variations used in this study

No	Substrate Variation
1	Rubber Seed Press-cake (control)
2	Rubber Seeds Press-cake + Glucose
3	Rubber Seed Press-cake + Olive Oil
4	Rubber Seeds Press-cake + Glucose + Olive Oil

Before pressing, rubber seeds are put into the cabinet for 24 h with the aim of getting more oil. The final results of the pressing treatment obtained coarse rubber seeds and oil. The rubber seeds from the pressing are finely blended until they become rubber seeds press-cakes. The rubber seeds press-cake was sieved with a particle size of 150–425 m to equalize the size [21]. The rubber seed press-cake was placed into ziplock plastic and placed in refrigerator 4 °C before used as a substrate.

Aspergillus aculeatus Ms.11 was propagated and cultured on PDA (Potato Dextrose Agar) slanted agar medium. This was done by dissolved of 3.9 g of PDA media, 1 g yeast extract and 100 mL of sterile aquadest in a 250 mL Erlenmeyer. Then PDA media was sterilized by autoclaving at a pressure of 1 atm 121 °C for 15 min [5].

2.2.2 Spore Count of *Aspergillus aculeatus* Ms.11

The isolates *Aspergillus aculeatus* Ms.11 that had been cultured on slanted medium by streak plate were spores count using a microscope. Before we count, the slanted PDA medium that had been overgrown with mold was added sterile 5 mL of distilled water and 0.1 mL of Triton-X 100 sterile. Then the spores were scraped slowly using a sterile loop needle. The spore suspension then transferred to a sterile glass bottle. Observation of spores using a microscope was carried out by diluting the spore suspension at the level of 10^{-1}. The results of a dilution of 1 mL of spore suspension were dripped using a micropipette on a hemocytometer to calculate spores under a light microscope [22]. The spore suspension was then inoculated into 14.5 mL of fermentation medium with various substrates and incubated for 0-to-7-day, 30 °C.

2.2.3 Lipase Enzyme Production with Substrate and Fermentation Time Variation

The production of lipase enzymes using solid substrate fermentation medium is carried out with variations in the components of the medium (substrate) and fermentation time (Table 1).

Lipase enzyme production is conducted using a method that refers to the research of Falony et al. [23]. 1.5% glucose and 1.5% olive oil in the fermentation medium were used in this study. Mineral Growth Medium (MGM) 14,28 ml which has been added 5 g of rubber seed press-cake was autoclaved (121 °C, 30 min). Then, spore suspension 0.071 ml was inoculated into MGM medium with various substrates: rubber seed press cake, rubber seed press cake + glucose, rubber seed press cake + olive oil, and

combination substrate (rubber seed press cake + glucose + olive oil) for 0, 2, 4, and 7 days, 30 °C. MGM medium consisted of ammonium Sulphate [$(NH_4)_2SO_4$] 0.0375 g (0.75%) and Urea (NH_2CONH_2) 0.017 g (0.34%) as a nitrogen source. Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) 0.00225 g (0.045%), sodium dihydrogen phosphate (NaH_2PO_4) 0.09 g (1.8%), calcium chloride ($CaCl_2$) 0.001875 g (0.0375%), and potassium dihydrogen phosphate (KH_2PO_4) 0.015 g (0.3%) were used as mineral salt in lipase production [24]; [23].

2.2.4 Extraction of Crude Enzyme

Extraction of crude enzyme was conducted by addition of distilled water 100 mL into a substrate that has been fermented. After that, it was agitated at 250 rpm at 25 °C for 30 min. The mixture then filtered using filter paper Whatman No. 1. The results were solid and liquid filtrate. The retained solid was used to biomass determination, whereas the liquid filtrate was used to glucose, lipase, and protein determination.

2.2.5 Lipase Enzyme Activity Test

Lipase enzyme activity can be calculated by preparing the substrate. First, we prepared 2 test tubes. Tube 1 was filled with 1.58 mL oleic acid and 8.42 mL isooctane, whereas tube 2 was filled with 0.29 mL ethanol and 9. 71 mL of isooctane. Then, the solutions in two tubes were mixed until homogeneous. After that, 100 μL crude extract filtrate of enzyme was taken and mixed with 1 mL isooctane and the substrate that we prepare previously. Vortex homogeneous and incubated in 30 °C, for 20 min. The test tube was placed in ice bath 5 min to stop the reaction.

