

Characterization of *Aspergillus Niger* 65i6 lipase from solid-state fermentation using *Jatropha* seed cake medium

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Abstract

Jatropha curcas seed cake contains a high amount of protein, and consequently has very high potential as a medium for lipase production. The objective of this research was to characterize lipase from *Aspergillus niger* 6516, which was produced by solid-state fermentation on *Jatropha curcas* seed cake as the medium. The effects of pH and temperature on enzyme activity were evaluated, along with substrate specificity and enzyme stability. Fermentation was performed at a water concentration of 63% and temperature of 30 °C for 7 days. The results showed that the optimum pH and temperature for *Aspergillus niger* 6516 lipase activities were 8.0 and 40 °C, respectively. The lipase had the substrate specificity to hydrolyze long-chain fatty acids and was stable in polar organic solvents. The lipase had a molecular weight, K_m and v_{max} about 19 kDa, 0.27 $\mu\text{mol/ml}$, and 52.63 $\mu\text{mol/ml/min}$, respectively. The results also suggested that the produced lipase from *Aspergillus niger* 6516 was an alkaline lipase. Based on these results, we conclude that *Jatropha* seed cake is a suitable medium for lipase production.

Keywords: Lipase, *Aspergillus niger*, *jatropha* cake, solid state fermentation

Introduction

Lipase (E.C.3.1.1.3) is an enzyme that catalyzes both the hydrolysis of esters (Bora *et al.*, 2011; Cho *et al.*, 2011), and under certain conditions the synthesis of esters (Akanbi *et al.*, 2015). Since the reaction is reversible, it can be used in many applications, such as food, chemicals, pharmaceuticals, biosensors, pesticides, leather, cosmetics, biodiesel, and detergents (Akanbi *et al.*, 2015; Hasan *et al.*, 2006; Berchmans and Hirata, 2008; Bisen *et al.*, 2010; Zang *et al.*, 2011).

Lipase can be found in plants, bacteria, and fungi. Plant lipase has been produced from *Jatropha* seeds (Abigor, 2002; Hidayat *et al.*, 2014), palm oil (Ebongue *et al.*, 2006), and rice bran (Chong *et al.*, 2007; Hidayat *et al.*, 2014). Bacterial lipases have been obtained from *Bacillus coagulans* and *Pseudomonas aeruginosa* (Alkan, 2007; Mahanta *et al.*, 2008). Meanwhile, yeast lipase has been produced mostly from *Candida rugosa* and *Candida antarctica* (Bussamara, 2010). Most lipases produced by fungi are from *Rhizopus sp.*, *Aspergillus sp.*, *Penicillium sp.*, and *Rhizomucor sp.* (Cihangir and Sarikaya, 2004; Gutarra *et al.*, 2009; Mahapatra *et al.*, 2011; Damaso *et al.*, 2008; Silva *et al.*, 2011; Kempka *et al.*, 2008). In general, the optimum pH for bacterial lipase is neutral or alkaline, and it is more thermostable than plant lipase. Yeast lipase is mostly in the form of isoenzyme and has a

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molecular weight of 33–65 kDa (Vokhlu and Kour, 2006).

In enzyme production, the cost of medium should also be taken into consideration. Agro-industrial wastes including wheat bran (Mahadik *et al.*, 2002; Mahapatra *et al.*, 2011), sugarcane bagasse (Rodriguez *et al.*, 2006), and babassu cake (Silva *et al.*, 2011) have been used as alternative media for the production of lipase using solid-state fermentation (SSF). *Jatropha curcas* seed cake is the by-product of oil extraction, containing a high concentration of protein and carbohydrates. It is very potential to be used as a source medium for C and N in the production of enzymes, especially lipase. Mahanta *et al.* (2008) used *Jatropha curcas* seed cake as a medium for lipase production from the bacteria *Pseudomonas aeruginosa*. However, fungi are much more suitable for solid-state fermentation since it creates a restricted condition that is almost similar to their natural environment.

