Effect of some variable in cellulase production by *Aspergillus niger* ITBCC L74 using solid state fermentation

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Abstract

Cellulase is a very important enzyme for ethanol production, food, papper, etc, from lignocellulose and others. Rice straw and corn cob are the largest agricultural waste in Indonesia, while the water hyacinth weed is a plant that has not been used optimally. The content of cellulose is high enough on rice straw, water hyacinth and corn cob so it can be used as a substrate in the production of cellulase to increase the economic value of the rice straw, hyacinth, and corn cob. As for the purpose of this study is to use the rice straw, water hyacinth, and corn cob as substrates of cellulase enzyme, determine the effect type of substrates, moisture content and fermentation time in production of cellulase enzyme and also determining the optimum conditions for production of cellulase enzymes. The method is solid fermentation system and using fungi *Aspergillus niger* ITBCC L74 as inoculum. The variable used were fermentation time is 2, 4, 6, 8 and 10 days, moisture content is 50, 60, 70, and 80%, as well as the type of substrate is rice straw, water hyacinth, and corn cob. The results showed that the highest protein content in the crude enzyme of the rice straw, water hyacinth and corn cob is 0.0153 mg/ml, 0.0194 mg/ml and 0.0146 mg/ml, respectively. The optimum enzyme activity were for the rice straw, water hyacinth and corn cobs @ 2.569 U/ml, 1.606 U/ml and 1.302 U/ml, respectively. The optimum moisture content were obtain for rice straw, water hyacinth and corn cob respectively 80%, 70% and 60%. And the optimum fermentation time for rice straw, corn cob, and water hyacinth is on the sixth day. In this study showed the highest enzyme activity on the type of rice straw substrate with a water content of 80% and fermentation time 6 day.

Keywords: Cellulase enzyme; *Aspergillus niger* ITBCC L74; rice straw; water hyacinth; corn cob

1. Introduction

Rice straw is the largest agricultural waste in Indonesia. There are around 20 million per year. According to BPS data in 2006, the paddy field area in Indonesia is 11.9 million ha. The tith of each hectare of rice fields can produce till 12-15 tons of dry material, it depends on location and varieties. So far, only 31-39% of rice straw use as fodder while another is about 36-62% used as fertilizer or be burned and about 7-16% used to industrial requiement [1].

Water hyacinth (*Eichhornia crassipes*) is a free-floating aquatic plants. Water hyacinth can grow and spread easily through the water supply to other water bodies. Water hyacinth has vegetative breeding speed rapidly, especially in the tropics and subtropics [2]. Because of the proliferation of water hyacinth which so quickly, this water hyacinth become weed for some areas in Indonesia. The amount
of water hyacinth has not been used optimally so many of researchers trying to develop the potential of water hyacinth into something that has high economic value.

According to BPS data on 2014, the production of dry-cornseed is about 19.03 million tons. The huge corn seed production create waste that called corncobs. Corncob usually dont used anymore or have low economic value.

Cellulase is one of the enzymes produced by microorganisms. Cellulase enzyme has an important role in the process of bioconversion of organic waste into glucose, single cell protein, animal feed, ethanol and others [3]. Cellulase is an enzyme that can hydrolyze β (1-4) in the cellulose. Cellulase enzymes can be obtained from various sources such as plants, insects, and microorganisms. Cellulase produced by fungi, bacteria, plants, and ruminants. Fungi are the main microorganisms that produce cellulase enzymes. Examples of filamentous fungi is Trichoderma and Aspergillus [4].

The purpose of this study is to use the straw, water hyacinth, and corn cobs as substrates for cellulase enzyme production, determine the effect type of substrates, moisture content and fermentation time in production of cellulase enzyme and also determine the optimum conditions for production of cellulase enzymes.