In this study, Colorimetric Cupric acetate pyridine (CAP) method was used to determine the amount of oleic acid in the reaction [25]. The first step is done by dissolving a 100 μL of sample with 1900 μL isooctane and 400 μL CAP. Then the sample vortexed 5 s and place for 20 min to 2 clear layers were formed. The bottom layer was named aqueous phase, whereas the top layer was named organic phase. The top layer that containing of free fatty acids was measured the absorbance at 715 nm. The converted of amount oleic acid (μmol/mL) to products per minute define as one U esterification [26].

The number of lipase unit per mL that multiplied with total filtrate volume (m) of medium was considered as total lipase unit (mL), whereas the number of total lipase unit per gram substrate was defined as yield (U/gr). The number of yields per day of fermentation time was defined as the productivity of lipase enzyme (Yield/day). The yield calculation also used the data of total lipase unit and yield data is also used in calculating enzyme productivity. The calculation was shown by the below formula.

$$\text{Total Lipase Unit (mL)} = \frac{\text{Lipase Unit (U)}}{\text{mL}} \times \text{Total Filtrate Volume (mL)} \quad (1)$$

$$\text{Yield (U/gr)} = \frac{\text{Lipase Unit (U)}}{\text{Rubber Seed Weight (gr)}} \quad (2)$$

$$\text{Productivity (Yield/day)} = \frac{\text{Yield (U/gr)}}{\text{time (day)}} \quad (3)$$

2.2.6 Determination of Specific Lipase Activity

The unit number of lipase relative activity per milligram protein was defined as the specific activity. Protein content was measured using the Lowry method [27]. The first step, 200 μL sample was taken, place into test tube, and added with Lowry's solution 1 mL. The second step, the solution was vortex and placed in the dark room (15 min). After that, Folin Ciocalteu (Lowry D) 100 μL was added into test tube in the dark and vortex until homogeneous. Next, the sample was incubated again in the dark room (30 min). The sample absorbance was measured at 750 nm. The calculation was shown by the formula.

$$\text{Enzyme Specific Activity (U/mg)} = \frac{\text{Enzyme Relative Activity (U/mL)}}{\text{Protein Concentration (mg/mL)}} \quad (4)$$

2.2.7 Reducing Sugar Test (Glucose Test)

The testing of reducing sugar of the sample was determined by the DNS (Dinitrosalicylic Acid) method [28]. The first step we have to prepare the DNS solution. The solution preparation was conducted by DNS powder 1 g added into 50 mL of distilled water in the beaker glass. Sodium potassium tertratetahydrate/KNa-tartrate 1.82 g was added little by little and homogenized slowly. The solution was place into volumetric flask and added with 20 mL of 2N NaOH. Next, distilled water was added until the volume reached the mark of the 100 mL volumetric flask. DNS reagent were stored in closed bottles at room temperature [29].

Reducing sugar test was done by placing a 200 μL sample into a test tube. DNS reagent 200 μL and distilled water 400 μL were added into the sample and vortexed until homogeneous. The sample heated in a water bath at 80 °C–90 °C, 10 min. This is to make the process faster and optimal. Next, the sample was cooled and added with distilled water 5 mL, vortexed until homogeneous. The absorbance was measured at a 540 nm [30].

2.2.8 Biomass

Measurement of biomass (constant dry weight) from the treatment of various substrates was carried out by filtering the media using filter paper Whatman No. 1. After that, the solids were transferred into Falcon bottles and dried at 100 °C. The solids are weighed until a constant weight is obtained.

2.3 Data Analysis

The data was analyzed using Microsoft Excel 2010 to calculate mean and standard deviation. Data visualization of glucose consumption, protein concentration, relative enzyme activity, enzyme-specific activity, lipase productivity, yield, and biomass of isolates *Aspergillus aculeatus* Ms. 11 is using OriginLab Corporation 2019 Data Analysis Software. All data obtained were analyzed using the DUNCAN One-Way ANOVA Post-hoc Test with 95% confidence level ($\alpha = 0.05$). Analysis of this data using the application SPSS v.25 (IBM SPSS Statistics20).

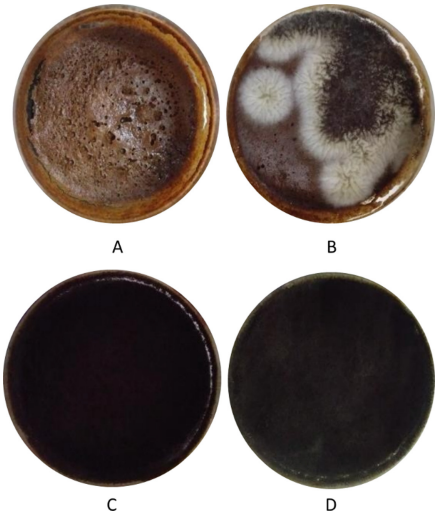

Fig. 2. Colony growth of *Aspergillus aculeatus* Ms.11 on rubber seed press cake fermentation substrate and MGM medium on day: (a) 0, (b) 2, (c) 4, and (d) 7.

