The effect of various substrate on production of cellobiose dehydrogenase enzyme by *Trametes versicolor*

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**Abstract.** Cellobiose dehydrogenase is an extracellular flavocytochrome which uses various oligosaccharide substrates as electron donors to reduce phenoxy radicals, radical or quinone compound. The flexible electrons transfer properties from CDH are currently exploited for many biomedical applications including wound dressing hydrogel [2,3]. Wound dressing application of CDH has a strong potential uses in medical cure for chronic wound which have become a major problem in medical world [4–6]. CDH could convert plant phenolic antioxidant compounds continuously into its original form after scavenging reactive oxygen species (ROS) from chronic wound [1,3]. Another uses is continuous supply of H₂O₂ as an antibacterial agent through Fenton reaction and prevent biofilm formation of bacteria [3].

CDH could be isolated and characterized from variety of white rot fungi, soft rot fungi, and brown rot fungi [7]. Some studies of CDH production carried out by *Trametes versicolor* white rot fungi [8], [9]. The CDH enzyme produced by *T. versicolor* has a molecular weight of around 79,324 Da for complex forms while its flavin domain has a molecular weight around 58,393 Da [8], pI of 4.2, optimum pH 5 with an optimum temperature of 50ºC [9]. CDH enzymes produced by *T. versicolor* are known to effectively degrade complex compounds including cellulose, hemicellulose, and lignin, which are commonly found in plants [10]. The function of this enzyme is not very clear on the fungi, but CDH involved on the degradation process of two biopolymers which are cellulose and lignin.

1. Introduction

Cellobiose dehydrogenase (CDH) is an extracellular flavocytochrome which uses various oligosaccharide substrates as electron donors to reduce phenoxy radicals, radical or quinone compound [1]. The flexible electrons transfer properties from CDH are currently exploited for many biomedical applications including wound dressing hydrogel [2,3]. Wound dressing application of CDH has a strong potential uses in medical cure for chronic wound which have become a major problem in medical world [4–6]. CDH could convert plant phenolic antioxidant compounds continuously into its original form after scavenging reactive oxygen species (ROS) from chronic wound [1,3]. Another uses is continuous supply of H₂O₂ as an antibacterial agent through Fenton reaction and prevent biofilm formation of bacteria [3].

CDH could be isolated and characterized from variety of white rot fungi, soft rot fungi, and brown rot fungi [7]. Some studies of CDH production carried out by *Trametes versicolor* white rot fungi [8], [9]. The CDH enzyme produced by *T. versicolor* has a molecular weight of around 79,324 Da for complex forms while its flavin domain has a molecular weight around 58,393 Da [8], pI of 4.2, optimum pH 5 with an optimum temperature of 50ºC [9]. CDH enzymes produced by *T. versicolor* are known to effectively degrade complex compounds including cellulose, hemicellulose, and lignin, which are commonly found in plants [10]. The function of this enzyme is not very clear on the fungi, but CDH involved on the degradation process of two biopolymers which are cellulose and lignin.
CDH could reduce Fe$^{3+}$ to Fe$^{2+}$ by the support of hydrogen peroxide (H$_2$O$_2$), producing highly reactive hydroxy radical to attacks lignin and cellulose on the wood [11].

In order to support the research about wound dressing hydrogel, the optimization of CDH production could be performed. Several substrates have been used in production culture of CDH from T. versicolor including cellulose [9,12]. Other substrates need to be used in order to find out the best yield of CDH enzyme production. The best yield of the CDH enzyme production optimization could support the best result on the wound dressing hydrogel research.

2. Material and Methods

2.1. Subculture of Trametes versicolor

Trametes versicolor isolates were obtained from the collection of the Microbiology and Biotechnology Laboratory, Department of Biology, Faculty of Science, Sepuluh Nopember Institute of Technology. Subculture of T. versicolor isolates was carried out by taking 1 loop of isolate to Petri dishes containing sterile solid potato dextrose agar (PDA) medium and incubated at room temperature for 7 days [13].

