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The Effect of Addition Mn^{2+} Metal Ions and Incubation Time to *Bacillus cereus* Cellulase Enzyme Activity from Endophytic Bacteria of *Curcuma Rhizome* (*Curcuma zanthorrhiza* Roxb.)

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Abstract

Cellulase is one of the most widely used enzymes in the industrial world and wastes decomposition process. *Bacillus cereus* is one of the bacteria that can produce cellulase enzymes that can hydrolyze cellulose to glucose. The addition of cofactors and incubation time can help determine the optimum conditions needed by cellulase enzymes to work optimally. This study aims to determine the effect of adding Mn^{2+} metal ions and incubation time to the activity of cellulase enzymes from *Bacillus cereus* endophytic bacteria. This research is experimentally used a Completely Randomized Design (CRD) factorial design with two factor treatments and 3 times repetitions. The first factor is variation of addition Mn^{2+} metal ions which are 5 mM, 10 mM, and 15 mM, the second is variation of incubation time which are 1 hour, 2 hours, 3 hours, 4 hours, and 5 hours. The data were analyzed using Analysis Of Variance (ANOVA) and if the data significantly affected the parameter, then it would be continued by Duncan Multiple Range Test (DMRT) with the fault level of 5%. The result showed that the interaction of addition Mn^{2+} metal ions and incubation time affected the cellulase enzyme activity of *Bacillus cereus* from endophytic bacteria. The highest cellulase enzyme activity obtained from interaction addition Mn^{2+} metal 10 mM and incubation time 3 hours with an activity 0,335 U/mL, while the lowest cellulase enzyme activity obtained from interaction addition Mn^{2+} metal 15 mM and incubation time 5 hours with an activity of 0,073 U/mL.

1. INTRODUCTION

Enzymes are also used in all industrial processes as biocatalysts. The enzyme can use repeatedly with immobilization technique, have high efficiency and effectiveness, and the reaction does not cause side effects. In industry sector, enzymes that are many used are cellulase enzymes (Wang, Cooney, Demain, Dunnill, & Humphrey, 1979).

Cellulase enzymes have large applications and potential for the industrial sector in agriculture, textile, food, biotechnology, paper, and animal feed digestibility. In addition, cellulase enzymes can also be used in the process of breaking down agricultural waste containing cellulose into economically valuable products, that is glucose (Sinatari, 2013).

The production of enzymes by microorganisms is affected by several factors that are temperature, pH, production media, incubation time, aeration, microbial strains used, and agitation (Trismilah & Waltam, 2016). Moreover, the high and low activity of enzymes is also affected by substrate concentration. Cellulase activity is also affected by the addition of metal ions as an activator in a certain amount and the incubation time (Poedjiadi & Supriyanti, 2006).

2. MATERIALS AND METHODS

CMC Agar and Liquid 1% Media

1 gram of CMC; 0.075 g KNO₃; 0.02g MgSO₄·7H₂O; 0.002 g FeSO₄·7H₂O; 0.05 g K₂HPO₄; 0.004 g CaCl₂·2H₂O; 0.2 g of yeast extract added into a 250 mL beaker glass and 100 mL of distilled water were added, heated on a hot plate until boiling and homogenized with a magnetic stirrer. Then put into an erlenmeyer and covered with cotton plugs, then sterilized for 15 minutes at 121 °C and a pressure of 1 atm in an autoclave. For CMC broth media, the composition of the media is the same as CMC media without adding bacto agar (Putri, 2014).

Nutrient Agar (NA) Media

Added nutrient Agar powder 2.3 g and dissolved with 100 mL of distilled water in erlenmeyer. Then it is heated to homogeneous using a hotplate-stirrer and put into a test tube as much as 5 mL. The test tube was closed and sterilized for 15 minutes with temperature of 121 °C and pressure of 1 atm in an autoclave (Lathifah, 2013).

DNS Reagents

Dissolved 3,5-Dinitrosalicylic acid 1 gr, 0.05 gr sodium sulfite, 1 gr sodium hydroxide, and 0.2 gr phenol in 100 mL with distilled water and beaten. Keep in a dark bottle (Miller, 1959).

KNa-Tartrat 40%

KNa-Tartrat 20 gr dissolved in 250 mL erlenmeyer with 50 mL of distilled water. Keep in a dark bottle (Miller, 1959).

Tris-HCl Buffer pH 9 0.05 M

For solvent A 0.2 M Tris (was made in 100 mL distilled water dissolved 2.103 g Tris) and for solvent B 0.1 M HCl (was made in 100 mL distilled water was dissolved 10 mL HCL 1 M). For pH 9, 50 mL of solvent A is needed and 10.6 mL of solvent B is added with distilled water to 200 mL (Kuhlmann, 2006).