3 Results and Discussion

3.1 Growth Profile *Aspergillus aculeatus* Ms.11 Using SSF on Rubber Seed Press Cake Fermentation Substrate

Based on the results of macroscopic observations, the growth of *Aspergillus aculeatus* Ms.11 isolate on rubber seed press cake fermentation substrate was dominated by *Aspergillus aculeatus* Ms.11 mold with white colony morphology that dominated the surface of the fermentation medium. However, the surface of the colony will change into black when the spores have formed on the surface of the media. Observations could be made after the isolates were inoculated into a fermentation medium containing rubber seed press cake and MGM medium and incubated at 30 °C. The growth profile of Ms.11 was shown in Fig. 2.

Based on Fig. 2, *Aspergillus aculeatus* Ms.11 isolates can grow on the surface of the rubber seed press cake substrate. This is indicating that the rubber seed press cake substrate contains the required nutrients for the growth of *Aspergillus aculeatus* Ms.11. especially proteins. Colonies of *Aspergillus aculeatus* Ms.11 isolates began to form white colonies on the 2nd day. It can be seen that on day 2 the colonies have started to form mycelium and spores on the surface of the media (Fig. 2b.). Mold spores will increase when incubation time increasing. This can be seen on day 2 to day 7 (Figs. 2b, 2c, and 2d) and on day 7 the mold begins to form new spores which are marked by the appearance of white fibers above the black surface of the spores.

Growth of isolates of *Aspergillus aculeatus* Ms.11. indicated by the growth of white mycelium colonies on the surface of the medium and the surface of the colony will change into black when mold spores have formed on the surface of the medium. This is in accordance with the research reported by Silva et al., [31] that in macroscopic, *Aspergillus*

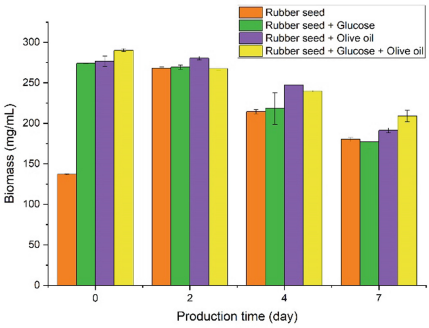

Fig. 3. Biomass on from growth using varied carbon source with production time of 0, 2, 4 and 7 days.

aculeatus has a white mycelium characteristic. *Aspergillus aculeatus* is included in the genus of niger group [19]; [31]. Strains that belong to the niger genus have conidia (spores) characteristics of dark-brown to black with a smooth black surface and have brightly pigmented hyphae at the apex [32].

Measurement of biomass (constant dry weight) from the treatment of substrate variations and fermentation time was carried out by obtaining constant dry weight of fermented solids. This is done by filtering the media using filter paper Whatman No. 1 into a Falcon bottle and drying at 100 °C. The solids were then weighed until a constant weight was obtained.

Biomass result on variations of solid substrate fermentation (rubber seed press cake, rubber seed press cake with added glucose, rubber seed press cake with added olive oil, and rubber seed press cake with added glucose and olive oil) and fermentation time treatment (day 0, 2, 4 and 7) with an incubation temperature of 30 °C as shown in Fig. 3.

Figure 3 was shown that the variation of substrate and fermentation time have affect the result of biomass. The highest biomass was obtained in the treatment with variations of rubber seed press cake + glucose + olive oil substrate on day 0 (290.27 mg/mL). This result was followed by the biomass was obtained in the treatment with variations of rubber seed press cake + olive oil substrate on day 2 (280.40 mg/mL). The lowest biomass was obtained in variations of rubber seed press cake substrate on day 0 (137.03 mg/mL). Biomass will decrease with increasing incubation time. Rubber seed press cake + glucose + olive oil Substrate on fermentation substrate showed the best biomass result.

In this study, culture media with various treatments of rubber seed press cake + glucose + olive oil showed the best biomass result in fermentation and biomass in all treatments (rubber seed press cake, rubber seed press cake + glucose, rubber seed press cake + olive oil and rubber seeds press cake + glucose + olive oil) will decrease with increasing fermentation time. This is showed that the mold isolates can use the carbon source as a nutrient to support growth and metabolism during fermentation which is characterized by a decrease substrate with increasing fermentation time. This study is accordance with research conducted by Cihangir & Sarikaya, [33] which showed that different carbon sources in each treatment will affect lipase activity. The result of biomass and production were influenced by carbon source. The higher addition of carbon

sources, biomass and enzyme production was higher obtained. However, the biomass will decrease with the fermentation time.

