Screening of agricultural wastes as a medium production of catalase for enzymatic fuel cell by *Neurospora crassa* InaCC F226

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Abstract. Explorations of local microorganisms from Indonesia that can produce of catalase are still limited. *Neurospora crassa* is a fungus which resulting of two kinds of catalase, namely catalase-1 and catalase-3. We studied the production of catalase by *Neurospora crassa* (no. F226) from Indonesia Culture Collection (InaCC) in Solid State Fermentation (SSF). Among four screened agro wastes (corn cob, rice straw, oil palm empty fruit bunches, and bagasse), rice straw and oil palm empty fruit bunches (OPEFB) were remarked as the most promising substrate suited for the excellent growth and adequate production of catalase. Based on the result, the method of solid state fermentation was suitable to production of catalase. It is caused that the medium served to maintain microbial growth and metabolism. The filamentous filament is more suitable for living on solid media because it has a high tolerance to low water activity, and it has a high potential to excrete hydrolytic enzymes that caused of its morphology. The filamentous filament morphology allows the fungus to form colonies and penetrate the solid substrates in order to obtain nutrients. The results showed that the highest catalase activity was obtained on rice straw and oil palm empty fruit bunches medium with catalase activity of 39.1 U/mL and 37.7 U/mL in 50% moisture content medium, respectively. Optimization of humidity and pH medium in the rice straw were investigated which is the highest activity obtained in 30% moisture content and pH medium of 6. The catalase activity was reached in the value of 53.761 U/mL and 56.903 U/mL by incubated 48 hours and 96 hours, respectively.

1. Introduction
Recently, energy from petroleum become a serious problem for the whole countries in the world, including Indonesia. Decreasing world’s petroleum stock make its price is very high. So, it is urgent to search other alternative energy sources [1]. The bio-refinery is a prospective key to solve the problem. The utilization of biomass in the bio-refinery concept to production of energy has been a focus on by researcher in the world. In the bio-refinery concept, the biomass has been converted to simple sugar, such as a glucose, which is used as a building block to result of bioethanol or biochemical by microbes or chemical treatment. Because there are several problems in the conversion of glucose to bioethanol by microbes, such as inhibitor furfural and HMF, or pollution of environmental by chemical treatment, the researchers have been looking for the best method to the conversion of the glucose to energy [2; 3].

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The most effective way to solve the problem is the development of cell bio-electricity by the concept of the enzymatic fuel cell. An enzymatic fuel cell is a device that can directly change the chemical into electrical energy through an electrochemical [4]. Chemical reactions in the enzymatic fuel cell may occur via direct electron transfer (DET), which electron transfer occurs directly between the enzyme and the electrode or through an intermediary in the form of mediated electron transfer (MET). The reaction between glucose with glucose oxidase will have resulted of a gluconolactone and hydrogen peroxide. The hydrogen peroxide that resulted from this reaction will be changed by the oxidoreductase groups, such as catalase, being water and oxygen. Two reactions in this process can be used as a principle of cell bio-electricity enzymatic based on biomass in the resulting of energy.

Catalase (hydrogen peroxide oxidoreductase, EC1.11.1.6) is one of the antioxidant enzymes, which deals with removal of the oxidative damage in cells. It has a double function; it catalyses the decomposition of hydrogen peroxide into oxygen and water (catalase activity) and also oxidizes electron donors such as ethanol, methanol, or phenols (peroxidative activity) [5;6]. It is classified into four groups. The first is monofunctional-heme (typical) catalases. This group is heme catalases, containing iron-protoporphyrin IX as a prosthetic group in their active sites. Monofunctional-heme (typical) catalases composed of two classes based on the size of the subunits: small-subunit catalases (<60 kDa) and large-subunit catalases (>75 kDa) and they have molecular masses of 200–340 kDa [7]. Various of microorganisms, such as bacteria, fungi and yeast, can be resulting of this enzyme. Diaz *et al.* [8] reported that the fungi of *Neurospora crassa* resulted in two kinds of catalase, namely catalase-1 and catalase-3 and it found in conidia at a very high concentration, tolerant in the high solvent and stable in the large of range pH.

