

The Production of Cellulase from *Bacillus sp.* BPPTCC-RK2
using OPEFB Substrate

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ABSTRACT

Cellulase enzyme is widely used in bioethanol industry. In bioethanol production, cellulase is used to hydrolyze lignocellulosic materials into glucose, where glucose is further converted into bioethanol through fermentation process. The demand of cellulase in Indonesia is quite high, however almost 99% of Indonesia industrial enzyme demands is fulfilled by imports. This situation might trigger a new opportunity to build local cellulase enzyme plant in Indonesia. One of the lignocellulosic biomass which has high potential as a substrate to produce cellulase enzyme is Oil Palm Empty Fruit Bunches (OPEFB) because it has pretty high cellulose content, which reaches up to 41,3 – 46,5%. The conventional method to for cellulase enzyme production is by utilizing lignocellulosic fungi, but it is known that cellulase also can be produced by bacteria with higher production rate. Several variables that affect enzyme activity are pH and temperature. Therefore, this research will examine how is the activity of cellulase enzyme produced by *Bacillus amyloliquefaciens* BPPTCC-RK 2 recombinant by varying temperature and pH. The method that is used in this observation is submerged fermentation (SMF). The measurement of cellulase activity is conducted by using DNS method, while the protein content is measured by using Lowry method. It has been shown that the highest activity of cellulase is at 24 hours. The optimum pH and temperature are 7,0 and 40^oC respectively. The Km and Vmax value are 0,11% and 2,907 U/mL respectively.

Keywords: Cellulase, OPEFB, *Bacillus sp.* BPPTCC-RK2, lignocellulosic

Introduction

Enzyme is a protein which functions as a biocatalyst in a chemical or biological reaction [1]. One of the enzyme that is widely used in industry is cellulase. Cellulase can catalyze cellulose hydrolysis reaction become glucose by breaking $\beta - (1,4) -$ glucosidic bond, where glucose can be processed further into bioethanol and other high value product. Nowadays, bioethanol production and consumption in Indonesia is quite high, especially due to new government's policy to increase the production and consumption of bioenergy. Therefore, it will also increase the demand of cellulase enzyme.

Meanwhile, Reference [2] shows that cellulase enzyme consumption for bioethanol, pulp and paper, textile, and detergent almost 99% fulfilled by import and the value is approximately 121,85 billion in 2013 with an average increasing rate of 6,67% per year. This huge demand of cellulase in local industry will open up a new opportunities for enzyme production in Indonesia.

Cellulase production needs cellulose as substrate. Commercial substrate such as Avicel and Solka Floc, or organic inducer like Carboxymethyl Cellulose (CMC) has expensive price. Oil Palm Empty Fruit Bunch (OPEFB) is one of the lignocellulosic material that has high content of cellulose, which is 41,3 – 46,5% of OPEFB dry weight [3]. OPEFB is abundantly available, not expensive and renewable, therefore OPEFB can be used as an alternative substrate to decrease raw material cost.

Indonesia produce high amount of oil palm, with the total of 9 million hectare with the production of CPO around 27,5 billion tonnes per year [4]. Each year, oil palm industry produce $27,5 \times 10^6$ tonnes solid waste such as OPEFB, fiber, and eggshell [3]. From the whole fresh bunches, will be produced 23% of OPEFB. Previously, OPEFB utilized as fuel for generator in oil palm grinding industry [5] and as fertilizer for oil palm plantation, however the utilization cost is quite high and the added value is relatively low, therefore it needs another alternative that is more efficient to utilize OPEFB. By utilizing OPEFB as a substrate to produce cellulase, the added value will be increased.

Several obstacles to produce cellulase are high cost, low growth rate of microorganisms and low yield. The conventional method to produce cellulase is by using lignocellulosic fungi such as *Aspergillus* sp., *Penicillium* sp., and *Rhizopus* sp. However, it has been known that cellulase can also be secreted by bacteria, such as *Bacillus* sp. Bacteria has faster growth rate than fungi [6], therefore bacteria can be one of the alternative to get a better cellulase production with OPEFB. Besides that, bacteria is also easier to genetically engineered to increase it's performance compared to fungi [7].