Most fungi lipases have been produced using solid-state fermentation, such as that produced by *Penicillium restrictum* (Gombert *et al.*, 1999), *Aspergillus niger* (Kamini *et al.*, 1998; Mahadik *et al.*, 2002), and *Rhizopus oligosporus* (ul-Haq *et al.*, 2002). It is a fermentation method that involves the growth of microorganisms in moist solid-substrate in the absence of free flowing water. The benefit of this low water requirement is a method that is simple, economical, and lower in energy demand and contamination risk, as well as superior in productivity.

In addition to the medium and fermentation conditions, lipase production also depends on the isolate. Microbial selections are very important, as well. The objective of this research was to investigate the characteristics of lipase from the selected fungi *Aspergillus niger* 6516 using solid-state fermentation on *Jatropha curcas* seed cake as the medium. The optimum pH and temperature for lipase activity, substrate specificity, and stability in organic solvents, were evaluated. Partial purification of the enzyme using

anion exchange chromatography was also performed.

Materials and Methods

Fungal strain

Aspergillus niger 6516 was obtained from the Laboratory of Biotechnology, Department of Food and Agricultural Product Technology, Universitas Gadjah Mada, Indonesia. The strain was maintained by monthly transfers to potato agar slant tubes, incubated at 30 °C and stored at 4 °C.

Materials

Jatropha seeds and palm oil were obtained from a local supplier in the Special Region of Yogyakarta, Indonesia. Methanol, pyridine, NaH₂PO₄, Na₂HPO₄, Cu acetate, sodium acetate, acetic acid, TRIS, acetone, n-hexane, and HCl were obtained from Merck KGaA (Germany). Olive oil, iso-octane, and tributyrin were obtained from Sigma-Aldrich (USA), and oleic acid from AppliChem (USA).

Fermentation Conditions

The medium for lipase production was prepared by pressing the *Jatropha* seeds using a press machine (Paul Weber Maschinen GmbH, Germany) at 140.6 kg/cm. The pressed *Jatropha* seed cake was ground and then defatted using an n-hexane solvent (1:6). The resulting defatted cake was dried and further ground into *Jatropha* seed powder, ready for use as a solid-state fermentation medium.

Fermentation was carried out according to the method of Falony *et al.* (2006). Medium containing 5 g of *Jatropha* cake powder and 2% olive oil (v/w) was autoclaved at 121 °C, and further inoculated with fungal cultures. Inoculum concentration was 10⁷ spores/ml while water concentration was 63%. The medium was incubated at room temperature for 7 days.

Enzyme Extraction

Extraction of lipase was performed according to the method of Gutarra *et al.*

(2009). Fermented medium was added into 0.1 M extraction buffer (5 ml/g substrate) and mixed in a rotary shaker at 30 °C and 100 rpm for 20 min. Solid phase was separated by filtration whereas liquid phase was further centrifuged at 3000 rpm for 30 min. The supernatant was used as a crude enzyme and stored at -18 °C for further analysis.

Enzyme Purification

The crude enzyme was added into an anion exchange column and washed with an adsorption buffer. The adsorbed lipase was eluted with an elution buffer containing 1 M NaCl, after which it was stored at -18 °C and used as a partially purified enzyme for further analysis.

Effect of pH on Lipase Activity

The effect of pH on lipase activity was studied by extracting the fermented medium with buffers at various pH levels (pH 4.0–9.0), namely acetate buffer (pH 4.0–5.0), phosphate buffer (pH 6.0–7.0), and Tris-HCl buffer (pH 8.0–9.0). The buffer was added into fermented seed cake (5 mL/g substrate) and mixed in a rotary shaker at 30 °C and 100 rpm for 20 min. The solid phase was separated by filtration and supernatant was centrifuged at 3000 rpm for 30 min. The supernatant was used as a crude enzyme. A partially purified enzyme was obtained by anion exchange purification. Lipase activity was further determined.