2. Material and method
2.1. Cellulosic materials

The raw material consists of water hyacinth which obtained from the river in Tirto Husodo Timur street Tembalang while corn cobs and straw obtained from Kecapi village Jepara. Before using, each substrat is chopped and sieved to a homogeneous size. While Aspergillus niger ITBCC L74 was obtained from the Laboratory of Microbiology and Bioprocess Bandung Institute of Technology.

2.2. Organism and culture condition

Culture of Aspergillus niger was maintained on PDA and incubated at 30 °C for 7 days [5,6,1].

2.3. Media preparation and enzyme production

Medium composition described by Mandles [22] and Weber was used for fermentation. The media contained (per liter of distilled water): urea 0.3 g, (NH₄)₂SO₄ 1.4 g, CaCl₂ 0.002 g, MgSO₄.7H₂O 0.3 g, KH₂PO₄ 2 g, FeSO₄.7H₂O 0.005 g, MnSO₄.7H₂O 0.0016 g, ZnSO₄.7H₂O 0.0014 g, tween-80 2 ml, and polypeptone 1 g. Production of cellulose enzym started by mixing each substrat and nutrient solution based on variable of moisture content. Adjusting the pH of fermentation and then sterilized by autoclaving 121°C for 15 min. This was cooled and inoculated with 1x1 cm² organism from PDA culture plates. Then incubated at ±30 °C and fermented with a predetermined time variables [5,6,1].

Fermented enzyme is extracted with distilled water so that would be obtained filtrate and solids. After that, the filtrate and solids are separated using a centrifuge. The filtrate obtained is used to test the protein and the activity of the enzyme.

2.4. Cellulase assay

The first analysis is the analysis of proteins by Lowry method. This analysis to indicate the levels of enzymes in the sample. Then, analysis for determine the activity of the enzyme produced is used CMC 1% is added to the enzyme extract, and glucose levels were tested by the method of DNS (3,5-dinitrosalicylic acid) [7].

3. Results and discussion
3.1. Raw material analysis for production of cellulase enzyme

This research was conducted on various types of substrates, namely rice straw, water hyacinth, and corn cobs. Characterization of the raw material of each substrate is presented in Table 1.
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content of the substrate is different from
18%, followed,

Table 1 Results of Raw Materials Characterization for Production of Cellulase Enzyme

| Parameter     | Rice Straw | Water Hyacinth | Corn Cob |
|---------------|------------|----------------|----------|
| **Cellulose (%)** | 35-45 (a)  | 17.8-31 (c)    | 32.3-45.46 (d) |
| **Hemicellulose (%)** | 30-40 (a) | 22-43.4 (c)    | 39-49 (d)    |
| **Lignin (%)**    | 5-20 (a)  | 7-26.36 (c)    | 6.7-13.9 (d) |
| **Protein (%)**   | 3-5 (b)   | 11.9-20 (c)    | 3.3-5.32 (e) |
| **Ash (%)**       | 7.8-15.6 (b) | 15-25.7 (c) | 2.77-2.99 (e) |

(a) [8]; (b) [1]; (c) [9]; (d) [10]; (e) [11]

Based on Table 1, it can be seen that there is a difference between the raw material content of the substrates used with that obtained from the literature. Such differences may be caused due to several factors such as climate, soil, pest / disease, as well as the type of the plant itself [12]. These factors certainly can’t be controlled, even though the same plant species, but other factors also depend on where they are grown also different and can cause the content of the substrate is different from the literature.