Up to now, the research of catalase, particularly in Indonesia, using indigenous microbe of Indonesia are still limited. By seeing of region of Indonesia as a tropical country with rich of biodiversity can be used to produce of catalase. Indonesia is a mega biodiversity country and the world second biggest biodiversity after Brazil. These bio-diversities include microbial diversity in soil and water. Today, Indonesia has isolated of various microbes, and it saved in the Indonesia Culture Collection (InaCC). InaCC has more than 10,000 isolates of species of bacteria, actinomycetes, fungi, yeast and microalgae [9]. Our previous research using *Neurospora crassa* InaCC F226 and production it in the solid medium of Vogel resulted in a high activity and stable as long as 2 hours in the temperature of 30-40°C [10]. Our current research is focused on the screening of an agricultural biomass that can be used as a medium to the production of catalase effectively in the solid state fermentation (SSF).

2. Material and methods

2.1. Materials
All of the chemicals were used from Merck and Sigma chemical unless otherwise stated.

2.2. Microorganisms and inoculum preparation
*Neurospora crassa* (no. F226) were obtained from the Indonesia Culture Collection. This fungus was isolated from the leaf litter at the Salak mountain, Bogor-Indonesia. It was grown from stocks of conidia on potato dextrose agar (PDA) slant at room temperature for 3 days and stored at 4°C. The spores were suspended by the addition of 5 mL of distilled water to the slant medium.

2.3. Physical treatment of agro-industrial residues
The corn cob, bagasse, rice straw and oil palm empty fruit branches used in this research were kindly provided by Biomaterial Laboratory of Indonesia Institute of Sciences, while the bagasse obtained from the sugar cane farmer in Kediri-West Java. These materials were ground with a machine to make the particle size being 100 mesh [11].
2.4. Screening of agricultural wastes to medium production of Catalase
Catalase production was produced in Solid State Fermentation (SSF) using corn cob, rice straw, oil palm empty fruit bunches (OPEFB) and bagasse with modification of Ayesha Sadaf & S. K Khare method [11]. The biomass was ground by machine until the size of biomass is 100 mesh, and each of agricultural wastes weighed as much as 2 grams. These medium were added of Vogel’s medium, 0.2% glucose, 0.5% yeast extract and 1% of glycerol [12]. The humidity and pH medium were adjusted being 50% and 5.5, respectively, and it was sterilised at temperature 121°C by 15 minutes. It was inoculated with 0.2 mL of Neurospora crassa InaCC F226 in the sterile of distilled water and incubated at room temperature for 120 hours. The sampling was done at 72 hours, 96 hours and 120 hours.

2.5. Moisture content optimization in the selected of medium production of catalase
The moisture content of medium production was studied in the range of 20-60% with interval 10% [4]. The media of rice straw (2 gram) was added with Vogel’s medium, 0.2% glucose, 0.5% yeast extract and 1% of glycerol [12]. The Vogel’s medium pH was adjusted to 5.5 before sterilization at 121°C for 15 minutes. The Neurospora crassa InaCC F226 has added aseptically into the medium as much as 0.2 mL and fermentation was allowed to proceed for 96. The sampling was done at 72 hours and 96 hours.

2.6. Optimization of pH medium in the selected of medium production
The optimum of pH medium for catalase production was determined using various pH medium ranging from 4.0 to 6.0 with interval 1. The Vogel’s medium was adjusted by 10% HCl to make the acidic medium. The rice straw (2 gram) with 30% moisture content was added of Vogel’s medium and sterilization at 121°C for 15 minutes. 0.2 mL of inoculum was aseptically inoculated into the medium and fermentation was allowed to proceed for 120 hours. The sampling was done at 72 hours, 96 hours, and 120 hours.

