Mannanase activity produced through fermentation of coconut flour at various pH by *Aspergillus niger*

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Abstract. Coconut flour is one of agriculture waste product, containing high concentration of mannan polysaccharide. This waste product can be used as a substrate to produce mannanase enzyme through fermentation by using inoculum of *Aspergillus niger*. The aim of the study was to produce mannanase enzyme with high activity from the non-lipid and non-protein coconut flour. The coconut flour was extracted by using 1M NaOH obtain free-protein coconut flour. The non protein coconut flour was then extracted by using hexane to release lipid. The non-protein and non-lipid substrates were then fermented with *Aspergillus niger*. A completely randomized design was used in this study with various pH (pH 6 7 8 and 9). The result of the study indicated that pH 9 of the substrate produce the highest mannanase activity, being 0.0313 U/ml. In conclusion, optimal activity of mannanase was reached when the pH of substrate was 9.

1. Introduction
Production of coconut milk is generally through wet processing. Coconut milk is a raw material for production of coconut oil and virgin coconut oil. The result of these processing is a by-product in the form of coconut flour. Mediati stated that every 100 coconuts to produce coconut oil generated 19.5 kg coconut flour as a by-product [1]. The utilization of coconut flour has been limited to animal feeding or used as a mixture of “tempeh bongkrek” (fermented coconut with *Rhizopus*) for human consumption. Miskiyah reported that the coconut flour contained 9.44% fat, 13.09% protein, 30.40% crude fiber, 5.92% ash, and water content of 13.35% [2]. Purawisstra stated that waste coconut contains 61% galactomannan and 26% mannan [3]. Both of these compounds can function as a prebiotic to lower pathogenic bacteria in the digestive tract of broiler, mycotoxin binder and lower blood cholesterol levels in poultry [4]. The presence of high enough galactomannan and mannan content in coconut flour can be utilized to produce the mannanase enzyme and cellulase enzyme [5, 6]. The mannanase enzyme were believed to be able to increase nutritive value of poultry feed [7, 8].

Production of enzymes can be done by fermentation using microorganisms, such as bacteria, *actinomycetes*, *yeasts* and fungi, isolated from natural environments [9]. Lee reported that *Aspergillus niger*, *Aspergilus wentii* TISTR 3075, *Aspergilus oryzae*, *Trichoderma reesei* TISTR3 3080 and *Penicillium spp* could produce mannanase enzyme, cellulase and xylanase using palm kernel cake as a substrate [10]. The enzyme produced by *Aspergillus niger* belongs to the extracellular enzyme that
serves to break down complex molecules into simple ones [11]. Kasmiran and Tirmizi reported that *Aspergillus niger* can produce the best cellulase enzyme when coconut flour was used as a substrate. Based on the results of a number of studies, *Aspergillus niger* could also produce cellulase, amylase, protease, phytase, mannanase and xylanase [12]. A study conducted by Indah indicated that *Aspergillus niger* can be used to produce lipase enzyme by isolating from moldy copra as a medium [13]. The ability of *Aspergillus niger* to produce lipase and protease enzymes needs an attempt of delipidation and deproteinisation to increase the productivity of the mannanase enzyme.

One of the ways used for the production of mannanase is by solid state fermentation method. The conversion of complex compound to a simple compound occurs during fermentation process with the release of water molecules [14]. There are several factors that can influence the fermentation process of mannanase enzyme such as water content, temperature, pH, and duration of fermentation. Seftiono explained that mannanase production from *Streptacidiphilus lutealbus* incubated at 50°C with pH of 6.5 generated enzyme with the activity of 0.0228 U/mL [15].

The activity of the mannanase enzyme was carried out using the Ludia method [16]. Determination of the quantity of manose was obtained quantitatively. Manose produced was measured using the DNS method miller based on the reduction and oxidation reactions (redox). The reaction process occurred the reduction process of the compound Dinitro Silicylate acid into 3-amino-5-nitrosalilate by manose so that the color changes into reddish. Concentration at 540 nm wavelength using spectrophotometer was then measured [17].

