OPTIMIZATION OF CELLULASE PRODUCTION BY CANDIDA G3.2 FROM THE RHIZOSPHERE OF GUNUNG ANYAR MANGROVE SURABAYA

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ABSTRACT

Microbial cellulases are the enzymes widely used in many biochemical industries. Yeast has potential to degrade cellulose because of cellulase activity. Yeast from the genus of Candida G3.2 has the ability to hydrolaze cellulose which tested by cellulolytic index. The purpose of this study is to determine the incubation time and the combination of physicochemical factors (pH and temperature) optimal for the production of cellulase enzymes by yeasts.The study design by factorial which analyzed by descriptive quantitative. This study begins with the optimization of cellulase production, starting with the optimization of the incubation period and followed by a combination of temperature optimization (room, 45°C, 55°C) and pH (4.5, 6, 8) then measure the cellulase activity. The results showed that the best incubation period for cellulase production is 72 hours (267 U/ml) with a combination of temperature 55°C and pH 6 (369.8 U/ml).

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1. INTRODUCTION

Yeast belongs to a group of microorganisms that are known to produce cellulases other than bacteria and fungi (Kirk et al., 2002). Yeast species such as *Pichia stipitis*, *Candida shehatae*, *Candida acidothermophilium*, *Kluvyveromyces marxianus*, *Trichosporon sp*., and *Pachysolen tannophilus* have an advantage in producing cellulase compared with other microorganisms. The advantage of the yeast group is the high catalytic reaction of the insoluble cellulose substrate, acting on a wide range of pH and temperature, having great tolerance to the inhibitor, and being able to use a sugar substrate with the amount of carbon bonds C5 and C6 (Agbogbo, 2008; Oikawa et al., 1998; Ballesteros et al., 2004; Golias et al., 2002; Kadam and Schmidt, 1997).

Cellulase (EC 3.2.1.4) include in the hydrolase enzyme class that catalyzed cellulose hydrolytic reaction into a simple form of a dissolved glucose monomer unit (Migardon et al., 2011; Romsaiyud et al., 2009). The complex of cellulose enzyme consists of three catalytic subunits; Endo-1,4-β-glucanase (EC 3.2.1.4), Exo-1,4-β-glucanase or cellobiohydrolase (EC 3.2.1.91), dan β-glucosidase (EC 3.2.1.21) that work synergistically. Cellulase hydrolyzes the β-1.4 glycosidic bond between D-glucose molecules (Goldschimdt, 2008). Cellulase has an important role in textile and fabric processing industries, paper recycling, detergents, fruit and vegetable extraction in the food industry, animal feed additives, ethanol, lignocellulosic waste treatment, agriculture, wine and beer industry, pharmaceutical and the use of this enzyme in the world's industry is the third largest (Sharada et al., 2014; Wilson, 2009; Zhang and Chi, 2007).

Cellulase production by microorganisms is influenced by various factors such as pH and temperature. The application of cellulase enzymes in the industry requires microorganisms that can survive at various pH and

temperature. This is because, during the fermentation process, the temperature and pH may change from the initial scale (Singh et al., 2009; Zhang et al., 2009).

Previous studies have found some isolates from Rhizosphere of Gunung Anyar Mangrove Surabaya. Some of these isolates have been tested the potential to degrade cellulose by hydrolytic test (Alami and Shovitri, 2015). Cellulose degradation can occur due to cellulase enzyme activity. So it can be said that this yeast is able to produce cellulase enzyme. Therefore, the aim of this research was to optimize cellulase production so that it has high enzyme activity value.

2. RESEARCH METHOD

Subcultures of Yeast Isolates

Yeast isolate collection of Microbiology and Biotechnology Laboratory, Biology Department,ITS, was subcultured on a slant YMEA (Yeast Malt Extract Agar) medium, and then was incubated for four days at room temperature. Yeast was isolated from Rhizosphere of Gunung Anyar mangrove Surabaya with G3.2 isolate code.