Bacillus cereus Confirmation Test on Qualitative Production of Cellulase Enzymes

The qualitative determination of cellulase enzyme activity was carried out by observing the clear zone formed on the CMC agar media 1%. 5 µl of bacterial isolates (24 hours) were grown by dropping into disc paper that had been placed on CMC agar media. Then incubated for 48 hours with a temperature of 28 °C. After incubation, the cup was soaked with 0.1% Congo Red solvent for 20 minutes and washed with 1 M NaCl. The clear zone formed around the colony on disc paper was measured using a calipers diameter (Chantarasiri, 2015). The cellulolytic index can be known by the formula (Kader & Omar, 1998):

$$\text{Cellulolytic index} = \frac{A-B}{B}$$

A is the diameter of the clear zone (mm) and B is the diameter of the colony (mm).

Bacterial Growth Curves and Cellulase Activity

Two loops of *Bacillus cereus* subculture inoculated to 200 mL of 1% CMC broth media. The inoculum was incubated in an incubator shaker at 150 rpm at 30 °C for 24 hours. Twenty mL of inoculum was taken and transferred into 200 mL of new CMC media. Four mL of inoculum were taken at 0, 24, 48, 96, 120, and 144 hours and then the number of cells ($\lambda = 660$ nm) and cell activity ($\lambda = 540$ nm) were measured. Determination of growth curves by making a plot between enzyme activity, absorbance, and time (Putri, 2014). Measuring reducing sugar (glucose) used the DNS method, cellulase activity can be determined (Miller, 1959).

Production of Cellulase Enzyme Crude Extract

Inoculated in 200 mL of 1% CMC broth medium of 2 loop *Bacillus cereus* and incubated in an incubator shaker at 150 rpm at 30 °C for 24 hours. Ten mL of inoculum was taken and transferred in 1% CMC broth medium as much as 100 mL. The inoculum was incubated in an incubator shaker up to a phase that produced a high cellulase enzyme determined from a growth curve at 30 °C (Putri, 2014). Crude extract of cellulase enzymes can be obtained by cold centrifugation of bacterial culture at 4 °C for 10 minutes at 10,000 rpm. Taken supernatant as a result of crude extract of cellulase enzymes (Jennifer & Thiruneelakandan, 2015). Then the enzyme activity was tested from the crude enzyme extract by DNS method.

Glucose Standard Curve

Each of the concentrations of glucose solvent that has been made is taken as much as 1 mL and put in a test tube. In each test tube

1 mL of DNS reagent was added and homogenized. The mouth of the tube is then covered with aluminum foil and then heated for 5-15 minutes in boiling water to form a red-brown color in the solvent. Next, 1 mL of Kna-tartrate is added and the tube is cooled. After chilling, each tube is added with distilled water until the volume is 10 mL. By using a spectrophotometer at $\lambda = 540$ nm, the absorbance of each solvent was measured. After each absorbance value is obtained, a glucose standard curve is made with each absorbance value, a glucose standard curve is created with Microsoft Excel. The equation of the line $y = ax + b$ obtained from the curve, is used to determine the concentration of glucose (x) from the sample to be measured absorbance (Rasyada, 2015).

Test of Cellulase Enzyme Activity with DNS Method

The quantitative testing of cellulase activity was carried out used 3,5-Dinitrosalisilic Acid (DNS) reagent based on the estimated amount of reducing sugar produced from 1% CMC broth media. 1 mL of 1% CMC broth media was added with 1 mL of crude cellulase enzyme extract and put in a tube. Then incubated with water bath for 15 minutes at a temperature of 55 °C. To stop the reaction, 1 mL of DNS reagent was added and boiled at 100 °C in a water bath for 15 minutes. Next, 1 mL Kna-tartrate was added. The test tube is cooled and added with distilled water to 10 mL in volume and homogenized. Spectrophotometer measurements with a wavelength (λ) of 540 nm were used to determine the amount of reducing sugar released (Jennifer & Thiruneelakandan, 2015).