In this study, we will use *Bacillus amyloliquefaciens* BPPT CC RK2

recombinant to get cellulase enzyme. The culture medium use LB broth with OPEFB as cellulose source. The cellulase enzyme activity will be examined in 24-48 hours every 3 hours, and the activity is also tested by varying temperature and pH.

MATERIALS AND METHODS

Pretreatment of OPEFB

OPEFB was obtained from Pekanbaru, Riau, Indonesia. The sample was stored in a dry place. The EFB is cut by using scissor until 2-4 mm, ground in a blender, and sieved until the size is 1-2 mm. EFB is pretreated by using sodium hydroxide (NaOH) 0,5 M for 4 hours. NaOH 0,5 M is made by dissolve 6 grams of solid NaOH into 300 mL distilled water. After that, it is washed thoroughly by using distilled water until the pH is neutral and dried in oven on 105°C overnight

Inoculum Preparation

1 -2 inoculation loop of *Bacillus amyloliquefaciens* BPPT CC RK2 is inoculated to Luria-Bertani (LB) slants agar. The inoculum was obtained from BPPT, Puspiptek, Tangerang, Indonesia. It is incubated at 37°C until 24 hours. The inoculum that has been prepared is used for culture stock for this study.

Cellulase Enzyme Production

a. Starter Medium Preparation

Starter medium is 10% of production medium. For instance if the total production medium is 50 mL, the starter medium is 5 mL. Starter medium consists of Luria Bertani broth of 25 g/L. The medium is autoclaved at 121°C and 1,2 atm for 15 minutes. The sterile medium is innoculated with 1 -2 loop of *B. amyloliquefaciens* BPPT CC RK2. The liquid culture is agitated in 150 rpm at 37°C for 6 hours.

b. Production Medium Preparation and Cellulase Production

Production medium consists of Luria Bertani broth and OPEFB substrate. The medium is autoclaved at 121°C and 1,2 atm for 15 minutes. The starter medium which already consisted of inoculum is inoculated into the production medium. It is agitated in 150 rpm at 37°C for 24 – 48 hours. After that, the optimum incubation time is evaluated. After determining optimum time, the cellulase is produced by varying pH of 6,0; 6,5; 7,0; 7,5; and 8,0. After obtaining optimum pH and incubation time, cellulase is produced by varying temperature of 25; 30; 35; 40; 45 and 50°C.

Cellulase Analysis

The crude enzyme of cellulase enzyme is obtained by using centrifugation at 4000 rpm for 15 minutes. For recombinant bacteria, the sample is first sonicated

for 15 minutes to break the cell and then centrifuged. The activity of cellulase enzyme is analyzed by using DNS (3,5-Dinitrosalicylic Acid) method, while the protein content is analyzed by using Lowry method.

a. Cellulase activity analysis

Cellulase existence can be known by determining the glucose content which formed as a result of hydrolysis. This analysis comprise of glucose content analysis and standard curve making. To measure glucose content, 100 μ L of enzyme (sample) is added by 900 μ L of CMC 1% which dissolved in 0,05 M phosphate buffer pH 7, so the total mixture is 1 mL. It is incubated at 50^oC. After 30 minutes, it is added by 1 mL of 3,5-dinitrosalicylic (DNS) solution. It is heated for 5 minutes so there will be a reaction between glucose and DNS. The mixture is cooled down and mixed by using vortex, and then the absorbance is measured at 540 nm. The activity is calculated by using this equation:

$$\text{Activity (U/mL)} = \frac{\text{mg glucose} \times 1000}{\text{Mr glucose} \times 30 \text{ min} \times 0,1 \text{ mL}} \quad (1)$$

mg glucose is the amount of glucose that is obtained by using the calculation of standard curve, Mr glucose is the molar mass of glucose which is 180 g/mol. 30 min is the incubation time, and 0,1 mL is the amount of cellulase sample that is analyzed.