Effect of Temperature on Lipase Activity

The fermented medium was extracted with a buffer (5 mL/g substrate) at pH 8.0. Partially purified lipase was obtained by anion exchange purification step. Furthermore, the crude and partially purified lipase activities were determined after incubation at various temperatures (30–70 °C) for 20 min.

Substrate specificity

The substrate specificity of lipase was determined by incubating the crude enzyme in different kinds of substrate (olive

oil, *Jatropha* oil, palm oil, and tributyrin) in iso-octane (60% w/v). The reaction mixture was incubated at 40 °C for 60 minutes. The reaction was stopped by placing the reaction mixture in an ice bath. About 200 µL aliquots were taken and added into the reaction tubes containing 1800 µL iso-octane and 400 µL of 5% cupric acetate pyridine pH 6.0. The content of free fatty acids in the mixtures was determined by comparing the absorbance of the mixture with standard curve of free fatty acids at 715 nm. One unit of lipase activity was defined as the amount of enzyme that released 1 µmol of fatty acids from acylglycerol per min.

Lipase Stability in Organic Solvents

Crude lipases were diluted in various solvents (methanol, acetone, and n-hexane) at a ratio of 1:1. Each mixture was then incubated in a waterbath at 37 °C at 60 rpm for 1 hour (Pera *et al.*, 2006). The remaining lipase activity in the solvent was then determined. Lipase stability was determined by comparing the activity of the lipase in the solvents and buffer.

Hydrolytic Activity Analysis

Lipase activity was determined according to Marseno *et al.* (1998). The substrate (60% olive oil in iso-octane) was prepared by mixing 60 ml of olive oil with 40 ml of iso-octane. The reaction was started by the addition of 200 µl of enzyme to 2 ml of the substrate. The mixture was incubated in a 30 °C shaker water bath at 120 rpm for 20 min. The reaction was stopped by placing the mixture in an ice bath. About 200 µl was added into reaction tubes containing 1800 µl of iso-octane and 400 µl of 5% cupric acetate pyridine (pH 6.0). The content of free fatty acids in the mixtures was determined by comparing the absorbance of the mixture with the standard curve of free fatty acids at 715 nm. One unit of lipase activity was defined as the amount of enzyme that released 1 µmol of fatty acid per min.

Esterification Activity Analysis

About 4 ml of medium containing oleic acid (0,5 M) and methanol (0,5 M) (molar ratio 1:1) in iso-octane was added into an Erlenmeyer flask, and adding 200 μ l of enzyme solution started the reaction. The mixture was incubated in a 30 °C water bath shaker at 120 rpm for 20 min, after that the reaction was stopped by placing the reaction mixture in an ice bath for 5 min. An aliquot of 200 μ l was taken and added to a mixture of 1800 μ l of iso-octane and 400 μ l cupric-acetate pyridine (CAP) (pH 6.0). Free fatty acids in the mixture were determined in the same manner as that described for the hydrolytic activity assay. One unit of lipase activity was defined as the amount of of free fatty acid (μ mol) that reacted with methanol per min.

Results and Discussion

Composition of *Jatropha* Solvent-defatted Press cake

Table 1 shows the composition of the *Jatropha* press cake and the solvent-defatted press cake. The dried *Jatropha* seeds were containing of 56.81% oil, which decreased 50% after oil extraction using a hydraulic press machine (Table 1). Furthermore solvent-based extraction of oil in the *Jatropha* press cake using n-hexane resulted in lower oil content (0.55%), as well as higher protein content (42.58%) because of the released

Table 1. Composition of *Jatropha* press cake and solvent-defatted *Jatropha* press cake.

No.	Composition	Press cake (%)	Solvent-defatted press cake (%)
1	Oil (%)	28.68	0.55
2	Protein (%)	30.55	42.58
3	Water (%)	10.43	15.59

protein from the seeds. As a consequence, nitrogen content in the medium increased without organic or inorganic nitrogen compounds being added to the fermentation medium.

