Production and Optimization of Lipase by Aspergillus niger using Coconut Pulp Waste in Solid State Fermentation

E Suyanto1,2*, E S Soetarto3 and M N Cahyanto4

1 Biology Department, Faculty of Mathematics and Natural Sciences Universitas Brawijaya, Malang, Indonesia
2 Research Centre for Smart Molecules of Natural Genetic Resources, Universitas Brawijaya, Malang, Indonesia
3 Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia
4 Faculty of Agricultural Technology, Universitas Gadjah Mada, Yogyakarta, Indonesia

*Corresponding author’s email: e.suyanto@ub.ac.id

Abstract. Lipase has high economical value and an attribute that treasures wide range applications. Among the microbes, fungi to be the best source for lipase production in agro-industrial residue using solid state fermentation (SSF). One of agro-industrial residue is coconut pulp waste. The aims of this study is to explore the possibility of coconut pulp waste for producing lipase using fungi isolate, Aspergillus niger and find the partial optimum condition of lipase production based on 2 parameters only. The crude enzyme was extracted by mixing fermented substrate with 50 mL sterile aquades pH 7 containing 0.01% Tween-80. The protein concentrations are determined by Bradford method using spectrophotometric. Lipase activity was followed the titrimetric method. The SSF was carried out with variation of initial spore concentration of 1x10⁵, 1x10⁶, 1x10⁷, and 1x10⁸ (spores.mL⁻¹) and crude enzyme was harvested by adding 25, 50, 75, 100, and 125 (mL) of extract solution. The result showed that initial spore concentration and extract solution affected of lipase production. The SSF in coconut pulp waste using A. niger has successfully produced lipase with optimum condition were achieved by adding spore concentration inoculum 1x10⁶ spore.mL⁻¹ in acidic condition at 30°C for 7 days and extracted by 75 mL of extract solution that was result 72.5 mL of crude enzyme with lipase activity 10.83 U.mL⁻¹ and protein content 0.074 mg/mL⁻¹.

Keywords: lipase, fungi, SSF, coconut pulp waste

1. Introduction
Lipase (triacylglycerol acyl hydrolases, E.C 3.1.1.3) has high economical value and also catalyse the hydrolysis of triglycerides to glycerol and free fatty acids [1]. The application of lipase including food additives, detergents, cosmetics, pharmaceuticals, leather processing, biodiesel, organic synthesis, medical area, treatment of domestic and industrial wastes [2],[3],[4]. Due to their distinctive catalytic potential, lipase is one of the most crucial industrial enzyme [5].

Lipase produced by plant, animal and microbe such as bacteria, yeast and fungi. One of the lipolytic fungi is Aspergillus producing lipase for industrial application [6]. Lipase originated from microbes are the better sources to provide high stability and offer ease of cultivation. Among the microbes, fungal lipases to be the best source for lipase production because thermally stable with high turnover number and easy recovery of extracellular enzymes [7].
Generally, lipase have been produced using submerged fermentation (SmF) method although it is high cost of equipment, media and higher probability of contamination. The alternative technology for producing lipase using solid state fermentation (SSF) to improve yield of enzyme and its activity which require less energy than SmF [8]. SSF also offers advantages including ease of extraction and resistance to contamination. SSF technology is currently the best method to obtain fungi spore on the non-soluble matrix that acts as a natural habitat for filamentous fungi [9]. Substrate of SSF achieved by using low cost accessible agro-industrial residues and provide nutrient source to microbial cells. These are being prospect in developing countries and problems associated with their disposal also important environmental concern. One of agro-industrial residue is coconut pulp waste prospective for developing as substrate of SSF.

Therefore, in the present study we explored the possibility of coconut pulp waste for producing of lipase using fungi isolate as *Aspergillus niger* and find the optimum condition of lipase production based on parameters such as initial spore concentration and volume of extract solution.