2.7. Extraction and assay enzyme
Neurospora crassa InaCC F226 grown in the agro-residues medium containing Vogel’s medium and incubated in the various times (72-120 hours). The medium containing Neurospora crassa InaCC F226 was added with 20 mL of Na-phosphate buffer (pH 7.0; 50 mM) containing 3% hydrogen peroxide. The medium was stirred at temperature 4°C for over night (16 hours), and the supernatant containing crude enzymes of catalase was separated from biomass and cells using centrifugation (Kubota 6200) at 13000 x g in 4°C for 15 minutes [10]. The commonly used procedure to investigate catalase activity conducted from modification of Santoso’s method [10]. The hydrogen peroxide was used as substrate and decrease of hydrogen peroxide concentration was measured spectrophotometrically at 240 nm. The catalase activity was measured at a controlled temperature (35°C) in 50 mM sodium phosphate buffer (pH 7.0). The free enzyme as much as 0.3 mL were mixed in 1.2 mL of 0.6 mg/mL hydrogen peroxide in buffer solution. The decrease of absorbance concentration hydrogen peroxide at 240 nm was monitored. Enzyme activity was determined using the initial rate of the reaction and the extinction coefficient for H₂O₂ of 39.4 M⁻¹ cm⁻¹ [13]. One enzyme unit was defined as the amount of enzyme that catalyzes the decomposition of 1 μmol hydrogen peroxide per minute at 35°C

2.8. Measurement of protein concentration
To measure of protein concentration on solution was used Bradford’s method [14]. A sample of 0.01 mL was added into 0.99 mL of Bradford solution in the test tube. It was incubated at room temperature for 10-15 min. The quantity of protein in solution was determined by spectrophotometer at wave length 595 nm using bovine serum albumin as standard protein
3. Result and discussion

3.1. Screening of agricultural biomass as a medium production of catalase

Agricultural biomass waste (Fig 1) can be used as a growth medium to produce of catalase by *Neurospora crassa* InaCC F226. There are two biomasses that it resulted in a good of catalase activity, namely Rice straw and oil palm empty fruit bunches (OPEFB). The highest catalase activity reaches in incubation of *Neurospora crassa* at 96 hours with activity value of 39.082 U/mL (Rice Straw). Incubation using rice straw at 72 hours and 120 hours yielded catalase activity of 33.016 U/mL and 34.306 U/mL (Figure 2), respectively. Production of catalase with oil palm empty fruit bunches gave the highest activity at incubation of 48 hours with an activity of 37.690 U/mL. The catalase activity began to decrease in incubation at 96 and 120 hours with activity 34.120 U/mL and 26.574 U/mL, respectively.

![Figure 1. The various of agricultural biomass; a. corn cob b. TKKS; c. rice straw; d. bagasse](image)

Utilization of corn cob and bagasse as a medium production of catalase still provides a pretty good catalase activity. It can be seen in Figure 1 that the highest catalase activity was produced by using bagasse in incubation of 96 hours (37.648 U/mL) and it was still given a good enough activity at 48 hours incubation with catalase activity 35.529 U/mL. The catalase activity at 120 h incubation provides an activity of 31.988 U/mL. Incubation of catalase using corn cobs yielded catalase activity at 96 h incubation (35.448 U/mL), while incubation at the 120 hours and 48 hours of catalase activity began to decrease with activity value of 32.183 U/mL and 30.947 U/mL, respectively.

Based on the result, the method of solid state fermentation (SSF) was suitable to production of catalase. Solid state fermentation (SSF) is defined as the growth of microorganisms on the dampened solid substrate, where the medium serves to maintain microbial growth and metabolism [19]. Generally, the filamentous filament is more suitable for living on solid media because it has a high tolerance to low water activity, and it has a high potential to excrete hydrolytic enzymes that caused of its morphology. The filamentous filament morphology allows the fungus to form colonies and penetrate the solid substrates in order to obtain nutrients.

