Production of crude enzyme from *Trichoderma* sp.

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Abstract. Cellulose enzymes can degrade and hydrolyze the β-1,4 glycosidic bond in cellulose, resulting in various products, including glucose. This study aimed to find a species of Trichoderma and determine the optimal growth day for producing cellulase enzymes with the highest activity. Trichoderma is a fungus that can produce cellulase enzymes. The study was conducted to test the cellulase enzyme activity produced by a specific species of Trichoderma to identify the species with the highest activity level. A Trichoderma species that can increase and have the best activity in producing cellulase enzymes is required. The stages of cellulase enzyme production include mycelium growth on PDA media, enzyme sampling, activity testing using the 3,5-Dinitrosalicylic acid (DNS) method, and absorbance testing using a UV-Vis spectrophotometer. Based on the results of the production and activity testing of crude cellulase enzyme from Trichoderma selection at the Palm Oil Research Center, Bogor Unit, it was found that Trichoderma polysporum on day 3 of growth had the highest activity of 0.0583 U/mL.

1 Introduction

Oil palm (Elaeis guineensis Jacq.) is one of the plantation types that is in the highest position in the agricultural sector, especially plantations. Indonesia is one of the countries that has oil palm plantations spread throughout its territory. As much as 95% of the production produced from these plantations is in crude palm oil [1]. Apart from products in the form of crude palm oil, palm oil plantations also produce production waste. Some examples of solid waste from palm oil production are empty palm fruit bunches (EFB), shells, fibers, and sludge or palm sludge. It is known that as much as 35% - 40% of the solid waste produced from total oil palm bunches is processed [2]. So, the availability of empty bunches is currently increasing in line with the increase in the number and capacity of palm oil mills.

Each empty bunch contains nutrients, and the nutrients from the empty bunches can be reused as organic fertilizer for oil palm plants. The nutrients in this EFB are 0.74-0.98% sodium, 0.06-0.07% phosphorus, 2.10-2.18% potassium, and 0.16-0.40% calcium, which play an important role in the plant composting process [3].

There is also one part of the empty palm fruit bunches, namely the fiber part contains 2% ash, 19% lignin, and 65% cellulose [4]. Lignin, cellulose, and hemicellulose are the primary

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sources of producing sugar products, carbon sources, and energy. Lignin ($C_9H_{10}O_2(OCH_3)n$) is a constituent of lignocellulosic biomass and cellulose and hemicellulose. Lignin cannot dissolve in water and is stable when found in nature as a link between cellulose and hemicellulose. Cellulose is a polysaccharide with the same formula as starch, namely ($C_6H_{10}O_5$)n. Like lignin, cellulose cannot dissolve in water because it is located in the cell walls of woody plants and is crystalline because of its linear structure. Besides that, cellulose is not easily degraded chemically or mechanically [5]. The cellulose found in waste is generally in the form of lignocellulose coated with lignin. Therefore, cellulase enzymes are needed to degrade cellulose.

Currently, empty palm fruit bunches are generally used as compost. Making fertilizer from OPEFB is a cheap and accessible technology and can reduce and utilize palm oil waste. However, decomposing compost from empty oil palm fruit bunches takes a long time until it can all be decomposed. The average decomposition time required for fertilizer from empty palm oil bunches is around 6-10 months after application [6]. The long process causes new problems, namely, empty oil palm fruit bunches will pile up, becoming a new habitat for insect pests of oil palm plants. Therefore, activators can be added to speed up the decomposition process of organic materials, especially empty oil palm fruit bunches.

The addition of this activator can improve soil structure and increase plant growth due to the rapid degradation process. One enzyme that can be used as an activator for TKKS fertilizer is the cellulase enzyme. Cellulase enzymes are the hydrolytic enzymes used to degrade biomass and hydrolyze β-1,4 glycosidic bonds in cellulose. This hydrolytic enzyme can inhibit the growth of plant pathogens. Because hydrolytic enzymes can hinder the growth of pathogens, they can be used to shorten the composting or decomposition process [7]. Cellulase enzymes that are usually commercialized are generally used in the food, biofuel, detergent, and paper industries.

One type of fungus can produce cellulase, namely the Trichoderma type. Trichoderma has several species including Trichoderma polysporus and Trichoderma viridae [8]. Trichoderma is a type of cellulolytic fungus that produces cellulase complex enzymes. It is known that the composting process carried out by Trichoderma only takes one month [9]. Trichoderma in the composting process can break down organic materials such as carbohydrates and cellulose with the help of the cellulase enzyme [10,11]. tr*ichoderma*is, a genus of fungi that has been widely used for cellulase production due to its high cellulolytic activity. However, selecting Trichoderma strains with the potential for high cellulolytic activity is essential for efficient cellulase enzyme production.