Plotted on the standard curve the absorbance value obtained from the previous procedure. The amount of μmol of glucose products produced by cellulase enzyme hydrolysis every one minute under test conditions is called one unit of cellulase enzyme activity. The value of cellulase activity

is determined based on the formula (Kombong, 2004):

$$AE = \frac{C}{BM \text{ glucose} \times t} \times \frac{H}{E}$$

Information:

AE = Enzyme Activity (Unit / mL)

C = Glucose Concentration

BM = Molecular Weight of Glucose (180 g / mol)

t = Incubation Time (minutes)

H = Total Enzyme-Substrate Volume (mL)

E = Enzyme Volume (mL)

Effect of Incubation Time and Addition of Mn²⁺ Metal Ion to Cellulase Activity

One mL of crude enzyme extract was put into each test tube, added 1 mL of CMC substrate with a concentration of 1.5%, conditioned at pH 9 (plus 0.05 M Tris-HCl buffer) and Mn²⁺ metal ion added with concentrations of 5 mM (Pachauri, More, Aranganathan, Sullia, & Deshmukh, 2018), 10 mM, and 15 mM (Zeng et al., 2016) were incubated in the shaker incubator at a speed of 120 rpm (Sulistyarsi & Ardhi, 2016) with a temperature of 30 °C with time variations 1 hour, 2 hours, 3 hours, 4 hours and 5 hours (Sulistyarsi & Ardhi, 2016). Cellulase enzyme activity with various effects of the addition of Mn²⁺ metal ions and the incubation time was measured by the DNS method according to the previous procedure (Miller, 1959).

To find out the effect of incubation time and the addition of Mn²⁺ metal ions to the value of cellulase activity, a factorial completely randomized design (RAL) was used with two factors, the incubation time and the addition of Mn²⁺ metal ions. Data on the effect of incubation time and the addition of Mn²⁺ metal ions to cellulase activity were analyzed with Analysis of Variance (ANOVA). If the treatment has a significant effect on the parameters, then proceed with the Duncan Multiple Test (DMRT).

3. RESULTS

Qualitative Confirmation of Cellulase Enzyme Activity Produced by *B. Cereus*

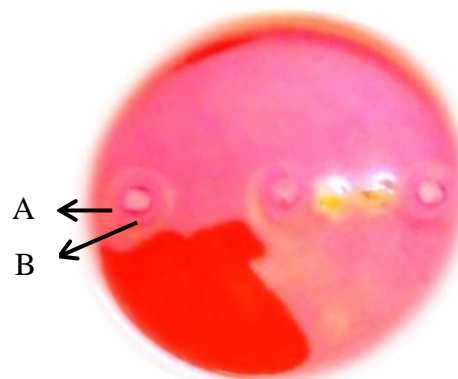


Figure 1. Clear zone produced by cellulase activity of *Bacillus cereus* isolates after staining using Congo Red 0,1% (A: clear zone, B: bacterial colony and paper disk (3 paper disk to show 3 repetitions))

Bacterial Growth Curves and Cellulase Activity

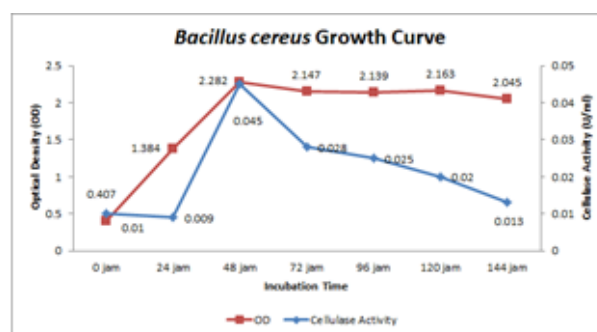


Figure 2. *Bacillus cereus* Growth Curve

Effect of Mn^{2+} Metal Addition and Incubation Time on Cellulase Enzyme Activity

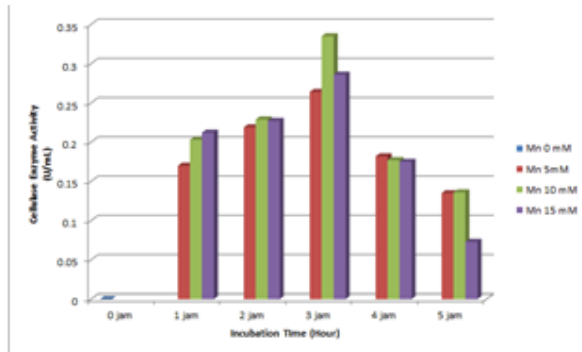


Figure 3. Graphic Effect of Interaction of Incubation Time (0 hour, 1 hour, 2 hours, 3 hours, 4 hours, and 5 hours) and Addition of Mn^{2+} Metals (0 mM, 5 mM, 10 mM, and 15 mM) to Cellulase Enzymes.