2. Materials and Methods

2.1. Microorganism and growth media

The filamentous fungi strains were isolated from leaf litter located in Arboretum (7°45’58.6"S 110°22’31.3"E), Yogyakarta, Indonesia. The fungi isolates were cultured in Potato Dextrose Agar (PDA) media with composition (g L⁻¹) 4-potato extract, 20-dextrose, 15-agar then incubated at 30°C for 7 days. We found KLC-33 isolate, *Aspergillus niger* so high for lipolytic enzyme on based on result of screening (qualitative method and quantitative method) that we used it for advanced study.

2.2. Solid substrates and chemicals

Coconut pulp waste was obtained from UD Barepan, Moyudan, Yogyakarta, Indonesia. Preparation of coconut pulp as substrate for SSF carried out by drying coconut pulp under the sun’s heat and then drying in incubator at 60°C for 3 h until the moisture content around 5-10%, grounded and sieved to provide particle size 250 μm prior to use. It was stored at room temperature. All the media components and chemicals were of analytical reagent grade.

2.3. SSF and enzyme extraction

The spores of *A. niger* on PDA slant were harvested using 0.01% Tween-80 solution then suspended in 3 mL of sterile distilled water with 0.01% Tween-80 then added 7 mL again for release spores from agar surface [10],[11]. The basal fermentation media of coconut pulp adjusted to pH 7 with moisture content to 60% then it sterilized. SSF was carried out by adding 1 mL of spores biomass into 5 g of coconut pulp then incubated at 30°C for 7 days. Crude enzyme was extracted by mixing fermented substrate with 50 mL sterile aquades pH 7 containing 0.01% Tween-80 on rotary shaker (150 rpm) at 30°C for 30 min. The suspension was filtered by sterile filter paper then filtrat was centrifuged (4000 rpm) at 4°C for 30 min. The supernatant (crude enzyme) was used for lipase assay and then stored at -20°C [12].

2.4. Enzyme assays

2.4.1. Determination of protein. Determination of protein of crude enzyme determined by Bradford method using Bovine Serum Albumin standard based on the reaction of the enzyme with 0.5 mL Bradford reagent at 30°C for 2 min. The absorbance read at λ 595 nm. The soluble protein was determined by converting the absorbance value into standard curve.

2.4.2. Lipase activity. Determination of lipase activity in crude enzyme followed the titrimetric method [13]. Lipase activity determined in substrate solution with ratio of arabic gum: 0.2 M phosphate buffer pH 7: coconut oil was 2:1:1. All components of substrate solution mixed until homogenous. It taken 0.1 mL and put into flask then added 1 mL of 1 M CaCl₂. The solution mixed with 0.01 mL of crude enzyme, homogenized by vortex and incubated on rotary shaker (150 rpm) at 30°C for 1 h. The reaction was stopped by adding 5 mL of acetone:ethanol (1:1) mixture after the incubation time was reached then
homogenized by shaking. Titration was performed with 0.01 N NaOH until the color of solution become pink. One unit (U) lipase activity was defined as the amount of enzymes which were used to produce one µmol of fatty acid equivalent per minute per mL under specified assay conditions.

\[
\text{Lipase activity} = \frac{\Delta V \times N \times 1000}{V_{\text{sample}} \times T}
\]

where \(\Delta V = V_2 - V_1\), \(V_1\) the volume of NaOH as control, \(V_2\) the volume of NaOH as experimental, \(N\) the normality of NaOH, \(V_{\text{sample}}\) the volume of enzyme and \(T\) incubation time.

2.5. Effect of initial spore concentration. The effect of spore concentrations for inoculum studied by using variation of spore concentration such as \(1\times10^5\), \(1\times10^6\), \(1\times10^7\), and \(1\times10^8\) (spores.mL\(^{-1}\)). It was inoculated into 5 g coconut pulp pH 7 with moisture content of 60% then the fermentation was incubated at 30°C for 7 days. Crude enzyme harvested by adding 50 mL of sterile distilled water pH 7 with 0.01% Tween-80. The optimum condition was used for further treatment.

2.6. Effect of extract solution. The effect of extract solution based on result of effect of spore concentration. The optimum of spore concentration was inoculated into 5 g coconut pulp pH 7 with moisture content of 60% then fermentation was incubated at 30°C for 7 days. Crude enzyme was harvested by adding 25, 50, 75, 100, and 125 (mL) of extract solution.