Therefore, this study aims to select Trichoderma strains with high cellulolytic activity and use them to produce cellulase enzymes from palm oil waste. This study will test various Trichoderma strains and see the most increased potential enzyme activity on a particular growing day.

2 Methods

2.1 Materials

Several tools and materials are used to produce cellulase enzymes from Trichoderma selection. The materials used include potatoes as the media, one liter of water, agar, and dextrose. Trichoderma sp; Trichoderma polysporum, and Trichoderma viridae owned by the Bogor Palm Oil Research Center as the isolate to be used. Citrate buffer solution, DNS (2,3-dinitrosalicylic acid), and substrate in filter paper (Whatman). The tools that will be used include a water bath (DLAB), Erlenmeyer and petri dish (IWAKI), Falcon, Microtips, and

micropippet (Thermo Scientific). Laminar Air Flow, autoclave (HIRAYAMA HVE-50), centrifuge (Eppendorf), and UV-Vis spectrophotometer (Thermo Scientific).

2.2 Research design

This research used a completely randomized design with repetition three times. This repetition was carried out for each Trichoderma species on the day of growth. Three types of Trichoderma are used, namely, Trichoderma sp, Trichoderma viridae, and Trichoderma polysporum. Cellulase activity was measured using a spectrophotometer on samples with a wavelength of 575 nm. This test was carried out on each Trichoderma species on growth days 3, 5, 7, 10, 12, and 14.

2.3 Media of Trichoderma from Potato Dextrose Agar (PDA)

Making potato PDA media begins with preparing ingredients like potatoes, agar, and dextrose. The three components were weighed with an analytical balance for 200g potatoes, 20g agar, and 20g dextrose. Prepare 1L of water and store it in a saucepan. Boil potatoes with water until boiling. After boiling, the cooking water is poured into 1L of duran. Add agar 20g and dextrose 20g, then homogenize. Once homogeneous, the media was sterilized in the autoclave at 121^{o}C for 15 minutes. If the media is stored at room temperature, when pouring it into a Petri dish, it must first be thawed so that it is not in agar or solid form.

2.4 Pre-culture and culture stages

At the pre-culture and culture stages, it is essential to pay attention to sterilizing the media that will be used. After preparing the PDA media, it was continued by preparing all isolates and then printing Trichoderma with cockbor. Transfer the printed Trichoderma using a sterile scalpel onto the PDA media. Cover and observe the growth diameter of Trichoderma for 7 days. After 7 days, transfer the isolate or Trichoderma into the PDB media which was previously poured into the Erlenmeyer. Observe the growth of isolates daily to prepare for activity tests on the 3rd, 5th, 7th, 10th, 12th, and 14th growth days.

2.5 Sampling method

At the sampling stage, the first step is to prepare isolates in Erlenmeyer. Pour the sample in the erlenmeyer into 10 mL of falcon, then name it according to the Trichoderma strain. Then centrifuged at 4°C at 10,000 rpm for 10 minutes [12].

2.6 Crude cellulase enzyme activity

The final step is to test the activity of crude cellulase enzymes from the selection of Trichoderma. The falcon that will be used is separated into 3 parts, namely, 1 falcon for the blank, 9 falcons for A0 as a control using filter paper substrate, and 9 falcons for UA as a test sample containing the cellulase enzyme from Trichoderma. The nine falcons are used to differentiate the types of Trichoderma and their repeats. Fill the UA with 500 µL citrate buffer and the blank with 1mL citrate buffer. Add the enzyme into A0 as much as 500 µL. Place A0 in a water bath at 100^{o}C for 15 minutes.

After that, add 500 µL of citrate buffer into falcon A0 and then homogenize with a vortex. The conditioning stage was carried out on all falcons in a water bath at a temperature of 50^{o}C for 10 minutes. After that, add 500 µL of enzyme to the falcon UA. Incubate all falcons in a

water bath at 50ºC for 1 hour. Then, DNS solution were added to all falcons with 3 mL each. Then the entire falcon was heated in a water bath at 100ºC for 5 minutes. Falcon is neutralized and can then be put into a microplate of 350 µL. After that, an activity test was carried out for each observation using a spectrophotometer with a wavelength of 575 nm. The specific activity level of the cellulase enzyme is searched. The equation to find the activity level is as follows:

$$\frac{\mathrm{U}}{\mathrm{mL}} = \frac{\frac{\mathrm{mg}}{\mathrm{mL}} \times \mathrm{Fp}}{\text{incubation time (menit)}} \times \frac{1000}{\text{Mr Glucose}} \quad (1)$$

3 Result and discussion

3.1 Growth of Trichoderma sp

Growth of Trichoderma sp. starting with preculture Trichoderma sp. on PDA media for 7 days. Basically, Trichoderma sp. is a fungus that can grow in various habitats and environments [13]. The preculture stage makes Trichoderma grow in media that is rich in nutrients, so that it can grow optimally. The propagation results from preculture (Figure 1a) are used as inoculum for PDB (Figure 1b). The growth of Trichoderma on day 7 on PDA and PDB media can be seen in Figure 1.