From Table 1, it can be seen that the corn cob contains cellulose which is the highest of 39.68%, followed by rice straw 38.26%, and 29.54% of water hyacinth. Corn cob hemicellulose also contains the highest of 40.58%, followed by rice straw 35.73%, and 35.71% of water hyacinth. Cellulose and hemicellulose in the raw materials are carbon source for the growth of Aspergillus niger for production of cellulase enzyme [13]. Water hyacinth has a lower cellulose content than straw or corn cobs, but also had the lowest lignin content is 7.46%, followed by rice straw 10.28% and 13.43% corn cobs. Lignin is a very complex molecules composed of units fenilpropan are connected in three-dimensional form that is difficult to be degraded. The higher proportion of lignin, the higher resistance to enzymatic degradation [14]. Because the carbon content is relatively high compared with cellulose and hemicellulose, lignin has a high energy content. Lignin naturally brown. If the substrate turns a little white color, it means there are some lignin missing. Lignin makes the substrate becomes hard and clay. If the substrate becomes softer, meaning its protector lignin already damaged [1]. Water hyacinth has the highest protein content is 12.18%, followed by rice straw 4.81%, and 4.61% corn cob. Protein is an amino acid that has the elements C, H, O, N as nutrients that get the highest enzyme [15]. Carbon acts as an enzyme cofactor [23], whereas nitrogen required in the fermentation process because it can affect the activity of Aspergillus niger [1]. In growth, carbon and nitrogen are used for the components of fungal cell of the body. This process occurs because during the fermentation of Aspergillus niger using nutrients for growth [16]. Water hyacinth also has the highest ash content is 15.21%, followed by rice straw 10.92%, and 2.7% of corn cob. The ash content in a material reflects mineral content, although the value of the ash can’t be used as an index to determine the number of elements. Fermentation requires an adequate intake of minerals to support the growth of mold (Aspergillus niger) so as to obtain optimal results [17].

3.2. Analysis of protein content

Based on the research that has been done by varying the variable type substrate, water content, as well as the long fermentation time, results for analysis of protein content are presented in Table 2.
3.2.1. Effect of fermentation time on protein content. According Ariyani et al (2014) [16], increased protein content in line with the growth of molds (fungi) due to the fungi body consists of elements containing nitrogen. Besides enzymes produced by the fungus is also a protein. This is also supported by Garraway and Evans (1984) [18], which states fungal cell wall contains 6.3% protein, while fungal cell membranes with hyphae contains 25-45% protein and 25-30% carbohydrates. In its growth, fungi using carbon and nitrogen for the fungus body cell components. This process occurs because during the fermentation of Aspergillus niger ITBCC L74 using nutrients (especially carbohydrates) for growth and increased protein content.

From Table 2 it can be seen that time of the fermentation affects the amount of protein content in the production of cellulase enzymes. On the third substrates highest protein content are produced on average are on the sixth day fermentation period. The highest protein content using substrates water hyacinth is 0.0194 mg/ml, followed by rice straw 0.0153 mg/ml, and corn cob 0.0146 mg/ml. After the sixth day, then the protein content decreased. It is because on the eighth day onwards Aspergillus niger ITBCC L74 are used currently on death phase, where the number of dead cells more than cells that grow [19].

3.2.2. Effect of water content on protein content. According Idiawati et al (2014) [5], the solid fermentation water content have an important role in the biosynthesis and secretion of many enzymes, especially cellulases. The fermentation process is closely related to microorganisms. Water has a very important function in the metabolism of organisms, including as a source of the element hydrogen and oxygen required for the biosynthesis of cell components, play an important role in the process of enzymatic hydrolysis and very important role in the transport of nutrients and products of metabolites through the cell membrane. Fermentation is a solid substrate fermentation using insoluble substrate but contain enough water for growth and development of microorganisms were inoculated into the substrate itself. Therefore, the presence of water affects the growth of Aspergillus niger ITBCC L74 to produce cellulase enzymes.

In Table 2 shows the water content can affect the protein content in the production of cellulase enzymes. Water content is too low leads to reduced solubility of nutrients in the substrate [1]. However, the optimum water content also depends on the type of substrate used. The highest protein content produced by using water hyacinth substrate with water content of 70% 0.0194 mg/ml, followed by rice straw with water content 80% 0.0153 mg/ml, and corn cob with water content 60% 0.0146 mg/ml.