Partial Purification of Lipase Using Anion Exchange Chromatography

Partial purification of *Aspergillus niger* 65I6 lipase was performed in order to achieve higher specific activity in the lipase. Table 2 shows the results of partial purification using anion exchange chromatography. One step purification using anion exchange chromatography resulted an increasing in specific activity from 34.82 U/mg to 421.97 U/mg. These results indicate that one step purification resulted in high enzyme purity, in which the purification factor was 12.1 and lipase yield 86.11%.

Effect of pH on *Aspergillus niger* 5I6 Lipase Activity

Fig. 1 shows that hydrolytic lipase activities increased 4.5 times with an increase in buffer pH from 4.0 to 8.0. Further increase to 9.0, however, resulted in lower hydrolytic activity by about 1.5 times. Lipase activity was not significantly different at buffer pHs of 5.0 to 7.0. Meanwhile, esterification activity increased about 4.9 times when the buffer pH increased from 6.0 to 8.0, but again decreased when the buffer pH was raised to 9.0. Therefore, the optimum buffer pH was pH 8.0. This suggests that *Aspergillus niger* 65I6 produced an alkaline lipase from the *Jatropha curcas* seed cake medium, contrasting other studies which report that most lipases produced by *Aspergillus* are acidic (Pera *et al.*, 2006; Shu *et al.*, 2009; Mhetras *et al.*, 2009; Toida *et al.*, 1998). Alkaline lipases have

Table 2. Partial purification of lipase from *Aspergillus niger* 65I6 using anion exchange chromatography.

No.	Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor (-)
1.	Crude	5.57	193.88	34.82	100	1
2.	Anion Exchange	0.39	166.9	421.97	86.11	12.1

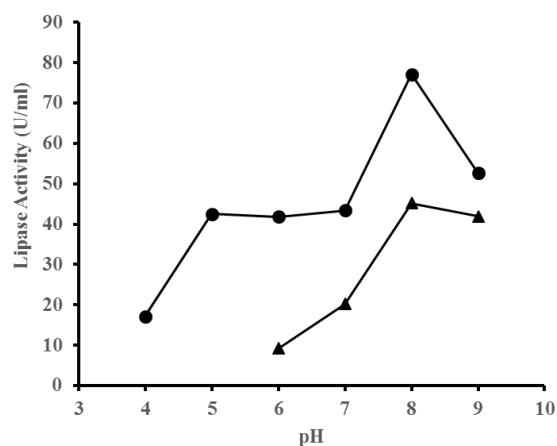


Fig. 1. Effect of pH buffer on hydrolytic (●) and esterification activity (▲) of *Aspergillus niger* 65I6 lipase.

been reported from other strains, however, including *Fusarium oxysporum* (Prazeres *et al.*, 2006; Rifaat *et al.*, 2010), *Fusarium solani* (Maia *et al.*, 1999), *Penicillium chrysogenum* (Cho *et al.*, 2007), and *Rhizopus chinensis* (Sun and Xu, 2009).

The differing characteristics of the *Aspergillus niger* 65I6 lipase is probably related to the physiology of the microorganism. According to Ward *et al.* (2006), the activity of enzymes at certain pH values corresponds with the environmental pH. It is to be expected that gene regulation is responsible for mediating this characteristic. Thus, the alkalinity of the lipase from *Aspergillus niger* 65I6 probably reflects its medium's environment, which supports its growth.

Effect of Temperature on *Aspergillus niger* 65I6 Lipase Activity

Fig. 2 shows the effect of temperature on lipase activities. Hydrolytic activities increased about 1.2 times when the temperature increased from 30 °C to 40 °C. However, increasing the temperature to 50 °C resulted in a decrease in hydrolytic activity by about 2.5 times, while an increase from 50 °C to 70 °C saw another slight decrease. Similarly, although to a smaller extent, esterification activity increased about 1.1 times when the temperature increased from 30 °C to 40 °C, but decreased by about 2 times