Data analysis was performed with the Anova statistical test (sig. < 0.05). The results of statistical tests, the biomass was no significant differences in the treatments. This is indicated that addition of glucose 1.5% and olive oil 1.5% in medium has no effect on biomass of *Aspergillus aculeatus* Ms.11 in lipase production.

3.2 Relative and Specific Activity of Lipase *Aspergillus aculeatus* Ms.11 Using SSF on Carbon Source Variation

Enzyme specific activity was calculated by compare the relative activity with protein concentration per milligram protein. Protein concentration was measured by Lowry method [27]. The principle of Lowry method was reacting the supernatant with Lowry's reagent and absorbance was measured at 750 nm.

Lipase relative activity obtained by calculating amount of Free Fatty Acid (FFA) that was reduced during activation divided by the time used when testing lipase activity using CAP reagent, so esterification activity was obtained.

Relative activity, specific activity, and protein concentration in various carbon source (rubber seed press cake, rubber seed press cake with added glucose, rubber seed press cake with added olive oil, and rubber seed press cake with added glucose and olive oil) and fermentation time treatments (days 0, 2, 4 and 7) with an incubation temperature of 30 °C is presented in Fig. 4.

Figure 4 was shown that substrate variation and fermentation time can affect the results of relative activity, specific activity, and protein concentration. The results show that the highest relative lipase activity was found in variation rubber seed press cake + olive oil substrate on the 2nd day (19.23 U/mL), while the lowest lipase relative activity was obtained in variation rubber seed press cake + glucose substrate at day 0 (8.11 U/mL). The highest lipase specific activity was found in variation rubber seed press cake + olive oil substrate on 7 day (1.16 U/mg), while the lowest lipase specific activity was obtained in variation treatment of rubber seed press cake + glucose substrate on 0 day (0.03 U/mg). The highest protein concentration was obtained in variation rubber seed press cake + glucose substrate on day 0 (321.29 mg/mL), whereas the lowest protein concentration was found in variation treatment rubber seed press cake + olive oil on day 7 (15.67 mg/mL). The substrate with the addition of olive oil (rubber seed press cake + olive oil) on the fermentation substrate showed the best relative activity results in lipase production. The substrate with variation rubber seed press cake with addition of olive oil and rubber seed press cake with addition of glucose was showed the best specific and protein concentration result in lipase production.

The rubber seed press cake with addition of olive oil on day 2 was shown the highest lipase relative activity. This is shown by the graph of the enzyme relative activity which is increasing from day 0 to day 2 then will decrease on day 4 and will rise again on day 7. This trend also applies to the various treatments of rubber seed press cake + glucose substrate, rubber seed press cake + olive oil, and rubber seed press cake + glucose + olive oil. In the variations treatment of rubber seed press cake substrate, the relative activity of the enzyme decreased on the 2nd day and increased again on the 4th day and then decreased again on the 7th day. Based on the results of statistical tests between days,