While the standard curve is done by making 10 variation of glucose concentration (from 0 – 0,4 mg/ml), and then analyzed by using DNS method. The absorbance also measured in 540 nm. The result is plotted in a graph of concentration versus absorbance.

b. Protein content analysis

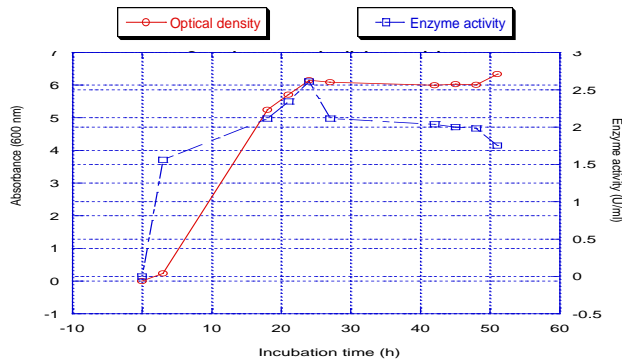
The protein content is measured by using Lowry method. 1 mL of enzyme sample is diluted 10 times. 1 mL of diluted samples is added by 2 mL of Lowry reagent. It is incubated at room temperature. After 10 minutes, it is added by 0,2 mL Folin reagent. It is incubated at room temperature for 30 minutes. And then, the absorbance is measured at 750 nm.

RESULTS AND DISCUSSION

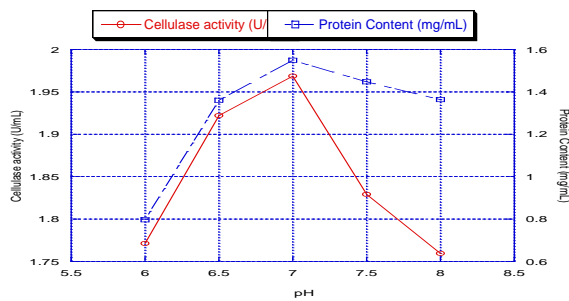
a. *Production of Cellulase Enzyme*

Cellulase enzyme is produced with *Bacillus amyloliquefaciens* BPPT CC RK2 by using oil palm empty fruit bunch substrate for 51 hours. It has been found that the highest cellulase activity reaches at 24 hours, which is around 2.6 U/mL. After knowing the optimum production time, the enzyme is evaluated at different pH and temperatures. The pH range that is examined for cellulase enzyme production are 6; 6,5; 7; 7,5 and 8,0. From the result, it has been shown that the optimum pH for cellulase enzyme is 7,0. After determining the optimum time and

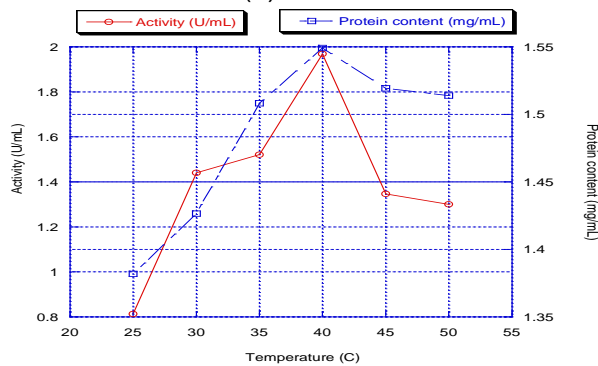
pH, we evaluate the optimum temperature. It has been shown that the optimum temperature for cellulase production is 40°C.