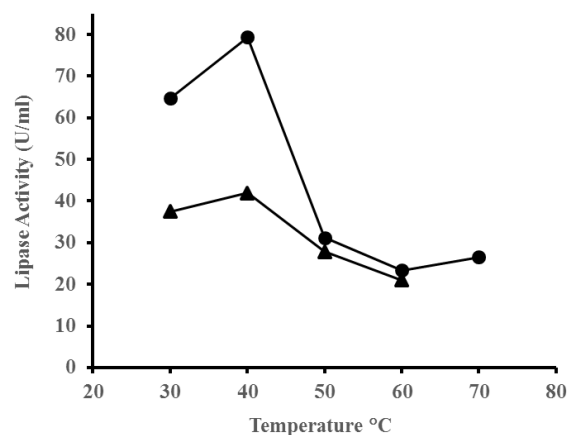


Fig. 2. Effect of temperature on the hydrolytic (●) and esterification activity (▲) of *Aspergillus niger* 65I6 lipase.

with a temperature increase of 40 °C to 60 °C. The optimum temperature for lipase activity was therefore 40 °C, suggesting that lipase from *Aspergillus niger* 65I6 is a mesophilic lipase. These results are similar to those of Falony *et al.* (2006), Ferrer *et al.* (2000), and Chahinian *et al.* (2000).

Temperature may affect the molecular structure of an enzyme along with its catalytic activity. The rate of enzymatic activity can double with every 10 °C increase in temperature (Copeland, 2000). However, the enzyme's activity rate will eventually decrease significantly as the result of denaturation. Thus, the decrease in lipase activity at temperatures above 40 °C was probably a reflection of thermal denaturation in the *Aspergillus niger* 65I6 lipase.

Substrate Specificity of Lipase

One of the characteristics of enzyme is substrate specificity. Since lipase has been used as a biocatalyst for the synthesis of biodiesel, the substrate specificity of the *Aspergillus niger* 65I6 lipase was evaluated. Fig. 3 shows that lipase hydrolyzed olive oil 2.06, 30, and 595 times faster compared with *Jatropha* oil, palm oil, and tributyrin, respectively. This indicates that *Aspergillus niger* 65I6 lipase has the specificity to hydrolyze long-chain triacylglycerols. The hydrolysis specificity can be seen from the

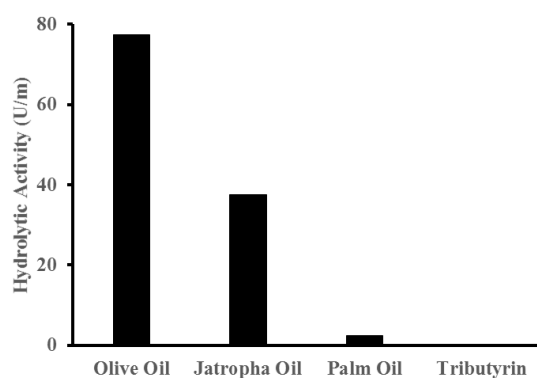


Fig. 3. Substrate specificity of lipase from *Aspergillus niger* 65I6.

fatty acids content of the triacylglycerols of the oils. The triacylglycerol of olive oil is composed mostly of long-chain fatty acids, containing 55–83% of oleic acid (C18:1) (Gunstone *et al.*, 2007), whereas *Jatropha* oil contains 34–45% of oleic acid and 29–44% of linoleic acid (C18:2). Palm oil contains middle-chain triacylglycerols, such as lauric acids (48%), while tributyrin contains short-chain triacylglycerols, such as butyric acid (C4). Tributyrin was the lowest substrate to be hydrolyzed by the *Aspergillus niger* 65I6 lipase. Thus, lipase from *Aspergillus niger* 65I6 exhibited higher specificity to hydrolyze long-chain fatty acids from olive oil than any other substrate (Fig. 3).