Preparation of the Yeast Growth Curve

Preparation of yeast growth curve aims to determine the age of culture so that it can be determined incubation time to make culture starter. The inoculum for the growth curve has an optical density (OD) of 0.5. After the OD value has reached 0.5, the suspension is taken as much as 20 ml to be inoculated into a 180 mL medium of Yeast Malt Extract Broth (YMEB) on a 500 mL Erlenmeyer (Kurtzman and Fell, 1998), then incubated in a rotary shaker at room temperature with a speed of 120 rpm (Kathiresan et al., 2011). Yeast growth was observed by the optical density value every day until the death phase using the method of turbidimetry with wavelength (λ) 600 nm. The method of turbidimetry was performed by inserting cultures into quvet as much as ± 2 mL and measured OD value using UV-Vis spectrophotometer. The obtained OD value data was used to generate a growth curve with the x-axis as time (t) and the y-axis as the OD value. Once known growth phase, then the age of starter can be determined, that is in the exponential phase (Suhartono, 1989).

Making of Selulase Production Medium

Cellulase enzyme is produced in CMC medium (Carboxyl Methyl Cellulose) Broth according to Apun (1995) by removing glucose composition. The medium is made by adding 2 gr / l of Carboxymethyl Cellulose (CMC), $NaNO_3$ 1 g / l, K_2HPO_4 1 g / l, $MgSO_4$. $7H_2O$ 0.5 g / l, KCl 1 g / l, yeast extract 5 g / l, $FeSO_4$ 0.01 g / l, and chloramphenicol 200 mg / l in 1 liter water. After that, the medium is homogenized and heated with a hot plate magnetic stirrer. The medium is then sterilized using an autoclave at a temperature of 121^0C and a pressure of 1.5 atm for 15 minutes (Apun, 1995).

Optimization of Cellulase Enzyme Production

Optimization of cellulase enzyme production begins by determining the optimum incubation time. The optimum incubation time is determined based on the highest cellulase activity results during the incubation time. After that proceed with temperature optimizer and pH. Crude enzyme and biomass in the production medium were separated by centrifugation method at 6000 rpm for 15 minutes. The supernatant is used to measure cellulase activity.

Optimization of Incubation Time

10 ml starter cultures were inoculated on 90 ml CMC Broth medium. The CMC Broth medium containing inoculum was incubated on the rotary shaker at an agitation of 120 rpm (Kathiresan et al., 2011) for 7 days at room temperature. The enzyme activity test was performed at 24, 48, 72, 96, 120, 144 and 168 hours to determine the effect of the incubation time (Ijaz, 2011). The control used was CMC Broth medium without the addition of culture.

Optimization of Temperature and pH

Temperature optimization and pH of cellulase enzyme production were done by inoculating 10 ml of starter culture on 90 ml CMC Broth medium with initial pH variation of 4.5, 6, and 8. The pH adjustment was

performed by adding different buffer ie 1 mol / L sodium acetate (pH 4.5) and 1 mol / L sodium phosphate (pH 6 and pH 8) (Rai et al., 2012). The culture medium with various variations of pH was then incubated at different temperatures; room temperature, 45 ° C, and 55 ° C. The culture medium was incubated using an incubator shaker at an agitation of 120 rpm according to the determined temperature (Kathiresan et al., 2011). The control used was CMC Broth medium without the addition of culture. The length of incubation time is determined based on the optimal incubation time of the previous stage.