3. Results and Discussion

3.1. Effect of variations of spore concentration

![Figure 1](image.png)

Figure 1. The correlation of lipase activity and protein in crude enzyme using different variation of spore concentration inoculum.

The effect of different concentration of spores is shown in Figure 1. It is shown that optimum of spore concentration for this fermentation was achieved at spore concentration of \(1\times10^6\) spore.mL\(^{-1}\) with lipase activity 10.83 U.mL\(^{-1}\) and protein content 0.067 mg.mL\(^{-1}\). In contrast, at spore concentration of \(1\times10^8\) was resulted lipase activity 8.33 U.mL\(^{-1}\) and protein content 0.068 mg.mL\(^{-1}\). Lipase activity was produced by the addition of spore concentration inoculum \(1\times10^6\) spore.mL\(^{-1}\) was higher than lipase activity of *Rhizopus pusillus*, 10.8 U.mL\(^{-1}\) on live-bagasse pulp at same of spore concentration [14]. Lipase production and lipase activity was influenced by microorganism, growth environment, substrate
[15], kind of carbon and nitrogen sources [4]. The result also showed that lipase activity was influenced by adding spore concentration inoculum at the beginning of SSF.

The effect of spore concentration to lipase activity and protein content in crude enzyme was not linear. Increasing of spore concentration inoculum tends to reduce lipase activity and protein content. In this case, there was an optimum condition of spore concentration inoculum to produce a high amount lipase activity. The additional spore concentration inoculum not only increase spore biomass but also decrease enzyme production along with limited nutrient in the substrate [16]. The result also showed that spore biomass production by A. niger was not linearly correlation with lipase activity (table 1) although the pH of substrate was gradually decreased during SSF using A. niger for all treatments of spore concentration inoculum (Figure 2).

Table 1. Spore biomass of A. niger after treated by adding inoculum of spore concentration into substrate at 30°C for 7 days.

| Isolate | Conc. (spores.mL⁻¹) | Spores biomass (spores.mL⁻¹) | Crude enzyme (mL) |
|---------|---------------------|-------------------------------|------------------|
| A. niger| 1x10⁵               | 1x10⁵                         | 0.44x10⁸         | 43.5  |
|         | 1x10⁶               | 1x10⁶                         | 0.51x10⁸         | 45    |
|         | 1x10⁷               | 1x10⁷                         | 0.85x10⁸         | 43    |
|         | 1x10⁸               | 1x10⁸                         | 1.19x10⁸         | 46    |

Figure 2. Change of pH in substrate of SSF was incubated at 30°C for 7 days with additional spore concentration inoculum of A. niger (spore.mL⁻¹).

3.2. Effect of extract solution
The effect of extract solution shown in Figure. 3. It shown that optimum of extract solution for this fermentation achieved at 75 mL with lipase activity 10.83 U.mL⁻¹ and protein content 0.074 mg.mL⁻¹. The result also showed that increasing of extract solution more than optimum limit of crude enzyme extraction of A. niger decreased protein concentration and lipase activity due to dilution factor.
Figure 3. The correlation of lipase activity and protein in crude enzyme using different of volume extract solution.

Figure 4. Spore biomass and volume of crude enzyme of A. niger after treated by adding gradually extraction solution into substrate fermentation.

The correlation of volume of extracting solution with spore biomass showed that the highest spore biomass was produced by treatment of 125 mL, 1.51x10^8 spore.mL^{-1} and crude enzyme volume 123 mL. The additional extract solution more highly able increase of spore biomass and crude enzyme production (Figure. 4) but decreased protein concentration and lipase activity due to dilution.

4. Conclusion
This paper demonstrated A. niger has successfully produced lipase with optimum condition were achieved by adding spore concentration inoculum 1x10^6 spore.mL^{-1} in acidic condition at 30°C for 7
days and extracted by 75 mL of extract solution that it resulted 72.5 mL of crude enzyme with lipase activity 10.83 U.mL⁻¹ and protein content 0.074 mg.mL⁻¹.

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