2.2. Production Optimization of Cellobiose Dehydrogenase

Every 100 mL T. versicolor production culture medium containing KCl 0.05 g, MgSO$_4$.7H$_2$O 0.01 g, KH$_2$PO$_4$.0.10 g, yeast extract 0.01 g, NH$_4$Cl 0.04 g, glucose 0.05 g, substrate 0.45 g, and trace metal solution 0.10 mL. The trace metal solution prepared in every 20 mL containing FeCl$_3$.6H$_2$O 0.1081 g, CuSO$_4$.5H$_2$O 0.0288 g, MnCl$_2$.4H$_2$O 0.0792 g, CoCl$_2$.6H$_2$O 0.0286 g, NiCl$_2$.6H$_2$O 0.0005 g, and (NH$_4$)$_6$Mo$_7$O$_24$.4H$_2$O 0.0124 g. The production media were prepared with six different types of substrates: carboxymethyl cellulose, lactose, methyl cellulose (Metolose), toilet paper, cellulose crystals (Vitacel), and Whatman filter paper No. 1.

Mold colonies which grown on PDA were taken about 2 cm$^2$ and put into 100 mL of production culture medium. The culture was incubated on rotary shaker 160 rpm at room temperature (pH 6.5). The culture were harvested every day about 1 mL from day 2 to day 14 to obtain enzyme activity data on every day of culture incubation. The harvested enzyme were centrifugated at 12,000 rpm for 30 minutes. Supernatant were concentrated using Whatman paper no. 1 and used as crude CDH enzyme extract.

2.3. Cellobiose Dehydrogenase Activity Assay

The DCIP standard curve was made by measuring the absorbance of DCIP 3 mM (in 10% ethanol) which is diluted in distilled water to a concentration of 0, 25, 50, 75, 100, 125, 150, 200, 225, and 250 µM. Absorbance was measured by UV Vis spectrophotometer at 520 nm wavelength. The absorbance value obtained were used to create a curve with equation $y = ax + b$, where $y$ is the absorbance value, $x$ is the DCIP concentration value, and $a$ is the slope value of the DCIP standard curve.

CDH crude enzyme activity known by measuring the absorbance decrease of 2,6-dichlorophenol-indophenol (DCIP) [14]. The CDH activity test was carried out by reacting 1 mL of the mixture (pH 4.5) containing 50 µL DCIP 3 mM (in 10% ethanol), 100 µL lactose 300 mM (in 100 mM buffer sodium acetate, pH 4.5), and 50 µL NaF 80 mM (in distilled water). The reaction starts by adding 10 µL of CDH enzyme. The decrease in absorbance was monitored every second for 5 minutes using a UV Vis spectrophotometer at a wavelength of 520 nm. The absorbance value obtained were used to form a curve with the equation $y = ax + b$, where $y$ is the absorbance value, $x$ is the incubation time (seconds), and $a$ is the slope value of the enzyme activity test. Calculation of enzyme activity was done using formula (1).

$$\text{Enzyme activity (U/mL/minute)} = \frac{\text{Slope value of enzyme activity} \times \frac{1}{\text{dilution factor}} \times \frac{1}{\text{incubation time (minute)}}}{\text{Slope value of DCIP standard curve}}$$ (1)
2.4. Protein Content Assay
CDH characterization was carried out by measuring protein content using bovine serum albumin (BSA) solution as a measurement standard [9]. The reagent was made based on Bradford method [15]. 100 mg of Coomasie Brilliant Blue G-250 (dissolved in 50 mL of 95% ethanol) and added by 100 mL of 85% H₃PO₄. The mixture were dissolved with distilled water until it reaches the final volume of 1 liter and were filtered using Whatman No. 1. Concentrated BSA solution was dissolved with distilled water with concentration (w/v) 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 mg/mL. An amount 0.1 mL of each BSA concentration was tested by adding 5 mL Bradford reagent, homogenized using vortex and its absorbance were measured at 595 nm wavelength. The absorbance value obtained were used to form a standard curve with the equation y = ax + b, where y is the absorbance value, x is the BSA standard concentration value, and a is the slope value of the BSA standard curve.

