The effect of straw substrate variation in production of cellulase enzyme by Serratia marcescens

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Abstract. Indonesia is a country with vast agricultural land, especially rice fields. Unfortunately, rice crops cause a problem of rice straw waste. The waste of rice straw consists of 38% cellulose, 24% hemicellulose and 8% lignin. The study aimed to assess the production of cellulose by Serratia marcescens in straw substrate medium. The research design was Completely Randomized Design. Variations of rice straw substrate were denoted by (S₀; S₁; S₂; S₃) within incubation period for 16 hours. Data analysis was analyzed using SPSS one-way ANOVA (α = 0.05). The result showed that variations of rice straw substrate concentrations correlated with the production of cellulose at 0.28 U/mL. The result of statistical analysis using DMRT Test showed a significant difference in every treatment (p≤0.056).

1. Introduction
Indonesia is an agricultural country with the main production of rice crop. Based on the Central Bureau of Statistics records, in 2014, Indonesia had 8,111,593 hectare of rice fields. Consequently, the crop resulted in increasing of rice straw waste, which might cause health problems and pollutions. Indonesia produced 180 tons of rice straw annually. The problems related to rice straw waste have become more complicated. Straw waste has not been utilized and appropriately used and the increasing amount of straw waste every year became our common problem. The stated rice straw waste had not been optimally utilized since it has been used for feeding kettle and the rest was left to rot or burn. This will lead to the production of pollutants (CO₂, NOₓ, SOₓ) which contributes to environmental damage and glass house effects. The waste of rice straw consists of 38% cellulose, 24% hemicellulose, and 8% lignin. The high cellulose content in straw can be used as a substrate in the production of cellulase enzyme by Serratia marcescens

Rice straw contains various components; hence the process of delignification for pretreatment should be conducted in order to get the expected component that is cellulose. According to Nenci (2012), delignification is a process to break lignin structure that binds and envelops cellulose. This process is required to facilitate cellulose hydrolysis. The stated that saponification of ester bond from the residue of lignin or hemicellulose opens the cellulose so that it will be easier to interact with the enzyme to ease the hydrolysis of cellulose into glucose. Pretreatment used in the study with delignification was NaOH 4% solution with the ratio 1:10 (w/v) [10].

Serratia marcescens is cellulolytic bacteria that has the ability to hydrolyze cellulose to simpler components like glucose. Serratia marcescens is negative Gram and form red pigment with the characteristics of alkaline acid in TSIA media with or without gas formation. This bacteria can ferment cellulose, inositol, and glycerol without producing gas, while arabinose is not fermented. Activities of
cellulase enzyme from *Serratia marcescens* should utilize cellulose in rice straw substrate, which was delignified by NaOH 4%. [8]

Cellulase (EC 3.2.1.4) is the enzyme to hydrolyze cellulose into simpler components like glucose. Cellulase requires cellulose a substrate to be systematically and structurally decomposed with H$_2$O. Components of cellulose are decomposed by cellulose enzyme so that simpler and smaller components. According to Nenci (2012) cellulose is a complex enzyme consisting of several enzymes that work gradually or together to decompose cellulose into glucose by hydrolyzing the bond of β-1,4 in cellulose. While the stated that cellulase (EC 3.2.1.4) is a complex of an enzyme with the ability to decompose cellulose into β-glucose, the enzyme is able to hydrolyze the bond of β-1,4-glycoside in cellulose [3]. The study aimed to assess the production of cellulase by *Serratia marcescens* in straw substrate medium.

2. Methods

2.1. Preparation of Rice Straw Substrate

Rice straw was soaked in 4% NaOH solution at acidity 10-11 using the ratio of straw and 4% NaOH solution at 1:10 (w/v). The straw was washed with distilled water to gain neutral acidity. Neutral acidity is the condition when the acidity of water is equal to the acidity of distilled water. The delignified straw was put into the oven to dry overnight at 50 °C. The next stage was mashing the straw using a blender and then sieved using a 125-mesh sieve to get straw powder. Next, the straw powder was weighed according to the treatment [4]. Distilled water was added into straw powder and boiled to get the extract. The extract was then kept in the refrigerator for subsequent use as substrate [2].

2.2. Starter culture

One ose of culture in lateral media was put into media starter. The starter was then incubated using rotary shaker at 120rpm for 18 hours., cell density was measured using spectrophotometer at λ 520 nm [2].

