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Lipase Production from *Aspergillus aculeatus* Ms.11 in Broth Medium with Variation of Agitation Speed

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Abstract. Lipase is an industrially important enzyme and is usually obtained from microorganisms, especially filamentous fungi, producing it in a high amount. The method commonly used to produce lipase from filamentous fungi is Submerged Fermentation (SmF) with agitation. However, agitation requires energy and high production cost. Hence, reducing the use of agitation is needed. This research aims to observe the influence of agitation speed toward colony growth, lipase productivity, and nutrient absorption from Indonesian *Aspergillus aculeatus* Ms.11 in broth medium. *Aspergillus aculeatus* Ms.11 was inoculated into 50 mL fermentation medium, which contains 1% of olive oil and glucose mixture with 1:1 comparison. Incubation was done using varied agitation speed (0, 50, 100, 150, and 200 rpm) at 30 °C for 96 h. Relative activity lipase productivity, enzyme-specific activity, and glucose and nitrogen consumption were determined after the incubation. The results show that the highest lipase activity (6.33 U/mL) and lipase specific activity (0.10 U/mg) are observed at 0 rpm, while the highest lipase productivity (0.43 U/mg) is observed at 50 rpm. We concluded that maximum lipase production from Ms. 11 can be obtained using low agitation speed. Further study to optimize carbon and nitrogen sources is suggested.

INTRODUCTION

The lipase enzyme in the triacylglycerol ester hydrolase (EC 3.1.1.3) family catalyzes long-chain triglyceride hydrolysis (and synthesis) into fatty acids, diacylglycerol, monoacylglycerol, and glycerol. In addition to hydrolysis activity, interesterification, esterification, aminolysis, and alcoholysis activity have proven to contribute to a broad range of industrial applications of the enzyme [1]. Lipase is generally produced from microorganisms, especially filamentous fungi, which are known to produce high extracellular enzymes [1,2]. One of the filamentous fungi widely used by industry to produce lipase is the genus *Aspergillus* [3]. Lipase from *Aspergillus sp.* present properties that important for industrial applications, such as pH and temperature stability and high enantioselectivity [4].

The widely used and most successful industrial method to produce lipase from fungi is Submerged Fermentation (SmF) [4]. The method has several advantages, such as ease in handling and reasonable operational control of the process [5]. SmF is greatly affected by media components and physical factors such as agitation, aeration, temperature, inoculum concentration, dissolved oxygen, and incubation time [6,7]. Apart from its advantages, the SmF method requires high energy consumption [8]. Agitation, as one of the required operational conditions, influences the energy consumption and costs for the fermentation process [9]. Reducing the agitation speed theoretically will reduce the energy consumption of the process, hence lower the production cost. However, agitation plays an essential role in oxygen transfer and nutrient distribution in the medium [10]. Therefore, a balance of low agitation speed and high production yield must be determined.

Our previous study has been able to isolate filamentous fungus from non-dairy creamer waste, identified as *Aspergillus aculeatus* Ms.11, which is a potential lipase producer when grown in liquid medium [11]. However, the effect of reducing agitation speed during lipase production from the strain is still unknown. Several studies have been done on the subject and showed that agitation directly influences enzyme productivity and fungal colony morphology [12-14]. Thus, this study examines the effect of agitation speed on fungal colony growth and lipase productivity. Moreover, the absorption of glucose and nitrogen is also observed in relation to agitation speed.

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EXPERIMENTAL DETAILS

Microorganism Maintenance and Inoculum Preparation

Fungal strain *Aspergillus aculeatus* Ms.11 were obtained from the Laboratory of Microbiology, Faculty of Biology, Universitas Gadjah Mada, Indonesia. The strain was grown on Potato Dextrose Agar (HiMedia) slant and maintained at 4 °C. Inoculum preparation was done by growing the fungal strain on slant PDA medium and incubated at 30 °C until sporulated. Then, five mL of sterile distilled water was added into the tube, and the fungal spores were scrapped gently using an inoculation needle. The spore suspension was collected and transferred into a sterile test tube. Spore concentration in the suspension was determined using a Neubauer counting chamber. The suspension was kept at 4 °C until further use.