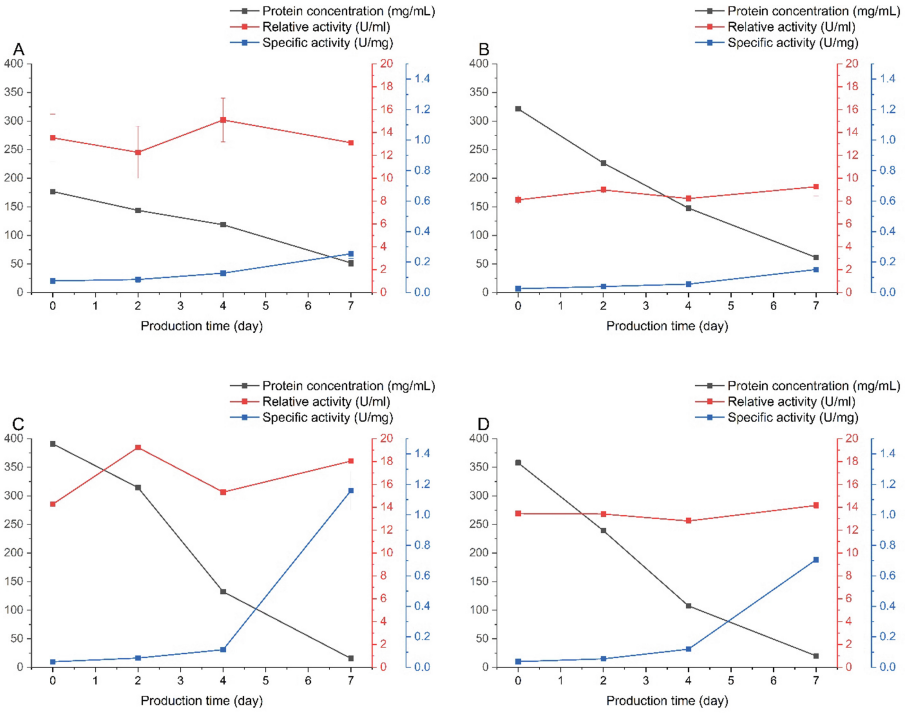

Fig. 4. Relative activity, specific activity, and protein concentration of enzymes in carbon source variations (a) rubber seed press cake, (b) rubber seed press cake + glucose, (c) rubber seed press cake + olive oil, (d) press cake rubber seeds + glucose + olive oil with time fermentation treatment on day 0, 2, 4 and 7.

it shows that there is no difference in the relative activity from day variations that we studied. This is indicating that the relative activity of the lipase enzyme is relatively stable for a long incubation time. The decrease of enzyme activity closely related to reduced nutrient during fermentation and denaturation of enzyme. Denaturation of enzyme can be caused by interactions between components with other or caused by pH changes [34].

The enzyme specific activity was increased and optimum on the 7^{th} day (1,16 U/mg) in all substrate variations with protein concentration decreased with increasing of fermentation time. The specific activity of lipase showed lower results than the study reported by Kamini et al. [35] of 11.06 U/mL protein and Mala et al. [36] of 17.48 U/mL protein. Lipolytic mold isolates were able to grow in a medium containing a carbon source and were able to produce exoenzymes (extracellular lipases). Lipase activity quantitatively is not linear with enzyme activity, this is because lipase activity depends on mold type and size colony [37].

The results of the research showed that the relative and specific enzyme activity increased along with the decrease of protein concentration during fermentation time. The relative lipase activity (19.23 U/mL) was optimum on day 2 with rubber seeds press cake substrate added with olive oil. This indicates that on day 2 the relative activity

of lipase is relatively stable and faster in lipase enzyme production, while the specific activity of the enzyme (1.16 U/mg) is optimum on day 7 with addition of olive oil in rubber seed press cake substrate. The enzyme specific activity was related to enzyme purity. The highest purity of enzyme protein, the higher activity of enzyme [38].

The amount of protein in this study was showed results with a trend tended to decrease with increasing fermentation time. The decreasing amount of protein indicates that this protein was protein that used by Ms.11 to produce lipase. However, the protein in the filtrate cannot shown the specific activity of enzyme. This is because the protein in the filtrate is a mixture of protein from subculture medium, culture medium, rubber seed press cake medium, and enzyme lipase protein. The decrease of protein concentration during fermentation in this study indicated that *Aspergillus aculeatus* Ms.11 was able to use the lipid and carbohydrate components of rubber seeds press cake as a carbon source [39] and can convert complex organic compounds into simpler compounds to lipase production.

Data analysis was performed with the Anova statistical test (sig. < 0.05). Based on the results of the statistical tests, the results for protein concentration and enzyme specific activity on variations in substrate fermentation showed no significant differences in all treatments. However, the results of statistical test of relative activity showed that there were significant differences. This indicates that the addition of glucose 1.5% and olive oil 1.5% in medium affected to the relative activity, but had no effect on protein concentration and specific enzyme activity of Ms.11 to produce lipase.

3.3 Productivity and Yield of Lipase *Aspergillus aculeatus* Ms.11 Using SSF on Carbon Source Variation

Lipase production of lipolytic molds was measured quantitatively by comparing the total lipase unit, lipase productivity, and yield with substrate variation and fermentation time. Lipase productivity was determined by SSF (solid-state fermentation) method with spore suspension of *Aspergillus aculeatus* Ms.11 was inoculated into a fermentation medium with substrates variation rubber seed press cake (control), rubber seed press cake with added glucose, rubber seed press cake with the addition of olive oil, and combination substrate (rubber seeds press cake with the addition of glucose and olive oil). The fermentation process was carried out for 0, 2, 4, and 7 days at 30 °C.