4. DISCUSSION

Qualitative Confirmation of Cellulase Enzyme Activity Produced by *B. Cereus*

Bacillus cereus is confirmed to be able to produce cellulase enzymes with the sign of the formation of a clear zone around the colony of *Bacillus cereus*. Thus, the tested *Bacillus cereus* isolate has the ability to degrade CMC media containing cellulose and then the enzyme harvesting process can be carried out.

Semi quantitative cellulase activity measurement based on clear zone formed around the colony of *Bacillus cereus* with calipers and calculated using the formula Cellulase Activity Index (CAI). The results of the measurement of the clear zone showed an index of cellulase activity 1.33 mm (Figure 1). Choi et al., (2005) stated that CAI values ranging from 1-2 mm are included in the medium ratio.

Bacterial Growth Curves and Cellulase Activity

The growth of *Bacillus cereus* can be observed from Optical Density (OD). OD can be calculated by

cloudy liquid the bacterial suspension. Based on the growth curve (Figure 2) obtained, the 0 to 48 hours are the exponential (logarithmic) phase of the growth of *Bacillus cereus*. This is indicated by the number of bacterial cells which increased rapidly from 0.407 nm to 2.282 nm. The exponential phase is characterized by a period of rapid growth. With a high number of bacterial cells, the value of reducing sugar productivity and cellulase enzyme activity also increases (Volk & Wheeler, 1988).

The stationary phase of *Bacillus cereus* growth occurs at the 48 to 120 hours. During this incubation period, the number of cells represented by optical density values tends to decrease to a constant. The stationary phase microorganisms have decreased the rate of the division until the number of bacteria that live and die tends to be constant (Volk & Wheeler, 1988).

The death phase is indicated in the incubation period of 120-144 hours. This is indicated by the decrease in optical density values which indicates the number of dead bacteria is far greater than the bacteria that grows. The death rate is faster than the growth rate, even the growth rate can be zero (Volk & Wheeler, 1988). In the *Bacillus cereus* growth curve, there is no adaptation phase (lag) because the bacterial inoculum has been previously cultured for 24 hours which is the adaptation phase for *Bacillus cereus*.

Based on the growth curve the optimum yield occurs at an incubation period of 48 hours. During the 48 hour incubation period, bacterial cell OD was 2.228 with enzyme activity of 0.045 U/ mL. With this result, the incubation process for 48 hours is used for the production of crude cellulase enzyme extracts, because at that hour *Bacillus cereus* is in the final stages of growth in the logarithmic phase which has optimum enzyme activity. The production of cellulase enzymes is the primary metabolite at the end of the logarithmic phase or the beginning of the stationary phase of the bacterium (Sonia & Kusnadi, 2015), also uses a 48-hour incubation process.

Effect of Mn^{2+} Metal Addition and Incubation Time on Cellulase Enzyme Activity

The influence of the addition of Mn^{2+} metal, incubation time, and their interaction with cellulase enzymes produced by *Bacillus cereus* endophytic bacteria were known by using statistical analysis in the form of ANOVA (Analysis of Variance). ANOVA analysis results show that there is an influence between the interaction of the two of them against cellulase enzymes produced by endophytic *Bacillus cereus* bacteria (Figure 3).

Then, to find out the best treatment of each treatment, tests were carried out used the DMRT (Duncan Multiple Range Test) tests. Based on the results of the DMRT test showed different notations which means that there is a real influence between treatments on enzyme activity.

The DMRT follow-up test results showed that the optimum cellulase enzyme activity of *Bacillus cereus* was in the interaction of the incubation time of 3 hours and Mn^{2+} 10 mM of 0.335 U/mL. While the minimum cellulase enzyme activity in the treatment of 5 hour interaction time and Mn^{2+} 15 mM was 0.073 U/mL. This result was not significantly different in all 5 hour interaction treatments with different Mn^{2+} (5 mM, 10 mM, and 15 mM).

The activity of cellulase enzymes produced by *Bacillus cereus* for Mn^{2+} 5 mM concentrations increased periodically and reached optimum performance at a 3 hour incubation time of 0.264 U/mL. The concentration of Mn^{2+} 10 mM also increased activity periodically and reached optimum at a 3 hour incubation time of 0.335 U/mL. The concentration Mn^{2+} 15 mM reached optimum activity at 3 hours incubation time of 0.286 U/mL. The longer incubation time will cause an increase in temperature and result in enzyme denaturation (Sulistyarsi & Ardhi, 2016).

The addition of incubation time will increase the activity of cellulase enzymes. The active side of the enzyme in binding the substrate optimally requires sufficient time. It is believed that if the active side of the enzyme

is not optimal in forming enzyme-substrate complexes, then it takes time to release the product. If in this condition the enzymatic process is stopped then the product has not been optimally produced. The reducing sugar products produced from the enzymatic reaction are proportional to the length of incubation time, but if the active side of the enzyme has been saturated by the substrate, then the incubation time addition has less effect on the amount of the product, where the product produced only experiences a relatively small increase (Susanti, 2011).