Stability of *Aspergillus niger* 65I6 Lipase in Organic Solvents

Lipases catalyze reversible reactions, especially the hydrolysis and synthesis of ester bonds. The reactions are controlled by the availability of water in the reaction mixture. The synthesis reaction will occur only when the water availability in the reaction mixture is relatively low. Sometimes, organic solvents are added to increase the solubility of hydrophobic substrates. However, the enzymes are generally unstable during the addition of these organic solvents.

Fig. 4 shows that incubation of lipase in acetone resulted in a decrease in enzyme activity by about 50%, while incubation in

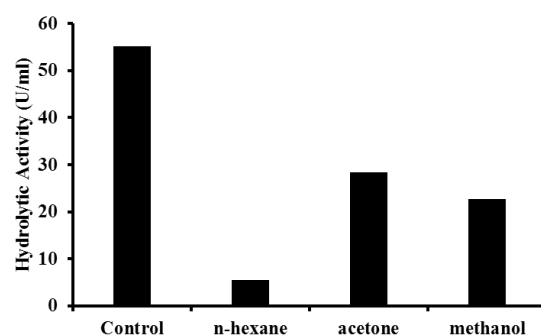


Fig. 4. Stability of lipase from *Aspergillus niger* 65I6 in various organic solvents.

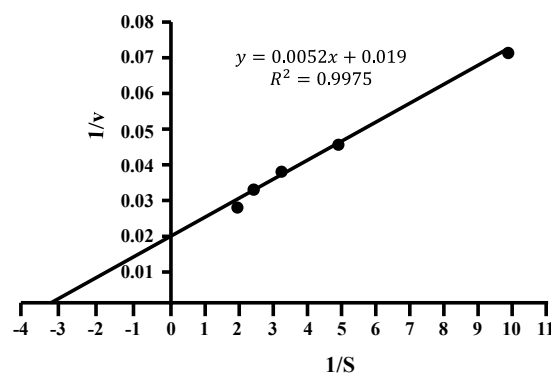


Fig. 5. Lineweaver-Burk or double reciprocal transformation of velocity against substrate concentration.

methanol resulted in a decrease in enzyme activity by about 60%. The addition of a non-polar solvent, n-hexane, resulted in a decrease in enzyme activity by 90%. These results show that lipase from *Aspergillus niger* 65I6 had higher stability in a polar organic solvent compared with a non-polar organic solvent.

In general, the addition of a polar organic solvent has a more destabilizing effect than a non-polar organic solvent (Rifaat *et al.*, 2010). Our findings were an exception, but in agreement with the results of Kukreja and Bera (2005), which showed that the addition of hexane might cause the loss of enzyme activity by up to 50%, whereas the addition of acetone and methanol results in a loss of only about 10% in lipase from *Pseudomonas aeruginosa*.

Kinetic Parameters

Fig. 5 shows Lineweaver-Burk or double reciprocal transformation of velocity substrate concentration. These types of plots generate linear regressions and enable easier determination of the Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}). The results indicate that lipase activities followed simple Michaelis-Menten kinetics. The apparent K_m and V_{max} were 0.27 $\mu\text{mol}/\text{ml}$ and 52.63 $\mu\text{mol}/\text{ml}/\text{min}$, respectively.

Conclusion

With *Jatropha curcas* seed cake as the medium, lipase from *Aspergillus niger* 65I6 was produced using solid-state fermentation. One step purification using anion exchange resulted in an increase in specific activity from 34.82 U/mg to 421.97 U/mg. The purification factor and lipase yield were 12.1 and 86.11%, respectively. The results further showed that the lipase from *Aspergillus niger* 65I6 was alkaline, and the optimum pH and temperature for lipase activities were pH 8.0 and 40 °C, respectively. The lipase had the specificity to hydrolyze long-chain fatty acids of acylglycerol. It was stable in the presence of polar organic solvents, specifically acetone of methanol. The apparent K_m and V_{max} of the *Aspergillus niger* 65I6 lipase were 0.27 $\mu\text{mol}/\text{ml}$ and 52.63 $\mu\text{mol}/\text{ml}/\text{min}$, respectively.

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