Total lipase unit were calculated by multiplying lipase unit per mL by total filtrate volume of production medium (mL). The data of total lipase unit can be used to calculate yield and productivity of lipase enzymes. The yield calculation is done by calculating the total lipase unit per weight of rubber seed (U/gr), while the lipase enzyme productivity is calculated by dividing the yield with the fermentation time (Yield/day). The yield and productivity of lipase by SSF with substrate variation and fermentation time is presented in Fig. 5.

Figure 5 was shown that the substrate and time fermentation can affect lipase yield and productivity. The highest yield and productivity were obtained in rubber seed press cake + olive oil on day 2, respectively at 98.93 U/g and 186.51 Yield/day. These results were followed by yield results obtained in the variations treatment of rubber seed press cake + olive oil substrate on the 7 day (72.22 U/g) and lipase productivity obtained in the variation treatment of rubber seed press cake + glucose + olive oil substrate on

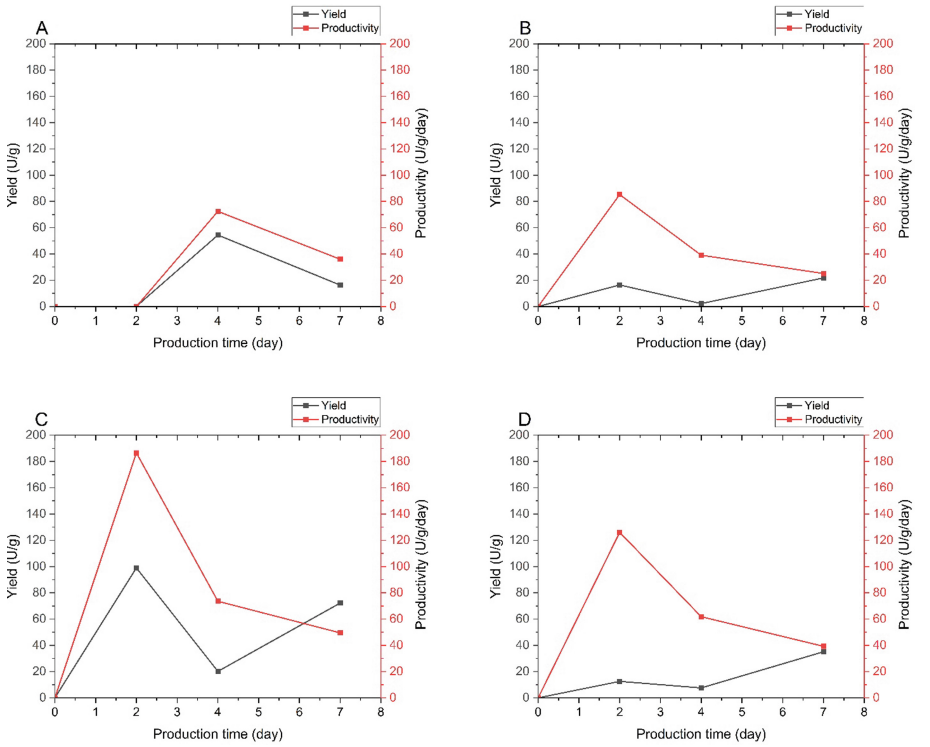

Fig. 5. Lipase yield and productivity in solid substrate fermentation with variations: (a) rubber seed press cake, (b) rubber seed press cake + glucose, (c) rubber seed press cake + olive oil, (d) press cake rubber seeds + glucose + olive oil with time fermentation treatment on days 0, 2, 4 and 7.

2 day (126.07 Yield/day). The lowest yield and productivity of lipase were obtained in rubber seed press cake, rubber seed press cake + glucose, rubber seed press cake + olive oil, and rubber seed press cake + glucose + olive oil on day 0. The substrate with the addition of olive oil (rubber seed press cake + olive oil) on the fermentation substrate showed the best results in lipase production, especially in increasing the amount of yield and lipase productivity.

The results showed that *Aspergillus aculeatus* Ms.11 could grow on the surface of the rubber seed press cake substrate. This indicates that *Aspergillus aculeatus* Ms.11 isolates can grow on rubber seed press cake substrate which contains biochemical compositions: water (%) 5.14, ash (%) 5.42, fat (%) 35.62, protein (%) 29.09, and carbohydrates (%) 24.73 based on proximate analysis (Table 2). These results indicate that the rubber seed press cake contains high fat enough, protein and carbohydrates to support growth. The protein content of rubber seed press cake that obtained was 29.09%. This value is higher than that reported by Giok et al. [40] of (27 g/100 g), Eka et al. [41] (17.41 g/100 g), and Oyekunle & Omode [42] (22.9%). Therefore, rubber seed press cake is a good protein source to support growth and isolate metabolism.