2.3. Cellulase Production Medium

Production medium consisted of MgSO$_4$.7H$_2$O 0,05 g, Na$_2$HPO$_4$.2H$_2$O 0,5 g, NaCl 0,23 g, yeast extract 0,2 g, dissolved into 100mL distilled water [12]. The treatment was as followed: 1g of CMC, S$_1$ added with 100mL straw extract 0,1g, S$_2$ added with 100mL straw extract 0,5 g, and S$_3$ added with 100mL straw extract 1 g.

2.4. Fermentation

5 mL starter was taken from 100 mL of production medium or 5% of the production medium. Culture media was incubated for 24 hours using rotary shaker at 120rpm. Every 4 hours, the culture media was taken to determine the growth and cellulase production.

2.5. Cell Growth Measurement

3 mL culture media was taken every 4 hours at intervals of 0 hours, 4 hours, 8 hours, 12 hours, 16 hours, 20 hours, 24 hours. The cell growth was measured using a Turbidimetry method, and the absorbance was measured using a spectrophotometer at λ 520 nm (growth curve).

2.6. Cellulase Production

1 mL culture medium was taken at 16 hours incubation time. Next, the sample was centrifuged at 3000 rpm for 10 minutes. Centrifugation process results in a Crude enzyme, which will be used for the determination of cellulase activity by sugar reduction method. The first tube was labeled as blanko, the second was labeled as control, and the third tube was labeled as a sample. Blanko tube was filled with 0,9 mL CMC substrate and 0,1 mL distilled water. A control tube was filled with 0,9 mL CMC substrate and 0,1 crude enzyme, and then the reaction was stopped by boiling in a water bath for 2-3 minutes. The sample tube was filled with 0,9 mL CMC substrate and 0,1 crude enzyme. The tubes were then incubated at 50°C for 30 minutes. The reaction was stopped by boiled in a water bath for 2-3
minutes and added by 1mL DNS reagent, then boiled for another 2-3 minutes. After that, 4mL of distilled water was added, and then, the tubes were measured for their absorbance by using spectrophotometer at λ 570 nm [11-12]

The cellulase activity is the result of the formation of reducing sugar. The concentration of reducing sugar can be measured based on the equation (Sample Absorbance-Blanko Absorbance) – (Control Absorbance-Blanko Absorbance). Cellulase activity measured in (U/mL) is measured by an equation. The definition of cellulase activity is based on the following formula [11-12]:

\[
\text{Cellulase activity (U/mL)} = \frac{\text{μg glucose} \times \text{dilution factor} \times 1000}{V \times \text{BM glucose} \times t}
\]

Where:
- Dilution factor: enzyme dilution
- \( V \): volume of enzyme (0.1 mL)
- \( t \): incubation time 3 minutes
- \( \text{BM glucose} \): BM glucose (180,2 Dalton)

3. Result and discussions

3.1. The growth of Serratia marcescens

*Seracia marsescen* needs nutrients for its growth process. Media does not contain nutrients will interfere with bacterial growth. Even the bacteria will die. Growth measurement is essential to observe if *Serratia marcescens* grows well in culture media. Cell growth is measured by Turbidimetry method and absorbance is measured by spectrophotometer at λ 520 nm. In general, growth is defined as the regular increase of all the components in living cells [7]. The increase in size caused by the increase of water in the cell cannot be considered as a growth. Cell propagation is a consequence of growth in multicellular microorganisms. Growth in cell growth also means the increase in the number of organisms that make up the population or culture.

Observation of growth showed that the starter increase growth process in the culture medium. The growth curve showed that, at 4-hour incubation time, the cell growth had entered the logarithmic phase, which was indicated by the increase of absorbency value shown as a drastic increase in the curve. Growth Curve of *Serratia marcescens* is presented in Figure 1. Starters speed up the fermentation process so that they can be more effective and efficient in the research process. So that it can be more effective and efficient. The starter resulted in a drastic increase of *Serratia marcescens* at 4-hour incubation period and entered the logarithmic phase. Log phase is a phase where the number of cells in bacteria grows significantly as a result of sufficient nutrition that supports metabolism. The bacteria grow rapidly so that the number of cells in the medium is very high. It can be indicated using Turbidimetry method that shows higher absorbance value compared to the previous value. It is that log phase is a phase where cells split rapidly and increase in line with a logarithmic curve [4-10]

![Figure 1. growth Curve of Serratia marcescens growth during the 24-hour incubation period on culture treated with rice straw substrate variations.](image-url)
The starter culture is a media containing microorganisms to fasten fermentation process. Starter culture should be free of contamination, fast growing, good texture and shape, resistant to bacteriophage and antibiotic. [10]

The next stage shown by standard curve (Figure 1) was stationer stage. The stage started at incubation period 4-20 hours characterized by the formation of the horizontal line in growth curve that was caused by balanced growth and death. There was no significant growth in the stage unlike what observed in the logarithmic stage. The stationer stage is the stage where the number of growing and dying cells are balanced.