Measurement of CDH protein content was carried out based on the BSA standard curve that had been made. As much as 0.1 mL of CDH added 5 mL of Bradford reagent then homogenized with vortex. The solution was measured for its absorbance at 595 nm wavelength using UV Vis spectrophotometer. Calculation of protein concentration carried out by the formula (2), whereas the specific enzyme activity was calculated by formula (3).

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\text{Protein concentration (mg/mL)} = \frac{\text{Absorbance of CDH crude enzyme of Bradford assay}}{\text{Slope value of BSA standard curve}} \quad (2)
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\text{Specific activity (U/mg/minute)} = \frac{\text{Enzyme activity (U/mL/minute)}}{\text{Protein content (mg/mL)}} \quad (3)
\]

2.5. Enzyme Activity Assay with Various Substrate Concentrations
The CDH activity test was carried out by reacting 1 mL of the mixture (pH 4.5) containing 50 µL DCIP 3 mM (in 10% ethanol), 50 µL NaF 80 mM (in distilled water) and lactose 300 mM (in 100 mM sodium acetate buffer, pH 4,5) dissolved to concentrations of 1, 3, 5, 7, 10, 40, 80, and 120 µM. The reaction starts with adding 10 µL of CDH enzyme. The decrease in absorbance was monitored every second for 5 minutes using a UV Vis spectrophotometer at a wavelength of 520 nm. The absorbance value obtained is made in the form of a curve with the equation y = ax + b, where y is the absorbance value, x is the incubation time (seconds), and a is the slope of the enzyme activity test. Calculation of enzyme activity with the final unit µM/mL/minute or U/mL/minute or U/mL is done by the formula (1).

3. Result and Discussion
3.1. Crude Celllobiose Dehydrogenase Enzyme Activity
Crude CDH enzyme extract from T. versicolor production culture with six different substrates showed that the enzyme had different activities over time of culture incubation (Figure 1). The activity of crude CDH enzyme extract with toilet paper substrate (TIS) had the highest activity by increasing of activity from second day culture incubation. The maximum activity of this extract was at 9th day. The activity of TIS crude CDH enzyme extract decreased activity to 240 U/mL/minute on the 11th day. However, the activity of these enzymes increased again on the 14th day.

The crude CDH enzyme extract which has moderate activity is Whatman No. 1 filter paper (WAT) substrate and crystalline cellulose (VIT) substrate. The activity of the crude CDH enzyme extract with both of substrates also increased over incubation time of the production culture. Crude CDH-WAT enzyme extract reached maximum enzyme activity on 9th day (120 U/mL/minute) and began to decline on 11th day. Whereas, CDH-VIT crude enzyme extract continued to show improvement of the activity that continued to rise until the last day of incubation culture (120 U/mL/minute). The activity of crude CDH enzyme extract which has low activity is lactose (LAK), methyl cellulose (MET), and carboxymethyl cellulose (CMC) substrate. Those substrates have fluctuating enzyme activity below 36 U/mL/minute.
Graph of CDH crude enzyme extract activity on the production medium everyday incubation of *T. versicolor* culture. CMC: carboxy methyl cellulose, LAK: lactose, MET: methyl cellulose, TIS: toilet paper, VIT: cellulose crystals, and WAT: Whatman No. filter paper.

### 3.2. Protein Content of Crude Celllobiose Dehydrogenase Enzyme

The measurement of protein concentration in the crude CDH enzyme extract on day 14 showed that crude enzyme extracts that had high activity had high protein content as well, namely the crude enzyme extract CDH TIS, WAT, and VIT. While crude enzyme extracts that have low activity also have low protein concentrations, namely LAK, CMC, and MET (Table 1).