Cellulase Activity Test

The cellulase activity was measured using the Miller method (1959) with CMC as the substrate. Measurement of enzyme activity was done by mixing 1 ml of crude enzyme and 1 ml of 0.5% CMC in sodium acetate buffer (pH 4.5) (Ferreira et al., 2009). The crude enzyme is a supernatant obtained from biomass separation on production medium using centrifugation method. Incubation was carried out in the water bath at 40 ° C for 10 minutes (Agarwal et al., 2014). The enzymatic activity was stopped by adding 1 ml of control reagent and heated in boiling water for 1 minute. Absorbance was measured using a spectrophotometer with a wavelength (λ) 540 nm (Adney and Baker, 1996). In control, the enzyme was inactivated by heating the enzyme in boiling water for 1 minute then reacted with the substrate without incubation (Chasanah et al., 2013). The unit of enzyme activity (SA) is expressed in units of U / ml or μM per minute per ml. A unit of cellulase activity is defined as the amount of cellulase in catalyzing the release reaction of 1 μmol glucose from the CMC substrate per minute (Lehninger et al., 2005). Measurement of cellulase enzyme activity (U / ml) is done by inserting the absorbance value obtained by the following formula [1], where x is absorbance of tested sample, V is total sample volume (enzyme volume and substrate) divided by volume of the enzyme (mL), t is enzymatic reaction time (minutes), and s is the constant value on the standard curve .

$$\text{Cellulase Enzyme Activity (U/ml)} = \frac{x.V}{t.s} \qquad [1]$$

3. RESULTS AND DISCUSSION

Growth Curve of *Candida* G3.2

The growth curve is needed to determine the optimum of incubation time of Candida G3.2 starter for the production of crude cellulase enzymes. Measurement of yeast growth was done by using the spectrophotometric method at 600 nm wavelength. The medium used for measuring the yeast growth curve is the Yeast Malt Extract Broth (YMEB). The results of the Candida G3.2 growth curve are presented in Figure 1. Based on Figure 1 there is no visible lag phase because the absorbance measurements were made at 12 hours after inoculation. The lag phase is the adaptation phase of yeast to its new environmental conditions in which the cell is biochemically active but not dividing (Fardiaz, 1992; Asaduzzaman, 2007).

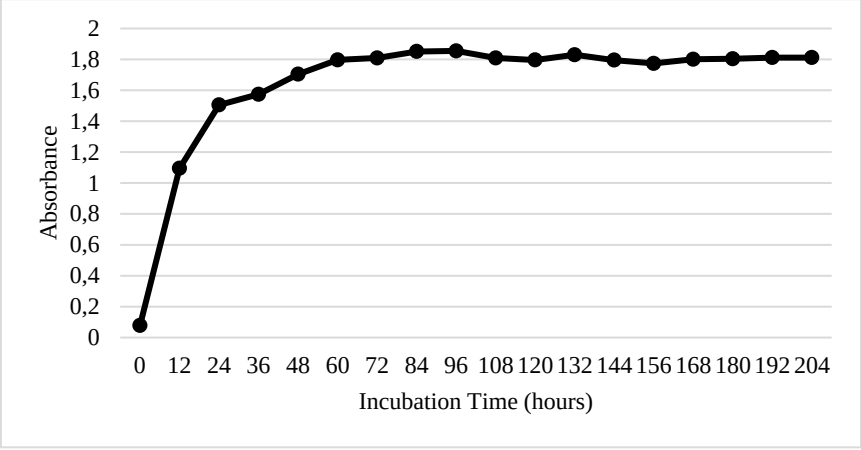

Figure 1. Growth curve of *Candida* G3.2

The exponential phase of *Candida* G3.2 ends at the 60 hours after inoculation. Upon entering the exponential phase, DNA replicates and cell division rapidly (Asaduzzaman, 2007). The yeast stationary phase begins after the 60th hours of inoculation. The yeast DNA does not replicate so that the yeast cells will not divide, and the speed of the transcription process slows two to fivefold compared to the exponential phase (Choder, 1991). The exponential phase is the most appropriate phase for the starter because it has the most active metabolic activity and the cells synthesize the component for its growth in constant amounts. So in this study, the age of starter used for cellulase enzyme production is 30 hours after inoculation which is an exponential phase.