This causes cells that grow together to die and cause balance. The last stage was the cell death stage that occurred during the incubation time at 20-24 hours of the treatment $S_1$, $S_2$, $S_3$. Treatment $S_0$ needed time before leading to cell death stage. It is presented in decreasing growth curve (Figure 1). All treatments showed a decrease that was identified as a cell death stage. The resulting decrease does not reach the death phase. This was caused by decreasing nutrition in culture media that is essential for cell growth. The cell death stage occurs because the source of nutrition for cell growth is decreasing. [9]

### 3.2. Production of Cellulase Enzyme by Serratia marcescens B

Cellulase is an enzyme that requires cellulose substrate to perform hydrolysis process. The process of hydrolysis without substrates in the form of cellulosic chemical processes will not be successful to decompose enzyme into simpler components such as glucose. The substrate used in the research was made of rice straw and CMC (Carboxymethyl cellulose). The rice straw was obtained from Bulusan Urban Village, Tembalang sub-district, Semarang. The concentrations of rice straw substrate were 0.1g; 0.5g; 1g and 1g of CMC as a positive control that was subsequently labeled as $S_1$; $S_2$; $S_3$; and $S_0$ as a positive control.

The result of analysis using one-way ANOVA showed that variations of rice straw substrate in production medium correlated to cellulase activities of *Serratia marcescens*. It is presented in Figure 2.

![Figure 2](image)

**Figure 2.** The result of Analysis of Variance (ANOVA)

Correlation to activities of cellulase enzyme produced by *Serratia marcescens* as shown by ANOVA test can be defined as rice straw substrate in cellulose can replace cellulose nutrition in common media that are usually used to produce cellulase enzyme produced by *Serratia marcescens*. Cellulase enzyme produced by *Serratia marcescens* decomposes cellulose by altering chemical bonds in cellulose into simpler components, like glucose. The cellulase (EC 3.2.1.4) is a complex enzyme having the ability to decompose cellulose into β-glucose. The enzyme also hydrolyzes the bond β-1,4-glycoside in cellulose [3]

Incubation period also correlates to the activities of cellulase since the incubation period is related to the growth of bacteria in particular condition or period of time that affects the cellulase activities. The bacteria growth during the exponential stage to stationer stage is the best stage for bacteria to produce cellulase. The production of an enzyme which acts as primary metabolite occurs from cell growth stage to the end of the exponential stage or stationer stage. In these stages, bacteria produce primary metabolite like cellulase to hydrolyze nutrition especially cellulose to be altered into essential components for the metabolism process [9]

Concerning the incubation period, shows that variations on incubation period correlate to cellulase activities [2]. The analysis using ANOVA resulted in the $F_{\text{count}}(\alpha = 0.05)$ from rice straw substrate, interaction (combination) between rice straw substrate and incubation period and incubation period
subsequently recorded as 0.53; 2.18; 8.00. The F table \((\alpha = 0.05)\) from rice straw substrate, interaction (combination) between rice straw substrate and incubation period and incubation period subsequently recorded as 2.99; 2.20; 3.39. The analysis using BNT 5% resulted in the highest incubation period of cellulase activities, which was recorded at 12-hour incubation with an average speed of 0.26 U/mL.

The results showed that the incubation time affected the cellulase activity. The results of research explain that the best incubation time is at 12 hours incubation time from the variations of incubation time 4, 8, 12 hours. The study was conducted in 16 hours incubation time because 16-hour incubation time is within the stationary phase.

The results of the ANOVA test should be tested further using the Duncan test with significance level 0.05. The result of statistical analysis through DMRT test at significance level 0.05 showed significant differences in all treatments used. The result of Duncan Test is presented in figure 3.

| Substrat_ Jerami | N  | Subset for alpha = 0.05 |
|------------------|----|------------------------|
| Duncan\(^a\)     | 3  | .26833                 |
| Jerami 1 g       | 3  | .27467                 |
| Jerami 0.5 g     | 3  | .27500                 |
| Tanpa Jerami     | 3  | .27900                 |
| Jerami 0.1 g     | 3  | .646                   |

Means for groups in homogeneous subsets are displayed.
\(\text{a. Uses Harmonic Mean Sample Size = 3.000.}\)

**Figure 3. Duncan Test**

4. Conclusions
Based on the results of the research, it can be concluded that variations of the straw substrate can be suggested as an alternative composition of CMC substitute in conventional media to produce cellulase produced by *Serratia marcescens*.

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