#### Table 1. Specific protein and activity concentrations of CDH crude enzyme extract on various substrate variations on day 14. CMC: carboxy methyl cellulose, LAK: lactose, MET: methyl cellulose, TIS: toilet paper, VIT: cellulose crystals, and WAT: Whatman No. 1 filter paper.

| Substrate | Crude Enzyme Activity (U/mL/min) | Protein concentration (mg/mL) | Specific activity (U/mg/min) |
|-----------|---------------------------------|------------------------------|------------------------------|
| TIS       | 360                             | 0.193                        | 1867.221                     |
| WAT       | 84                              | 0.130                        | 647.795                      |
| LAK       | 12                              | 0.029                        | 413.718                      |
| VIT       | 120                             | 0.107                        | 1116.381                     |
| CMC       | 12                              | 0.015                        | 781.467                      |
| MET       | 24                              | 0.024                        | 1004.743                     |

The substrate which has the highest activity is toilet paper. This material is pulp made from plant fibers. Plant fibers are formed by three structural polymers such as cellulose, hemicellulose, and lignin, as well as several small components such as proteins, extractive substances, and minerals [16]. Pulping processes through several processes that can degrade components from plant fibers. The pulping process includes wood handling, boiling, selection, delignification, and bleaching [17]. The delignification and bleaching process could reduce lignin in the pulp. Pulp as a substrate supplies cellulose, hemicellulose and lignin for *T. versicolor* culture nutrients. These would induce the
formation of other enzymes to degrade the substances. *T. versicolor* can produce cellulase enzymes, xylanase [18], laccase, manganese peroxidase, versatile peroxidase related to Mn, versatile peroxidase not related to Mn [19], and CDH enzymes [12, 20]. These enzymes can simplify the form of polymers from long chain compounds in plant fibers and pulp. Simple compounds make compounds with long polymers can turn into oligomers which are more easily recognized as specific substrates in other extracellular enzymes. Cellulase enzymes can simplify the form of p-nitrophenyl-β-D-cellobioside to cellobiose which can be utilized by the CDH enzyme to reduce its shape to cellubionolactone[21].

Cellulose substrate is a common substrate used for CDH production by *T. versicolor*. Some studies use Solka-Floc microcrystalline cellulose to produce CDH [9,12]. Alpha cellulose and cellulose crystals produce CDH crude enzyme extract with moderate activity. The utilization of cellulose derivative substrates namely CMC and methyl cellulose did not have a good effect on CDH enzyme activity. Both of ingredients produce low enzyme activity. This proved that the *T. versicolor* could not use derivative substrates from cellulose as a substrate to produce the CDH enzyme optimally.

The CDH enzyme produced by *T. versicolor* can efficiently catalyze oxidation in disaccharides or oligosaccharides which have glucose as a reducing unit and the second sugar unit is bound to the reducing group by 1,4 beta bonds [22]. There have been no reports of the use of lactose in CDH production cultures in *T. versicolor*. The use of lactose in CDH production culture by *T. versicolor* produces low enzyme activity. This shows that lactose is not a substrate that is essential for the growth of *T. versicolor*.

The key factor affecting the activity rate of the reaction catalyzed by the enzyme is the concentration of the substrate. The activity of CDH TIS crude enzyme extract at 1 mM lactose substrate concentration showed activity of 60 U/mL. Activity increased at lactose substrate concentration of 3 mM to 120 U/mL. At a concentration of 5-120 mM the activity increased to 240 U/mL and did not experience an increase (Figure 2 a). In this condition the maximum reaction rate ($V_{\text{max}}$) of CDH enzyme extract can be estimated by calculating the equation in the Lineweaver-Burk diagram. From the calculation, the maximum reaction rate was 303.03 µM/minute. While the Michaelis-Menten constant ($K_m$) of the CDH enzyme extract is 4 where $K_m$ is worth half of the maximum reaction speed (Figure 2 b) [23].

**Figure 2.** Crude CDH-TIS enzyme reaction rate with various lactose substrate concentration, a. Michaelis-Menten chart, b. Lineweaver-Burk chart.
4. Conclusion

Optimization of CDH enzyme production using several substrates shows that toilet paper is the best substrate in the production culture of cellobiose dehydrogenase (CDH) enzyme by *Trametes versicolor*. The usage of alpha cellulose substrate and crystalline cellulose can also produce CDH enzymes with lower activity. While the use of cellulose derivative substrates (methyl cellulose and carboxymethyl cellulose) and lactose produce CDH enzymes with low enzyme activity.

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