### 2. Experimental

#### 2.1. Equipments and material

**2.1.1. Equipments.** The equipments used in this research were a 60 mesh sieve, 10-1000 ml measuring flask, buchner funnel, water bath, micro pipette, dropper pipette, stirring rod, erlenmeyer 250 ml and 300 ml, 50-2000 ml glass, porcelain cup, one set of reflux appliance, oven, desiccator, 10-1000 ml measuring cup, analytic balance, laminar, autoclave, ose wire, UV-Vis spectrophotometer, microscope and other glass tools commonly used in Laboratory.

**2.1.2. Materials.** The main ingredients used in this study were Coconut flour, K$_2$HPO$_4$, MgSO$_4$.7H$_2$O, CaCl$_2$, NaNO$_3$, FeSO$_4$.7H$_2$O, ZnSO$_4$, MnSO$_4$, Phenol, Sodium Metabisulfite, LBG, Manosa, PDA, GDP, Sodium Potassium Tartrate, Alcohol 70%, phosphate buffer (pH 6, 7, 8 and 9), Aspergillus niger, N-hexane, NaOH.

#### 2.2. Research design

This experiment used a Completely Randomized Design (RAL) where each treatment was repeated 3 times so that there were 12 experimental units. The treatments were pH levels of fermentation medium consisting of pH 6, 7, 8 and 9. The parameters observed were mannose level and mannanase enzyme activity.

#### 2.3. Research Procedures

**2.3.1. Sample preparation.** Coconut flour was sun-dried for 5 days. The sun-dried coconut flour was then ground and sieved with the size of 60 mesh. The ground coconut flour was measured for water content. Coconut flour was extracted to release fat using hexane solvent by maceration. The mixture of hexane and coconut flour were stirred for 24 hours at room temperature. The fat content of coconut flour was determined by the method of socletation. Fat free coconut flour was liberated its protein by using 1M NaOH solution. The protein content of coconut flour was determined by the kjedhal method.

**2.3.2. Production of Aspergillus niger.** Petri dish was filled with Media Potato Dextrose Agar (PDA) of 39 gr. A total of 30 ml destilled water was to dissolve PDA. The mixture was allowed to keep the
medium solid for 3-4 hours. Process of breeding Aspergillus niger using scratching method was initiated. Scratching technique was done in a zigzag way from top to bottom. Then it was incubated for 5 days.

2.4. Mannanase Production

2.4.1. pH of medium fermentation. The procedure used in this study was modified from Meryandini et al. [18]. A total of 20 g of free fat and free protein of coconut flour was used and added into a buffer solution in various variations of pH (6, 7, 8 and 9). The mixtures were then autoclaved at 1 atm pressure for 30 min. The mixture was stirred uniformly with mineral substances (0.306 g of K$_2$HPO$_4$, 0.066 g of MgSO$_4$.7H$_2$O, 0.066 g of CaCl$_2$, 0.066 g of NaNO$_3$, 0.012 g of FeSO$_4$.7H$_2$O, 0.0026 g of ZnSO$_4$, 0.016 g of MnSO$_4$) and $1.192 \times 10^8$ Aspergillu niger with ratio moisture content of 50%. The mixtures were then stirred until evenly distributed and incubated for 120 hours. The enzyme produced in the fermentation process was extracted using aquades to obtain a crude enzyme filtrate. Each treatment was tested its mannanase activity using LBG substrate and DNS method.

2.5. Determination of Mannanase Activity

2.5.1. Determination of levels of mannose by DNS method: Creation of DNS reagents. The method used in determination of levels of mannose by DNS method (Chafid and Kusumawardani) [19]. The DNS reagent was prepared by dissolving 1.06 g of 3,5-dinitrosalicylic acid and 1.98 g of NaOH into 141.6 mL of aquadest. These substances were added to the solution of 30.6 g potassium calcium tartaric, 0.76 mL phenol (melted on 50°C) and 0.83 g of sodium metabisulfite [17].