Besides the incubation time, another factor that influences the activity of the *Bacillus cereus* cellulase enzyme tested is the addition of Mn^{2+} . Cellulase activity at concentrations of 5 mM and 10 mM Mn^{2+} showed greater activity compared to activities at concentrations of 15 mM Mn^{2+} . However, the graph shows that the optimum activity is at an incubation time of 3 hours with a concentration of Mn^{2+} 10 mM. Metal ions are positive moderators that cause conformational changes in the catalytic side of the enzyme, which will facilitate interaction between the enzyme and the substrate thereby increasing the catalytic activity of the enzyme (Dini, 2014).

Metal ions can carry more than one positive charge whose effect is stronger than protons (Zeng et al., 2016). In addition, metal ions have complex actions that can keep the concentration of the solvent stable. In this research, the intended metal ion is Mn^{2+} . Optimal Mn^{2+} metal ion concentration will help increase cellulase enzyme activity. If the concentration used is below the optimum, the increase in cellulase enzyme activity is relatively small. Increased cellulase enzyme activity will occur if the concentration is used in optimum quantities. Concentrations that exceed the optimum will cause a decrease in interaction between the enzyme and the substrate, causing the enzyme catalytic activity to decrease. In other words, the low cellulase activity at Mn^{2+} concentrations of 15 mM is due to decreased interaction between enzymes

and substrate, causing a decrease in enzymatic catalytic activity.

Each metal ion has its own optimum level (Zeng et al., 2016). This depends on the bacteria used, the substrate, temperature, pH, and incubation time. Mn^{2+} with a concentration of 10 mM in this study showed optimum cellulase enzyme activity compared to a concentration of 15 mM. This is because the Mn^{2+} concentration of 10 mM is the optimum concentration for *Bacillus cereus*.

Based on the overall activity value of the treatment, the interaction of incubation time of 3 hours and the concentration of Mn^{2+} 10 mM is the optimum cellulase activity of *Bacillus cereus* compared to other interaction treatments. This is because at the optimum Mn^{2+} concentration, cellulase from *Bacillus cereus* has an active side conformation that is suitable for the substrate so that it can form the appropriate enzyme-substrate complex and produce maximum activity. In addition, conditions of optimum incubation time can increase the kinetic energy that can accelerate the movement of enzymes and substrates, thereby increasing the chances of an enzyme reaction with the substrate (Sharma, Buragohain, & Kaushal, 2013). Collisions that often occur between this enzyme and substrate will form an enzyme-substrate product complex that causes a large number of products to be formed.

The results of the study by (Zeng et al., 2016) stated that the optimum *Bacillus cereus* cellulase activity at a concentration of 10 mM Mn^{2+} with a 4-hour incubation time of 0.12 U/mL. Yin et al., explain that the optimum cellulase activity is 0,1 U/mL at 10 mM concentration of Mn^{2+} and 2 hours incubation time. From the results of these studies, it can be interpreted that the optimum *Bacillus cereus* bacteria at Mn^{2+} 10 mM concentration and various incubation times. This is because cellulase activity can increase with the addition of certain metal ions and the optimum incubation time. The difference in incubation

time is due to the different types of bacteria used.

The results of the minimum cellulase activity of the whole treatment on the interaction of incubation time of 5 hours and the concentration of Mn^{2+} 15 mM were 0.073 U/mL. The addition of Mn^{2+} with a concentration of 15 mM caused a decrease in cellulase enzyme activity (Zeng et al., 2016). This is because excessive addition of metal ions causes the interaction between enzymes and substrate to decrease. The optimum incubation time is needed so that cellulase activity increases (Sulistiyarsi & Ardhi, 2016). Because the longer incubation time will cause temperatures to rise due to strong interactions between substrate molecules and enzymes, resulting in enzyme denaturation.

5. CONCLUSION

The effect of adding Mn^{2+} metal ions and the incubation time on cellulase enzyme activity of endophytic bacteria *Bacillus cereus* from temulawak rhizome (*Curcuma zanthorrhiza* Roxb.) obtaining the optimum cellulase activity in the concentration of 10 mM Mn^{2+} metal interactions and incubation time of 3 hours with 0.335 U/mL. While the minimum cellulase enzyme activity was obtained in the treatment of Mn^{2+} 15 mM metal interactions and a 5-hour incubation time of 0.073 U/mL.

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