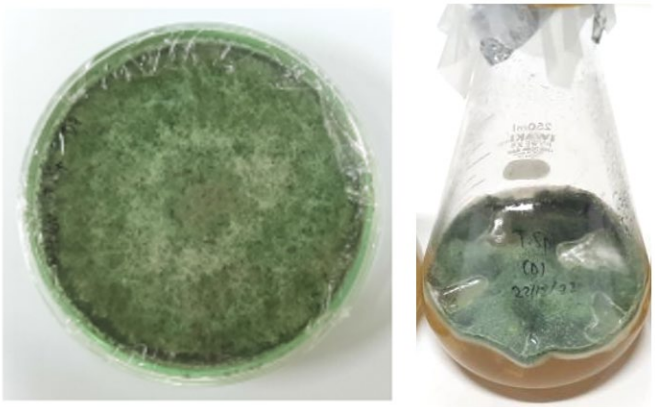

Fig. 1. The growth of Trichoderma sp. days 7 at different media: (a) PDA; (b) PDB

Trichoderma sp. can produce cellulase enzymes so that this fungus can degrade media containing cellulose such as EFB [14]. So, the prospect of Trichoderma sp. as a biodecomposer whose function is to break down organic materials very well. Accelerated growth with the highest enzyme activity values is the key to becoming a good decomposer.

3.2 Glucose standards

Determination of the level of activity of the cellulase enzymes produced by Trichoderma sp, Trichoderma viridae, Trichoderma polysporum was determined by the level of absorbance resulting from the spectrophotometer and the calibration curve of glucose standard measurements. The glucose standard curve states the relationship between glucose concentration and the optical density level at a specific wavelength [12].

The process for making a standard series solution test is the same as the activity test procedure using the DNS method and a UV-Vis spectrophotometer. It can be seen that the linearity of the concentration of the standard solution with the resulting absorbance forms the

equation y = 1.9296x - 0.0292. Where Y is the absorbance and x is the concentration of glucose [12]. The value of a is the slope or slope of 1.9296 and b or intercept is the response value to the blank with an ideal value of zero of -0.0292.The R-value is the correlation coefficient between analyte concentration and response or absorbance [15] namely 0.9886.

3.3 The enzyme activity of crude cellulose

Based on the activity test stages carried out previously, the absorbance value of each sample was obtained, namely the cellulase enzyme in each Trichoderma. This test was carried out on Trichoderma sp, Trichoderma viridae, and Trichoderma polysporum periodically on growth days 3, 5, 7, 10, 12, and 14 (Table 1). Measurement results were obtained using a UV-Vis spectrophotometer with a wavelength of 575 nm.

Table 1. Data from absorbance test results with a wavelength of 575 nm.

Cellulase Enzyme Absorbance Test at a Wavelength of 575 nm							
Parameter	**Test**	**Growth Day Comparison**					
		3	**5**	**7**	**10**	**12**	**14**
Trichoderma sp	1	0.0600	0.3269	0.0639	0.0767	0.0467	0.1005
	2	0.1247	0.3100	0.0733	0.0596	0.0336	0.0683
	3	0.2588	0.2398	0.0545	0.0555	0.0703	0.0843
Trichoderma polysporum	1	0.1304	0.3096	0.0407	0.0562	0.0272	0.0696
	2	0.0953	0.3080	0.0513	0.0563	0.0453	0.0855
	3	0.1748	0.5846	0.1095	0.0824	0.0348	0.0874
Trichoderma viridae	1	0.1865	0.0164	0.0280	0.0560	0.0766	0.0481
	2	0.1710	0.0110	0.0370	0.0834	0.0846	0.0549
	3	0.1492	0.0054	0.0403	0.0798	0.0418	0.0521

Cellulase enzyme activity values from Trichoderma sp. The various types of isolates owned by PPKS Bogor can be seen in Table 2.

Table 2. Average Trichoderma cellulase enzyme activity test results (U/mL).