Lipase Production on Varied Agitation Speed

Production media was made according to Adham & Ahmed [15]. The media consisted of olive oil 0.5% (v/v), glucose [Merck] 0.5% (w/v), peptone [Oxoid] 3% (w/v), $MgSO_4.7H_2O$ [BDH] 0.05% (w/v), KCl [J.T.Baker] 0.05% (w/v), and $K_2H_2PO_4$ [Merck] 0.2% (w/v). Fifty mL of sterile media in 250 mL Erlenmeyer flask was inoculated with Ms.11 spore suspension until the final concentration reached 10^6 spores/mL. The flasks were incubated at 30 °C for four days with varied agitation speed (0, 50, 100, 150, and 200 rpm). All experiments were done in triplicate.

Biomass Weight and Pellet Diameter Measurement

Biomass from the lipase production was obtained by filtering the content of incubated flasks using filter paper of known weight. The diameter of fungal pellets that were trapped on the filter paper was measured using a digital calliper. Then, the filter paper was weighed and washed using sterile distilled water. After that, the paper is dried at 70 ℃ and then weighed again. The difference between the dried filter paperweight with the initial weight was considered as biomass weight.

Lipase Activity Assay

The enzyme-substrate was prepared by mixing 1.58 mL of oleic acid in 8.42 ml of isooctane and 0.29 ml of ethanol in 9.71 mL of isooctane. Lipase was activated by adding 100 µL of crude filtrate into 1 mL substrate in a test tube and incubated at 30 °C for 20 min. The reaction was stopped by placing the test tube on the ice bath for 5 min and let the aqueous phase settled down at the bottom of the tube. For control, a similar reaction mixture was stopped at 0 minutes. The amount of oleic acid before and after the reaction was determined by the cupric acetate pyridine (CAP) colorimetric assay [16]. A 100 µL of the top (organic) phase from the enzyme reaction mixture was added into 1900 µL isooctane and 400 µL CAP reagent. The mixture was vortexed for 5 sec and allowed to stand for 20 min. The absorbance value of the top (organic) phase was measured using a spectrophotometer at a wavelength of 715 nm. One unit of esterification activity (U) was defined as the amount of oleic acid (µmol/mL), which was converted into products per minute by 1 mL of the filtrate containing the enzyme [17].

Lipase Specific Activity and Lipase Productivity Calculation

Lipase specific activity shows the amount of lipase enzyme activity unit per milligram of protein in the filtrate. Protein content was measured using the method based on Lowry [18]. The specific activity of the enzyme was calculated using the following Equation 1.

$$\text{Enzyme specific activity (U/mg)} = \frac{\text{enzyme activity (U/mL)}}{\text{protein concentration (mg/mL)}} \quad (1)$$

Lipase productivity was defined as the number of enzyme-specific units per milligram of biomass. The productivity was calculated using the following Equation 2.

$$\text{Productivity} = \frac{\text{enzyme specific activity (U/mg protein)}}{\text{biomass (mg)}} \quad (2)$$

Determination of Residual Glucose and Nitrogen

The residual glucose in the filtrated fermentation medium was determined using the dinitro salicylic acid (DNS) method [19]. While the residual nitrogen was determined using the Kjeldahl method [20].

Data Analysis

The data obtained were analyzed using One-Way ANOVA Post-Hoc Test with a confidence level of 95% (α = 0.05). Analysis of this data was carried out using the SPSS v.23 application (IBM SPSS Statistics 20).