Optimization of Incubation Time

The incubation time optimization was performed in order to obtain the optimum incubation time for *Candida* G3.2 in producing cellulase with the highest enzyme activity. *Candida* G3.2 was incubated on CMC Broth medium with an incubation time of 0 to 168 h at room temperature. The highest cellulase activity based on Figure 2 was obtained at the 72 hours of incubation with an activity of 267 U / ml. The high value of cellulase activity is thought to be due to a large number of cells and cellulase secretions by *Candida* G3.2. If cell numbers increase then cellulase secretion will also increase, according to Das (2013), where the secretion of enzymes depends on the number of cells. Large cellulase secretion will increase cellulase activity. This is confirmed by the statement Pelczar (1986), when the concentration of enzymes increases, the enzyme activity also increases.
Based on Figure 2 it can be seen that there is an increase in cellulase activity from hours 24 to 72 hours where the highest activity is at 72 hours. The cellulase activity decreased gradually from the 96th hour to the 168th hour. The decrease of enzyme activity is suspected due to decreasing of CMC substrate concentration in the fermentation medium. As the incubation time increases, the degree of hydrolysis of the CMC substrate into glucose by the secreted cellulase also increases. Glucose concentration increases with increasing incubation time. According to Johri et al. (1990) and Ates et al. (1997), the availability of glucose as a large carbon source will decrease the rate of cell growth itself so that the secretion of the enzyme will also decrease. Decreased levels of cellulase enzyme secretion lead to decreased activity of the enzyme. In addition, decreased enzyme activity occurs along with cell autolysis (Ire et al., 2011). Depletion of a number of nutrients in the production medium will affect the level of enzyme secretion (Nochure et al., 1993). One source of nutrients needed by microorganisms is the trace element. Trace elements found in the CMC Broth production medium are Na^+, K^+, Mg^{2+}, and Fe^{2+} ions in salt form. The ions are cellulase cofactors that activate enzymatic reactions (Chi et al., 2009). Another factor that affects the decrease in enzyme activity is the crude enzyme itself which becomes unstable if left too long in the production medium (Rabelo et al., 2011). Thus, the 72 hours is the optimum incubation period for cellulase enzyme extraction.

In Figure 2 there is a value on the control (the cultureless medium) that is lower than the results obtained from the treatment with the addition of culture. The value of the control is caused by the detection of glucose on the CMC substrate used in the measurement of activity.

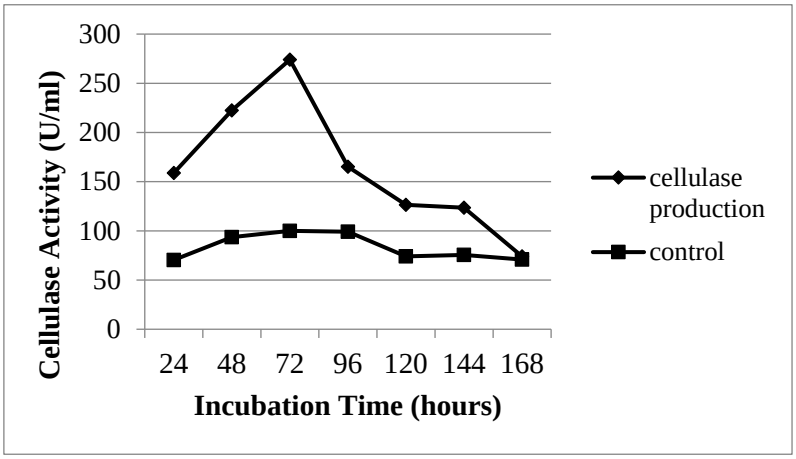

Figure 2. The correlation of incubation time against cellulase activity

Temperature Optimization and pH

Temperature optimization and pH are performed in order to obtain a combination of temperature and pH with the highest cellulase activity values in the cellulase enzyme production process. The cellulase enzyme activity for temperature and pH was measured after the culture medium was incubated for 72 hours in accordance with the results obtained from the optimization of the incubation time. Based on the descriptive analysis, the optimum physicochemical factor condition was achieved by a combination of 55 ° C and pH 6 (369,8 U / ml) (Fig. 3). In accordance with the descriptive analysis based on data that can be seen in Figure 3, where the temperature 55 ° C has cellulase activity value 2 times greater than other temperatures.