(a)



(b)



(c)

Fig. 1. (a) Production of cellulase for 51 hours, and production of cellulase by varying operating (a) pH and (b) temperature

b. Michaelis – Menten Parameters Characterization

Based on the previous part, it has been shown that cellulase activity is highest at 24 hours, with operating pH of 7,0 and operating temperature of 40°C. This result will be used for cellulase enzyme characterization. The parameter that is

characterized is Michaelis – Menten parameter. This parameter will evaluate the value of K_m (michaelis – menten constant) and v_{max} (maximum rate of reaction) of cellulase enzyme.

From the result, it has been shown that substrate concentration affect the cellulase activity. In an enzymatic reaction, when substrate concentration is increased until specified concentration while the other condition is remain unganggung, then the rate of reaction (v) will increases until a limit called as maximum rate of reaction (v_{max}). Therefore, v_{max} can also be called as the enzymatic rate when enzyme is saturated with substrate. While K_m can be defined as a rate of reaction when enzyme reach half of it's maximum rate [8]. From the result, it is shown that the enzymatic rate starts to be constant at around 2,3 U/ml. When the substrate concentration is increased, the activity is not increased much. It meas that cellulase enzyme's binding site is already filled out by substrate.

The graph is then linearized by using Lineweaver-Burk fitting. With the R^2 value of 0,997, the graph is linear enough and the data can be interpreted. The K_m value is 0,11% and v_{max} value is 2,907 U/mL. It means that when the substrate is 0,11%, cellulase will reach half of it's maximum activity, and the maximum rate of reaction is 2,907 U/mL.

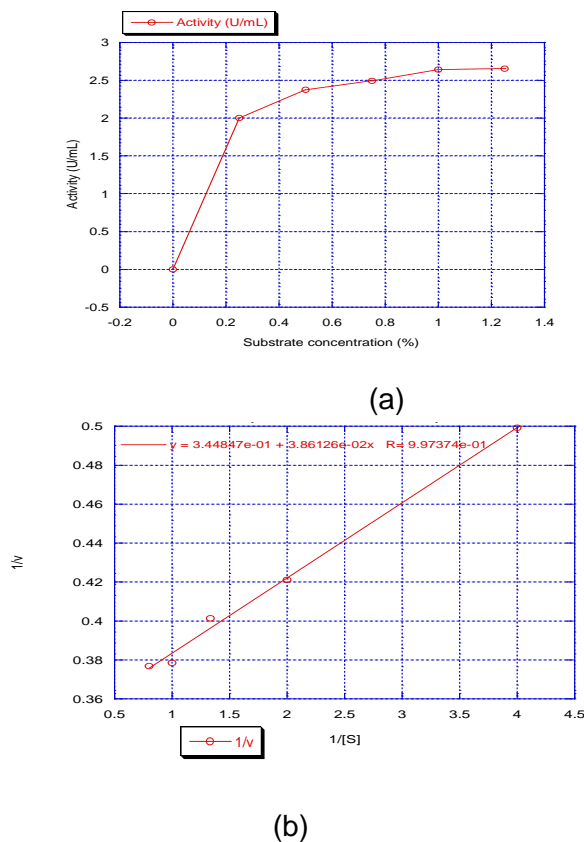


Fig. 2. (a) Effects of substrate concentration on cellulase activity, (b) Lineweaver-burk linearization of cellulase enzyme kinetics

CONCLUSION

OPEFB contains quite high amount of cellulose, which is up to 41,3 – 46,5%. With it's abundance in Indonesia and low price, it is very beneficial to make OPEFB as a substrate for cellulase production. This cellulase can be used for bioethanol industry. Bacteria is known to have faster growth rate than fungi, one of the known cellulase-producing bacteria in Indonesia is *Bacillus amyloliquefaciens* BPPTCC-RK2.

Cellulase from *B. amyloliquefaciens* BPPTCC-RK2 can be produced by using OPEFB with highest activity at 24 hours. The optimum pH and temperature are 7,0 and 40⁰C respectively. The Km and v_{max} value are 0,11% and 2,907% respectively.

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