Average Trichoderma Cellulase Enzyme Activity Test Results (U/mL)			
Growing Up Days	***Trichoderma sp***	***Trichoderma polysporum***	***Trichoderma viridae***
3	0.0464	0.0583	0.0514
5	0.0019	0.0156	0.0072
7	0.0075	0.0084	0.0061
10	0.0063	0.0046	0.0047
12	0.0054	0.0401	0.0052
14	0.0064	0.0058	0.0045

Trichoderma sp. on the 3rd growth day it had the highest cellulase activity of 0.0464 U/mL. Then the activity value of Trichoderma sp. continues to decline. Trichoderma polysporum which had the highest activity, namely on the 3rd growth day, had cellulase activity of 0.0583 U/mL. Then it decreased until the 10th growth day. On the 12th growth day of Trichoderma polysporum cellulase activity again increased by 0.0401 U/mL. Trichoderma viridae which had the highest activity, namely on the 3rd growth day, had cellulase activity of 0.0514 U/mL. Furthermore, the activity value of Trichoderma viridae continues to decline.

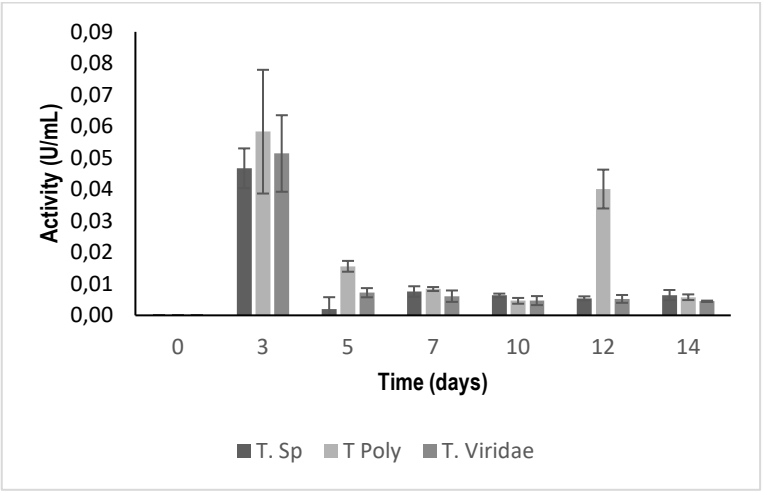

Fig. 2. Average trichoderma cellulase enzyme activity test results.

Each activity will depend on the type of Trichoderma species and also the growing days of the fungus. The resulting graph shows that Trichoderma polysporum has the highest average growth. It can be seen that Trichoderma polysporum on the 3rd growth day has the highest activity level, namely 0.0583 U/mL. After that, a few days later the activity of the cellulase enzyme decreased. However, on the 12th day of growth, the activity of Trichoderma polysporum increased again to 0.0401 U/mL and then decreased again. Trichoderma sp. showed that the highest activity was on the 3rd growth day, namely 0.0464 U/mL, the same as Trichoderma viridae which showed the highest activity, namely on the 3rd growth day, 0.0514 U/mL.

The level of activity of an enzyme can decrease, this is because there is a struggle for nutrients between microorganisms so that the growth of microorganisms is hampered [16]. Enzyme activity can be affected by many factors such as temperature, pH, substrate concentration, presence of cofactors or coenzymes, activators, inhibitors, etc [17-19]. In some cases, changes in enzyme activity may occur naturally during the life cycle or in response to environmental changes. For example, in some types of enzymes the activity can fluctuate during the daily cycle due to regulation by internal factors and external factors such as light and temperature [20]. For example, in enzymes involved in glucose metabolism such as glucokinase and phosphofructokinase [21].

Based on this activity test, the level of cellulase enzyme activity in all types of Trichoderma is below one and close to zero. These results can be said that the cellulase enzyme activity is still small. However, a low or inactive enzyme activity test value does not always mean that the enzyme does not have the potential to increase its activity. Further research is needed, especially in optimizing conditions to increase production efficiency and cellulase enzyme activity.

4 Conclusion

The treatments carried out show that Trichoderma polysporum on the 3rd growth day had the highest activity level, namely 0.0583 U/mL. After that a few days later the cellulase enzyme activity decreased. However, on the 12th day of growth, the activity of Trichoderma polysporum again increased to 0.0401 U/mL. However, suppose you look at the results of the activity test, which overall are below one and close to zero. In that case, it can be said that the cellulase enzyme from Trichoderma polysporum has a small level of activity when

used as an activator. However, a low or inactive enzyme activity test value does not mean that the enzyme does not have the potential to increase its activity [22]. Therefore, further research is needed, especially in optimizing conditions to increase production efficiency and cellulase enzyme activity

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