RESULTS

Growth of *Aspergillus aculeatus* Ms.11 SmF

The growth of *Aspergillus aculeatus* Ms.11 isolates can be seen from the colony diameter and weight of the biomass produced after the fermentation process. One indicator that affects the size of the colony diameter and biomass weight is the speed of agitation. The diameter of the colony was obtained by measuring the retained biomass after filtration using digital callipers. Biomass was also weighted and expressed in mg per mL of fermentation media. The appearance of the measured colony can be seen in Figure 1, while the diameter and weight of the biomass can be seen in Figure 2**.**

FIGURE 1. Fungal colony appearance grew on different agitation speed: (a) 0, (b) 50, (c) 100, (d) 150, and (e) 200 rpm, with incubation at 30 °C for four days.

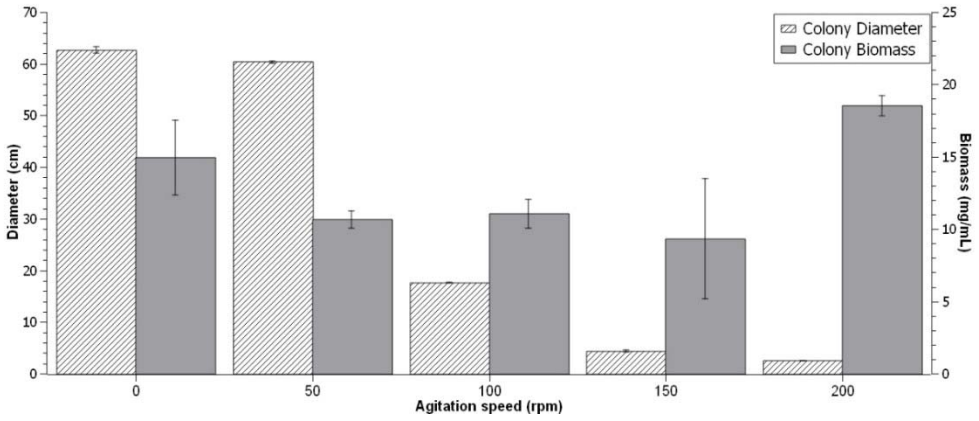

FIGURE 2. Effect of agitation speed on fungal biomass and fungal colony diameter, with incubation at 30 °C for four days.

Based on Figure 1, the colony forms one large pellicle at the agitation speed of 0 rpm and 50 rpm and grows on the surface of the medium. On the other hand, the colony forms round pellets at agitation 100, 150, and 200 rpm. It appears that the pellets' size decreased at a higher agitation speed. Figure 2 shows that the biomass and colony diameter is influenced by agitation speed. The colony diameter is decreased along with the increase of the agitation speed; the smallest colony is 2.52 cm at 200 rpm, and the largest was 62.68 cm at 0 rpm. On the other hand, the total biomass produced is fluctuating; the highest biomass is obtained at 200 rpm agitation speed of 18.7 mg/mL, while the lowest biomass is obtained at 150 rpm agitation speed of 9.4 mg/mL.

Lipase Productivity of *Aspergillus aculeatus* Ms.11

The relative enzyme activity was calculated by detecting the reduction of Free Fatty Acid (FFA) per reaction time and sample volume during enzyme activation using CAP reagent. Lipase productivity was calculated by dividing the relative activity of enzymes with fungal biomass. Figure 3 shows that agitation speeds affect the relative activity and lipase productivity. The lowest relative enzyme activity and lipase productivity are obtained at an agitation speed of 200 rpm, respectively, 0.60 U/mL and 0.03 U/mg. The highest relative enzyme activity (6.21 U/mL) is obtained at 0 rpm, and the highest lipase productivity (0.43 U/mg) is obtained at 50 rpm.