Table 2. Proximate analysis of rubber seed press cake

Components	Analysis Results
Water (%)	5.14 ± 0.00
Ash (%)	5.425 ± 0.005
Fat (%)	35.635 ± 0.015
Protein, fk: 6.25 (%)	29.12 ± 0.03
Carbohydrates (%)	24.68 ± 0.05

The fat content in this study was 35.62%. The value are lower but not different from those value that reported by Oyekunle & Omode [42] (44.00%). However, the value is lower than the value that reported by Eka et al. [41] (68.53%). The carbohydrate content in this study was (24.73%). This value is higher than the value reported by Oyekunle & Omode [42] (4.8%) and Eka et al. [41] (6.99%). The fats and carbohydrates support growth and metabolism of molds.

Inorganic level of the sample was indicated by ash content. The ash content in this study was 5.42%. This value is not different from the value that reported by Oyekunle & Omode (5.00%) [42]. The differences in fat, protein, and carbohydrate content as well as the results of other proximate analyzes in this study when compared with the results from other studies are caused by differences in rubber tree species, climatic conditions, and soil conditions.

Lipase production *Aspergillus aculeatus* Ms.11 on rubber seed press cake with different fermentation substrate (rubber seed press cake (control), rubber seed press cake with addition glucose, rubber seed press cake with addition olive oil, and rubber seed press cake with addition glucose and olive oil) was showed a difference. This is quite large in its lipolytic activity (Fig. 5). This is indicated by the results of total lipase units, yields and high lipase productivity in fermentation. Yield percentage is linear with total lipase units. As the fermentation time increased, the carbon content in the culture media will decreased. Therefore, lipase production will decrease along with decrease of yield. The highest total lipase unit and yield were obtained in rubber seed press cake + olive oil substrate on day 2, respectively at 1865.14 U/mL and 98.93 U/g. This is shows that lipase production is more significant in culture media with olive oil (lipid) addition as a carbon source than in culture media without lipid addition as supplementation. It is also shows that *Aspergillus aculeatus* Ms.11 can use olive oil efficiently for enzyme synthesis and produce biomass. This study is consistent with the study that reported by Mahadik et al. [43] who explained the highest yield was obtained with olive oil as a substrate. The substrate with addition of olive oil also showed optimum lipase activity. The lower glucose concentrations do not suppress enzyme production, but if the concentrations are above 1.0% it can inhibit growth and enzyme production [44]; [45].

Many studies have reported positive results of substrates combination treatment to optimum lipase production [23]. In the study by Triyaswati & Ilmi, [19], it was explained that the best lipase production obtained by medium with containing of glucose 1% + olive oil 1%. This is because glucose is used by molds as an initial source of nutrients for

cell growth and lipase production. However, when the initial nutrient source (glucose) has been exhausted, the mold will use olive oil as a next nutrients source. So, that at 96 h of incubation, the highest lipase activity was obtained which characterized by high biomass.

In this study, carbon compounds such as glucose and olive oil were used. The percentage of carbon source used in medium was olive oil 1.5% and glucose 1.5%. The addition is intended so that the essential components needed for mold growth are met, so that more lipases are obtained. However, this study shows the different results. In this study, it was indicated that the substrate with addition of olive oil is optimal support to produce lipase. The glucose supplemented substrate only supported fungal growth, but did not support lipase synthesis activity Ms.11. This can happen when the main carbon source has been exhausted [39]. Oil on the fermentation substrate will create a suitable environment. The suitable environment will support lipase producing microbes to induced easily, so the lipase can be produced [37].

Based on research that reported by Muralidhar et al., [46] olive oil is a better carbon source than glucose in lipase production. This is supported by research reported by Mobarak-Qamsari [47], Fadiloglu and Erkhmen [48] and Kanimozhi and Perinbam [49], who state that olive oil is the most suitable carbon source and inducer in lipase production.

This study is also in accordance with study that reported by Palilu et al. [50] who explained that olive oil 2% as carbon source showed the maximum lipase production (24.56 ± 1.30 U/mg biomass). The research that conducted by El-Batal et al. [51] also explained that olive oil has a maximum lipase activity (24.0 U/mL) when compared to other carbon sources such as palm oil, corn oil, cotton seed oil, sunflower oil, and glucose.

Data analysis was performed with the Anova statistical test (sig. < 0.05). Based on the results of statistical tests, yield show that there were significant differences in all treatments. However, the lipase productivity showed that there were no significant differences. This is indicating that the addition of glucose 1.5% and olive oil 1.5% in culture medium affected the yield, but did not affect the lipase productivity *A. aculeatus* Ms.11 in produce lipase.