Determination of Manosa Levels. Mannose levels were determined by using the regression line equation obtained from standard curves made from glucose solution. Glucose solution was made with various concentrations of 10, 50, 100, 150 and 200 ppm. Furthermore, the absorbance of standard glucose solution was measured at a wavelength of 540 nm. Each concentration of solution was taken 1 mL and put into the reaction tube and then added 3 mL of control reagent. All tubes were heated at 100°C for 15 minutes and cooled at room temperature. For blanks, it was used 1 mL of aquadest with the same treatment in preparation of glucose solution.

2.5.2. Determination of enzyme activity. Procedure of determination of enzyme activity was done in accordance with the procedure of Ludia [16]. The enzyme solution was pipette 1 ml into the test tube, heated for 5 minutes in a water bath with a temperature of 40°C. It was then added 1 ml of a 0.5% locust bean gum heated at 40°C and stirred with vortex. The mixed solution was incubated at 40°C for 30 minutes, added 3 mL of the DNS reagent and then stirred with vortex. The solutions were boiled at 100°C for 15 minutes. After being cooled, the color solutions was measured the absorbance with a spectrophotometer at a wavelength of 540 nm. The blank was made by mixing 1 ml of buffer solution with 3ml DNS. The solution was then added with 1 ml of locust bean gum substrate, stirred with vortex, then boiling at 100°C for 15 minutes. The same thing was done to the control treatment. Enzyme activity can be calculated using the formula:

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\text{Enzyme activity} = \frac{\text{Mannose produced} \times \text{factor dilution} \times 1000}{\text{Molecule weight of mannose} \times \text{duration of incubation}} = \text{U/mL}
\]

2.6. Statistical analysis

Collected data was analyzed by One-way Anova using SPSS (SPSS 32, SPSS Inc. Chicago, USA) to identify effect of pH of fermentation medium on enzyme activity. Duncan Multiple Range test were conducted to determine the differences among treatments.
3. Results and Discussions

3.1. Content of coconut flour free from fat and protein
The fat content of coconut flour before the process of delipidation was 37.13%. After the maceration process using hexane solvent, the amount of fat present in the coconut flour was reduced to 0.77%. This finding has met the requirements for the fat-free condition. According to Mappiratu when the food had less than 1% fat, the food could be regarded as fat-free. It is nearly impossible to release the fat in the sample up to 100% [13].

The protein content of the coconut pulp was dissolved with NaOH as it was done by Kusakabe and Takashi [20]. This was intended that all dissolved proteins present in the coconut flour would be extracted into NaOH solution. This is in accordance with research conducted by many other researchers [21]. Protein dissolved in food could be extracted with NaOH. Coconut flour that had been extracted its protein had 1.62% crude protein content.

3.2. Production of Aspergillus niger
Growth of Aspergillus niger was characterized by the presence of mycelium and conidia on agar media. Mold growth during fermentation can be seen in figure 1.

At the time of 0 to 24 hours, the growth of Aspergillus niger has not been initiated. It was suspected that Aspergillus niger in the fermentation medium was still in the adaptation stage. Furthermore, after the first day until the fifth day of fermentation, the growth of the mold cell increased with the increasing number of spores growing on the surface of the substrate. This finding is in accordance with the research conducted by Kompiang et al. and Zaenudin et al. [22, 23]. Based on the calculation of the number of spores which is generated after 5 days of growth using a counting chamber method, it was obtained that the number of spores was 11.92 x 10⁷ cell / mL. The obtained spores were then converted to concentrations 10⁶ for the purpose of the use in the fermentation process to produce mannanase.