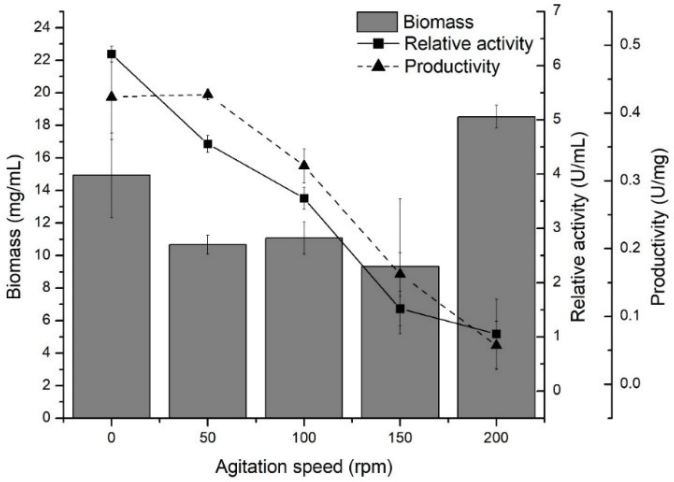

FIGURE 3. Effect of agitation speed on biomass, lipase relative activity, and lipase productivity, with incubation at 30 °C for four days.

Specific Activity of *Aspergillus aculeatus* Ms.11 Lipase

Enzyme specific activity was calculated by dividing the relative activity of the enzyme with protein concentration and expressed in units per milligram of protein. Based on Figure 4, enzyme activity decreases along with the increase of the agitation speed. The lowest relative and specific enzyme activities observed at 200 rpm are 0.60 U/mL and 0.01 U/mg, respectively. On the other hand, the highest relative and specific enzyme activities observed at 0 rpm are 6.21 U/mL and 0.09 U/mg, respectively.

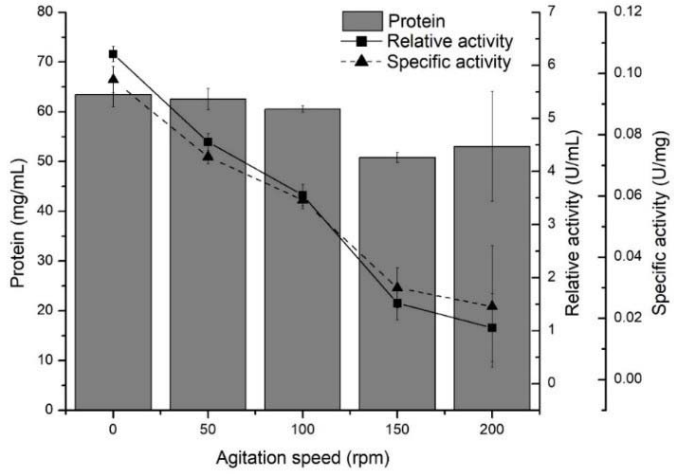

FIGURE 4. Comparison of protein concentration, lipase relative activity, and lipase specific activity produced using various agitation speeds, with incubation at 30 °C for four days.

Nutrient Consumption in Various Agitation Speed

Olive oil and glucose were used as a carbon source, while peptone was used as a nitrogen source. Media that are supplemented with triglycerides such as olive oil are indispensable for lipase production. Moreover, the addition of glucose to the medium can also increase lipase production [21]. Glucose was also used as the initial carbon source for the fungi before utilizing the olive oil. Figure 5 shows that the highest glucose consumption is 96.09% at 50 rpm agitation, while the lowest glucose consumption of 92.59% is found at 150 rpm agitation. The amount of glucose consumption at agitation 50 and 100 rpm is not significantly different ($p \geq 0.05$), while at agitation 0, 150, and 200 rpm are significantly different ($p \leq 0.05$). The highest yield of nitrogen consumption is 60.95% at 200 rpm agitation, while the lowest is 51.46% at 100 rpm agitation.

DISCUSSION

Filamentous fungi can produce extracellular lipase which is excreted through the cell membrane into the medium [22]. Cihangir & Sarikaya added that if the medium is supplied with olive oil as a carbon source and peptone as a source of nitrogen, the obtained lipase yield becomes higher [21]. The fermentation medium used in this study contained 1% olive oil and 1% glucose. Triglycerides such as olive oil are needed in the fermentation medium to increase lipase production [20]. In addition, the peptone in the medium acts as an organic nitrogen source, which can be directly used by filamentous fungi to produce lipases [15].