3.4 Glucose Consumption *Aspergillus aculeatus* Ms.11 Using SSF on Carbon Source Variation

The sugar reducing test can be calculated by DNS (Dinitrosalicylic Acid) method. Glucose consumption in isolates of *Aspergillus aculeatus* Ms.11 was obtained from the determination of glucose levels. Glucose levels before and after fermentation were obtained from measurements using the Dinitrosalicylic Acid (DNS) method.

The results of glucose consumption on solid substrate fermentation variations (rubber seed press cake, rubber seed press cake with addition glucose, rubber seed press cake with addition olive oil, and rubber seed press cake with addition glucose and olive oil) and fermentation time treatment (days to 0, 2, 4 and 7) with an incubation temperature of 30 °C is presented in Fig. 6.

Figure 6 was shown that the substrate variation and fermentation time can affect the results of glucose consumption. The highest glucose consumption was obtained in rubber seed press cake + glucose + olive oil substrate on the 7th day (96.37%). These results

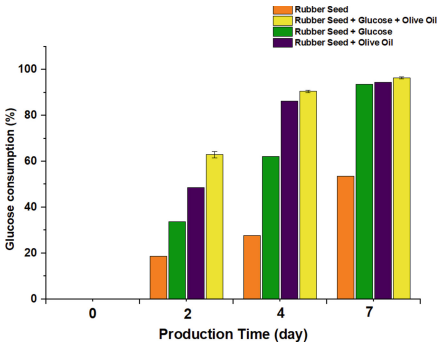

Fig. 6. Glucose consumption in solid substrate fermentation with variations rubber seed press cake, rubber seed press cake + glucose, rubber seed press cake + olive oil, press cake rubber seeds + glucose + olive oil with time fermentation treatment on days 0, 2, 4 and 7.

were followed by the results of glucose consumption obtained in rubber seed press cake + olive oil substrate on the 7th day (94.39%). The lowest glucose consumption results were obtained in rubber seed press cake, rubber seed press cake + glucose, rubber seed press cake + olive oil, and rubber seed press cake + glucose + olive oil substrates on day 0. The substrate with the addition of glucose and olive oil (rubber seed press cake + glucose + olive oil) on the fermentation substrate showed the best results in glucose consumption.

The highest glucose consumption was obtained when treated with variation rubber seed press cake + glucose + olive oil on day 7. The results of glucose consumption showed that the *Aspergillus aculeatus* Ms.11 isolate could use the carbon source in optimal in the substrate combination of rubber seed press cake, glucose and olive oil during the fermentation time to produce high lipase. This study is also accordance with the research that reported by Melliawati et al. [52] which showed that the mold isolate R.6F.18 produced the highest reducing sugar, as well as the enzyme activity it produced. This reducing sugar is closely related to enzyme activity. The enzyme activity increases along with increasing the reducing sugar produced during fermentation.

Glucose and olive oil in this study act as carbon sources. Carbon source is the major component of the fermentative substrate that serve an energy source for microorganisms in lipase production [49]. Glucose is the primary carbon source for lipase production, while olive oil is the secondary carbon source and inducer of lipase synthesis during fermentation. Glucose consumption increased with increasing fermentation time and the level addition carbon and nitrogen (C/N) sources can affect microbial metabolic pathways [53]. Medium containing a combination of glucose and olive oil showed an increase in lipase activity [48]. The addition is intended so that the essential components needed for mold growth are met, so that more lipases are obtained.

Data analysis was performed with the Anova statistical test (sig. < 0.05). Based on the results of statistical tests, glucose consumption shows that there were no significant differences in all treatments. This is indicating that the addition of glucose 1.5% and olive oil 1.5% in culture medium not affected to glucose consumption of *A. aculeatus* Ms.11 in produce lipase.

Based on the research that has been done, we can conclude that rubber seed press cake can be used as a fermentation substrate for *Aspergillus aculeatus* Ms.11 to lipase production. The production of lipase with addition of olive oil on fermentation substrate will be higher than the substrate that is not added with carbon source. The growth profile of *Aspergillus aculeatus* Ms.11 on rubber seed press cake began to grow and could be observed on day 2, while the maximum lipase production of Ms.11 can be obtained using rubber seed press cake with addition of olive oil.

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Authors' Contributions. MN conducted the experiments, analyzed the data that obtained, and wrote the research paper. MI designed the trial to be conducted, supervised the study, provided critical review of the paper, and approved the final version of the manuscript for publication. All authors agree to be responsible for the content contained therein. All authors read and approved the final manuscript.

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