3.3. Analysis of mannose levels
The mannanase enzyme extract obtained by fermentation on the pH variation was reacted with the substrate of Legum Bean Gum. The mannose compounds produced from the reaction were determined its concentration by spectrophotometer method at 540 nm wavelength. Substances used to produce color were dinitro salisilate (DNS) compounds in the form of reduction and oxidation reactions. The amount of reduced DNS is directly proportional to the concentration of mannose produced in the
hydrolysis process of the enzyme reaction. The color change occurred in the DNS reagent after reacting with reducing sugar so that the original color of yellow turns reddish orange. The levels of mannose obtained showed the activity of the mannanase enzyme produced in the fermentation stage. Mannose concentration obtained were calculated based on the standard regression with the equation \( Y = 0.0042 + 0.0782x \). The results of calculations from mannose generated on various pH were shown in figure 2.

![Figure 2. Mannose production at various fermentation medium pH.](image)

Figure 2. showed that the highest concentration of mannose were obtained at the pH 9 of the fermentation medium. This indicates that the concentration of mannanase enzymes produced under these conditions was the highest. This finding supports the previous research indicating that the best conditions to produce the enzyme mannanase were in the pH 9 is 171.3 ppm. It can be speculated that the mannose amount is going to increase as the pH of medium increased up pH 10.

3.4. The enzyme activity at various pH

The mannanase activity was calculated based on the amount of mannose produced using the legume bean gum substrate (LBG). The enzyme used in this study was from enzyme extract produced at various fermentation pH and from control treatment. The result of calculating the number of mannose produced can be seen in figure 3 where the activity of the mannanase enzyme extracted from fat-free and protein free of coconut flour at various pH.

Aspergillus niger mold growth is influenced by several factors such as pH media, temperature etc. Acidity (pH) needs to be regulated so that the growth of fungus can grow well. The way to control acidity was usually with the addition of buffer solution according to the desired condition. The buffer solution was not only to regulate the level of acidity, but also to maintain the pH changes due to the presence of compounds produced during the fermentation process. One common buffer used for alkaline conditions is phosphate buffer while acidic conditions uses citric acid buffer. This is because most fungi can grow in the pH range of 2-8.5. Better growth occurs under acidic conditions or base pH. In addition, the water content of the material also greatly affected the rate of enzymatic reactions, high water content inhibits the action of enzymes or substrates, consequently hydrolysis occurs only on the substrate directly related to the enzyme. So it needs a phosphate buffer with low water content to maintain the condition of the medium where the fungi grows.
Figure 3. Effect of fermentation medium pH on enzyme activity. Values are means and standard deviation.

The results of the present study showed that the maximum enzyme activity was 0.0313 Unit / mL and it was found when the media was at pH 9. These results of the enzyme activity were lower than the results found by Felicia et.al. [24], who reported that mananase can be produced from various species of Aspergillus niger using a solid fermentation method. The authors found 1.21 U/mg for crude enzymes to 19.091 U/mg after purification. It is suspected that the small amount of nitrogen source in the coconut flour due to deproteination was the reason. Production of enzyme through fermentation by microorganisms required a source of nitrogen to produce amino acids and enzyme protein.

According to Christakopoulus [25], that either high or low pH allowed the occurrence of denaturation and this would result in decreased enzyme activity. Since enzymes are proteins, changes in pH will cause the change of ionisation in protein molecules [25]. This change would result in the change of its three dimensional structure. Accordingly, its catalytic function is disrupted. This can be seen from the figure 3 and figure 4, the enzyme activity at the pH 6 was lower than at pH 9. In addition, the optimum mannanase enzyme activity from Bacillus sp were produced at pH 9 with enzyme activity of 48.62 U/mL. It is probably going to increase when the pH of medium increased up to 10.

The result of statistical analysis showed that various pH of fermentation medium had significant effect on enzyme activity. The Duncan’s Multiple Range Test showed that each fermentation medium pH had significantly different to enzyme activity (data not shown). The highest production of mannose was obtained on medium fermentation with pH 9.

4. Conclusion
According to result research can conclusion that pH of fermentation medium is better to acquire enzyme activity maximum is pH 9 with activity 0.0313 U/ml.

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