Aspergillus aculeatus Ms.11 was used in this study to evaluate the production of lipases using submerged fermentation (SmF) for 96 h at 30 °C using glucose and olive oil (1% each) with variations in agitation speed 0, 50, 100, 150, and 200 rpm. The medium composition and incubation temperature and time were chosen based on the previous study [11]. Lipase production in units per milligram of biomass was calculated by comparison between the relative activity of enzymes (U/mL) and filamentous fungi biomass (U/mg).

The variation of agitation speed in this study aims to determine its effect on cell growth, lipase productivity, and consumption of nutrients by filamentous fungi. Based on Figure 2, the biomass weight and colony diameter are influenced by agitation speed. The largest colony diameter is obtained at the lowest agitation speed, which is 0 rpm. The diameter of the colony decreases significantly with increasing agitation speed, from 62.68 to 2.52 cm (Figure 2). These results are consistent with the study by Fomina & Gadd, which shows the lower intensity of agitation causes an increase in the rate of formation of rounded and large colonies [23]. Although colony diameter at agitation speed 0 and 50 rpm are similar, there are differences in the colony density. Colony, which is formed at 50 rpm agitation speed, is more porous than the one formed at 0 rpm (Figure 1), hence the biomass of the later is higher (Figure 2).

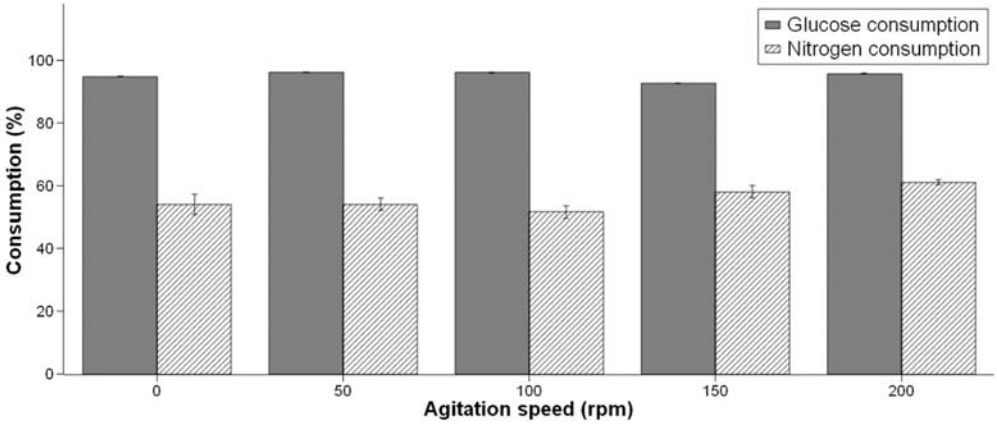

FIGURE 5. Effect of agitation speed on glucose and nitrogen consumption, with incubation at 30 °C for four days.

On average, glucose is depleted in all variations of agitation (Figure 5). The highest glucose consumption (94.66%) is at 50 rpm agitation speed, whereas the lowest consumption (91.23%) can be seen at the agitation speed of 150 rpm. This is not in accordance with the results of Paul *et al*., which states that the larger pellets produced during fermentation resulted in lower glucose utilization rates [24]. They added that lower glucose utilization was inversely proportional to the decrease in the growth rate of biomass pellet diameter.

Based on Figure 2, the lowest pellet diameter is at 200 rpm agitation, where the highest biomass is obtained at the agitation. When the agitation speed is too high, it causes contact and collision between cells so that the pellets formed are denser and single rounded. In addition, high agitation results in better nutrient transfer and higher dissolved oxygen pressure [25], hence the rate of agitation can affect homogeneity in the fermentation medium, and nutrient availability can be distributed evenly, which can result in growth cells run faster, and biomass weight tends to rise [13].