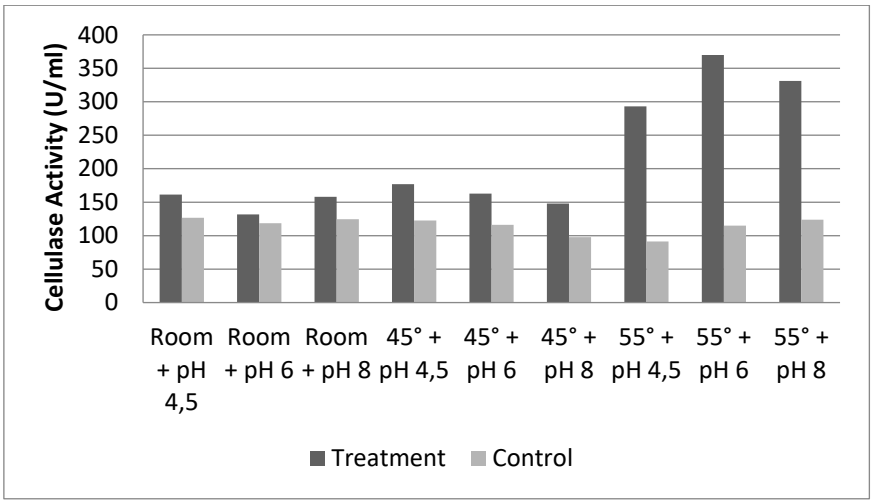

Figure 3. Effect of Temperature and pH on Production of Cellulase

The temperature of 55 ° C is the optimum temperature for cellulase production by *Candida* G3.2 with the highest activity value. The optimum temperature can regulate the synthesis and secretion of extracellular enzymes (Ray et al., 1992). Temperature is able to regulate enzyme synthesis at the mRNA transcription stage and is possible at the translation stage (Votruba et al., 1991). For extracellular enzymes, the temperature may affect the secretion of enzymes by altering the physical properties of cell membranes (Rahman et al., 2004). The level of secretion of the resulting enzyme will be directly proportional to the value of its activity.Murao et al. (1988) and Lu et al. (2003) report that the optimum temperature for cellulase production depends on the strain of the microorganism itself. Thus, the optimum temperature for the production of enzymes produced by a microorganism is different from one another. The results of Rai et al. (2012) shows the optimum temperature for cellulase production by *Candida* is 50 ° C. Taha et al. (2015) states cellulase activity of *Trichoderma viride* optimum at 50 ° C. Based on the results of some of these studies, it is seen that the optimum temperature for cellulase production is 50 ° C - 60 ° C. Mandels et al., 1976 states that above the optimum temperature there will be a decrease in enzyme activity that correlates with the denatured enzyme protein. Figure 3 shows that at the same temperature, the difference in the value of cellulase activity at each pH is not much different. Thus, it can be said that the production of cellulase by *Candida* G3.2 has a wide pH range. In accordance with the study of Harshvardhan et al. (2013) in which the cellulase enzyme can be active in the pH range 3 to 9. In this study, it was shown that the highest enzyme activity at room temperature and temperature 45 ° C was achieved at pH 4.5, while at 55 ° C achieved at pH 6. According to Rai et al. (2012), the optimum pH for cellulase production by *Candida* is 5.5. Ijaz et al. (2011) show that pH 6 is the optimum pH for cellulase production by *Alternaria alternata*. Taha et al. (2015) state cellulase activity of *Trichoderma viride* optimum at pH 6. The effect of pH on the enzyme activity is to influence the ion charge on the active side of the enzyme by changing the conformation of the active side (Englard and Sifters, 1990).

CONCLUSION

The result showed that the best incubation period for cellulase production is 72 hours (267 U/ml) with a combination of temperature 55°C and pH 6 (369.8 U/ml).

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