| Substrate       | Moisture Content | 2 days | 4 days | 6 days | 8 days | 10 days |
|-----------------|------------------|--------|--------|--------|--------|---------|
| Rice Straw      | 50%              | 0.0068 | 0.0056 | 0.0122 | 0.0117 | 0.0086  |
|                 | 60%              | 0.0063 | 0.0055 | 0.0112 | 0.0109 | 0.0078  |
|                 | 70%              | 0.0108 | 0.0076 | 0.0129 | 0.0124 | 0.0104  |
|                 | 80%              | 0.0144 | 0.0116 | 0.0153 | 0.0147 | 0.0142  |
| Water Hyacinth  | 50%              | 0.0102 | 0.0083 | 0.0140 | 0.0133 | 0.0097  |
|                 | 60%              | 0.0171 | 0.0109 | 0.0161 | 0.0141 | 0.0124  |
|                 | 70%              | 0.0183 | 0.0139 | 0.0194 | 0.0156 | 0.0146  |
|                 | 80%              | 0.0038 | 0.0019 | 0.0089 | 0.0081 | 0.0073  |
| Corn Cob        | 50%              | 0.0121 | 0.0087 | 0.0144 | 0.0136 | 0.0114  |
|                 | 60%              | 0.0132 | 0.0095 | 0.0146 | 0.0140 | 0.0115  |
|                 | 70%              | 0.0102 | 0.0074 | 0.0118 | 0.0119 | 0.0093  |
|                 | 80%              | 0.0049 | 0.0046 | 0.0097 | 0.0092 | 0.0075  |
3.2.3. Effect of Substrate Type on Protein Content. Based on Table 2, the type of substrate can affect the production of cellulase enzymes. It can be seen from Table 1 on the content of each substrate. Water hyacinth has a lower cellulose content than straw or corn cobs, but also has a low lignin content. Lignin is a very complex molecules composed of units phenyl propane are connected in three-dimensional form that is difficult to be degraded. The higher the proportion of lignin, the higher the resistance to chemical and enzymatic degradation [14]. Because the carbon content is relatively high compared with cellulose and hemicellulose, lignin has a high energy content. Lignin naturally brown. If the substrate turns a little white color, it means there are some lignin damaged. Lignin makes the substrate becomes hard and clay. If the substrate becomes softer, it means the protector lignin already damaged [1]. In Table 1 shows that the corn cobs have a structure tougher than straw and water hyacinth for the lignin content most among the three substrate. Instead, the water hyacinth has a softer structure because the lignin content least of the third substrate. The highest protein content produced by using a substrate of water hyacinth 0.0194 mg/ml, followed by rice straw 0.0153 mg/ml, and corn cobs 0.0146 mg/ml.

3.3. Analysis of enzyme activity

The enzyme activity is expressed in U/ml. 1 unit is the amount of enzyme needed to break down cellulose into 1 mole of reducing sugar per minute. Based on the research that has been done by varying the variable type substrate, water content, as well as the long fermentation time, the enzyme activity analysis results are presented in Table 3.

3.3.1. Effect of fermentation time on enzyme activity. Based on Table 3, the fermentation time relationship with the activity of the enzyme can be expressed in Figure 1.