The biomass weight obtained is fluctuating at variations in agitation speed (Figure 2). The highest biomass is attained at an agitation speed of 200 rpm of 18.7 mg/mL with a lipase activity of 1.075 U/mL, while the lowest biomass is obtained at an agitation speed of 150 rpm of 9.4 mg/mL with a lipase activity of 1.548 U/mL. These results are consistent with the results of research conducted by Dalmau *et al*. using *Candida rugosa*, which shows that high biomass does not necessarily indicate high lipase activity [26]. Whereas at 150 rpm agitation, the lowest biomass weight is obtained. This is because the agitation speed of 150 rpm limits oxygen levels, damages the homogenization culture media, and reduces the availability of lipids consumed by cells, resulting in the weight of the biomass being low [27].

Figures 3 and 4 show that lipase productivity and enzyme-specific activity decreases with increasing agitation speed. Under static conditions during 96 hours of incubation, lipase productivity and enzyme-specific activity are high, namely 0.432 U/mg and 0.101 U/mg. Ibrahim *et al*. explain that oxygen solubility is significant in providing energy for cellular activities, such as lipase production [13]. The rate of agitation affects the level of homogeneity in the fermentation media so that the nutrient availability is fulfilled. At lower agitation speeds, oxygen is insufficient in the media, so biomass growth is small. However, the results of lipase activity are high because when compared with the results of lipase productivity and specific activity at 200 rpm, static conditions can produce higher lipases within 96 h than those of 200 rpm. While the results of lipase productivity and enzyme-specific activity at the agitation speed of 200 rpm are the lowest at 0.033 U/mg and 0.025 U/mg, but at the agitation speed of 200 rpm the largest biomass yield is 18.7 mg/mL (Figure 3). Paul *et al*. explain that biomass concentrations increase with higher agitation speeds [24]. At agitation speeds of 50, 100, and 150 rpm, biomass results are not significantly different. This shows that the agitation process is not enough to flatten oxygen and nutrients into the entire fermentation or homogenization media is not going well.

Lipase production by filamentous fungi is strongly affected by carbon and nitrogen sources in the medium [28]. At the highest agitation speed (200 rpm), low specific enzyme activity is obtained (Figure 4). According to Figure 4, the results of protein concentration tend to be the same. The results show that protein concentration in the variation of agitation is not significantly different in all variations of agitation. This can happen, possibly because peptone as a source of too much nitrogen is given in the medium. Cihangir & Sarikaya explain that *Aspergillus* sp. show a good level of lipolytic activity in peptone 1% medium [21].

The highest agitation speed (200 rpm) shows that the aeration is going well so that the growth of filamentous fungi produced is maximum. There is low lipase productivity with high biomass at 200 rpm agitation (Figure 3). This is because the lipase activity produced at 200 rpm for 96 hours of incubation has entered the second stationary phase, at which time the carbon source derived from glucose in the first 24 to 48 h has been used to produce lipase (Figure 5). This is in accordance with the research of Mangunwardoyo *et al.* that at the 20th-h, lipase activity is obtained [22]. Glucose as a carbon source has been used up in the initial 20 hours of incubation, and cells begin to secrete lipolytic enzymes to break down the lipid substrate as another carbon source. This is supported by high biomass growth at 200 rpm agitation (Figure 3) and glucose consumption, which tends to run out at 96 h of incubation with the same initial glucose administration in all agitation variations (Figure 5).

Pereira-Meirelles *et al*. explain that only lipases in the basal level will be detected in the medium because lipase production at the beginning of growth is cell-bound lipase [29]. The possible causes of decreased lipase activity are during the second stationary phase. Extracellular lipase begins to be secreted into the medium when about 50% of the carbon source (glucose and olive oil) has been consumed by filamentous fungi to produce lipase and will reach at the end of the stationary phase.

SUMMARY

Based on research that has been done, it can be concluded that the speed of agitation is very influential on the growth of *Aspergillus aculeatus* Ms.11. The higher agitation speed causes, the denser the pellets formed. However, variations in agitation speed produce fluctuating biomass. On the other hand, lipase production decreases with higher agitation speed. The absorption of nutrients in all variations of agitation tends to be exhausted by giving the same initial glucose.

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