Increased production of hydrolytic enzymes in solid-state fermentation is due to increased aerial hypha growth. Aerial hyphae play an important role in the respiration process during fungi growth. Rahardjo *et al.* [15] reported that the aerial hyphae of *Aspergillus oryzae* grown on wheat flour substrate gave 75% oxygen intake. The amount of oxygen consumed is proportional to the amount of α-amylase produced by *Aspergillus orizae*. This means that the aerial hyphae can accelerate the oxygen uptake so that the formation of the aerial mycelium will be abundant. Abundant aerial formation of mycelium may increase the production of hydrolytic enzymes. In addition to high enzyme productivity, using solid media fermentation also provides high product stability and lower catabolic repression [16;17; 18; 19] reported that tanase production on solid media achieved the highest activity at 96 hours of incubation time while for media Liquid, the highest tanase activity is produced after 120 hours of incubation time. This means the use of solid media is more efficient for the production of
enzyme tanase than liquid media. Aguilar et al. [20] also reported that the protease activity in the media was submerged 10 times higher than in solid media. With the low proteolytic activity of proteases in the media solid, then tanase production becomes high.

![Figure 2](image)

**Figure 2.** Production of catalase in the various of agricultural biomass

3.2. **Optimazation of moisture content and pH medium production of catalase**

Humidity of medium was studied in this research 20; 30; 40; and 60%. The catalase activity decreases in the moisture content and incubation time. The results in Figure 3 show the activity on a medium with 30% moisture content resulting high catalase activity of 53.761 U/mL (incubation 48 hours). Then, the moisture content of 40% produced an activity of 44.454 U/mL, catalase activity began to decrease in humidity 20; 50 and 60% with significant values of 26.768 U/mL; 26.848 U/mL; And 27.432 U/mL (incubation 48 h). The catalase activity with an incubation time of 96 hours resulted in activity of 28.701 U/mL (20%); 30.931 U/mL (30%); 31.146 U/mL (40%); 29.958 U/mL (50%); And 28.232 U/mL (60%).

![Figure 3](image)

**Figure 3.** The effect of moisture content in medium production of catalase
The acidity (pH) on medium production can be increasing of catalase activity. It can be seen from Figure 4 that the highest catalase activity obtained on pH medium of 6 by the activity of 56.903 U/mL in 96 hours incubation while the incubation at 48 hours and 120 hours resulted in the activity 56.514 U/mL and 55.989 U/mL, respectively. Meanwhile, the catalase activity on pH medium of 5 increased in the incubation of 48 hours and 96 hours by the activity of 47.225 and 55.385 U/mL, respectively, but there was a small decrease in the incubation of 120 hours with activity 55.799 U/mL. The incubation of Neurospora crassa in the pH medium of 4 was resulting in the activity 45.630 U/mL for incubation of 48 hours, 49.107 U/mL for 96 hours, and 54.281 U/mL for 120 hours. pH values can affect structure and function of the protein so that enzymes present in the system will be inactive if microbes grow at too high or too low pH. In addition, if the pH growth is too low, it will disrupt the proton gradient between environments Outside and inside the cell so as to interfere with the cell's ability to produce energy [20].

![Figure 4. The effect of pH medium on catalase activity](image)

4. Conclusion

Agricultural waste (corn cob, rice straw, oil palm empty fruit bunches and bagasse) can be used as solid state fermentation to the production of catalase. The results showed that the highest catalase activity was obtained on rice straw medium and oil palm empty palm fruit bunches (OPEFB) with catalase activity of 39.1 U/mL (rice straw, 50% moisture content) and 37.7 U/mL (OPEFB, 50% moisture media). Variations of medium humidity and pH of the medium resulted in a 30% moisture content of rice straw medium by resulting catalase activity of 53.8 U/mL (48 hours incubation) and catalase activity at pH 6 resulting 56.903 U/mL on 96 hours incubation.

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