| Substrate     | Moisture Content | 2 days | 4 days | 6 days | 8 days | 10 days |
|---------------|-----------------|--------|--------|--------|--------|---------|
| Rice Straw    | 50%             | 0.517  | 0.694  | 1.267  | 0.643  | 0.491   |
|               | 60%             | 0.967  | 1.312  | 1.673  | 0.782  | 0.394   |
|               | 70%             | 1.226  | 1.353  | 1.783  | 1.117  | 0.536   |
|               | 80%             | 1.092  | 1.581  | 2.569  | 2.214  | 1.226   |
| Water Hyacinth| 50%             | 0.591  | 0.679  | 1.073  | 1.055  | 0.269   |
|               | 60%             | 0.517  | 0.973  | 1.125  | 0.794  | 0.205   |
|               | 70%             | 0.001  | 1.555  | 1.631  | 1.606  | 0.730   |
|               | 80%             | 0.617  | 0.657  | 0.986  | 0.637  | 0.442   |
| Corn Cob      | 50%             | 0.086  | 0.238  | 0.364  | 0.111  | 0.061   |
|               | 60%             | 0.694  | 1.023  | 1.302  | 1.251  | 0.719   |
|               | 70%             | 0.415  | 0.651  | 1.023  | 0.466  | 0.086   |
|               | 80%             | 0.035  | 0.086  | 0.466  | 0.339  | 0.010   |
Time of fermentation to produce cellulase enzyme is one of the essential aspects for microbial growth affecting the production of enzymes. In Table 3 and Figure 1 shows the effect of variations in fermentation time on the activity of enzyme cellulase. The enzyme activity will increase along with increasing fermentation time. However, the enzyme activity decreased on the eighth day and the tenth day. It is caused by the interaction between the enzyme cellulase to cellulose began to decline due to the accumulation of the product formed on the previous day, causing inhibition of the enzyme cellulase [20]. Besides the growth of mold is also influenced by the availability of nutrients [5]. If the availability of nutrients many lifetimes, the microorganisms are also getting longer. Conversely, if the availability of nutrients less than the lifetime of microorganisms will be more brief. The enzyme activity highest of rice straw 2.569 U/ml, followed by water hyacinth 1.631 U/ml, and corn cob 1.302 U/ml.

3.3.2. Effect of water content on enzyme activity. Based on Table 3, effect of water content on activity of the enzyme can be expressed in Figure 2.

The production of cellulase enzymes is influenced by factors such as keeping the water content. On Solid State Fermentation (SSF) water content has an important role in the biosynthesis and secretion of enzymes, especially cellulases [5]. The fermentation process is closely related to microorganisms. Water has an important function in microorganisms of which play a role in the metabolism of an organism as well as in the transport of nutrients. Solid State Fermentation (SSF) is fermented using substrates that are not soluble but contain enough water for growth and development of microorganisms were inoculated into the substrate. Therefore, the presence of water affects the growth of Aspergillus niger ITBCC L74 to produce cellulase enzymes. In Solid State Fermentation (SSF), the water content maximum is around 50% - 80% [16].

The growth of microorganisms is affected by the water content in the solid substrate. If the water content in low numbers it will reduce nutrient diffusion and solubility of the solid substance. Causing insufficient supply of nutrients to the growth of microorganisms and enzymes to be disrupted as well as lower production of the enzyme [20]. However, if the moisture content high, it will cause a decrease in porosity and gas volume, but it will increase the contamination causing a decrease in the production of the enzyme [21]. In the solid fermentation requires sufficient water conditions for mold growth, but not too wet because it will encourage the growth of bacteria.
Based on Table 3 and Figure 2 are shows that the water content effect on enzyme activity. The highest enzyme activity was obtained at the straw with water content of 80% followed by water hyacinth of 70% and corn cob of 60% with enzyme activity of 2.569, 1.631, 1.302 U/ml, respectively.

3.3.3. Effect of substrate type on enzyme activity. Based on Table 1 and 3, Figure 1 and 2 show that the enzyme activity was effected mainly cellulose content in substrate due to as a carbon source for growth of Aspergillus niger ITBCC L74 to produce cellulase enzymes. The cellulose content of rice straw and corn cob almost similar (38.26 and 39.68%) with enzyme activity of 2.569 and 1.302 U/ml, Although the water hyacinth has lower cellulose content (29.54%) than corn cob, but the enzyme activity was higher (1.631 U/ml), it might be due to protein and mineral content more higher than other was needed for growth as macro and micro element. Lignin content in substrate also affect to the enzyme activity.

4. Conclusion
The optimum fermentation time to produce cellulase enzymes obtained was six days. Based on the analysis of enzyme activity in the to the long fermentation time, obtained. The highest enzyme activity obtained was 2.569 U/ml by using rice straw as substrate with a water content of 80%. Followed by water hyacinth and corn cob with enzyme activity of 1.631 and 1.302 U/ml at water content of 70% and 60%, respectively.

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