Production and characterization of cellulase from the newly isolated Bacillus subtilis A8 on rice bran and corncob

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Abstract. Rice bran and corncob are the agricultural waste with the high cellulose content. The research was carried out to utilize rice bran and corncob as media/substrate to produce cellulase enzyme by means of the bacteria fermentation process. Bacteria that used to the fermentation process isolated from the shrimp paste origin Bonang, Samarinda, East Kalimantan. Bacteria identified molecularly based on 16S rDNA sequence. The results of identification showed the bacteria is Bacillus subtilis A8. In the research showed the cellulase enzyme activity of B. subtilis A8 on the rice bran media have optimum of incubation time 3 days at pH 6.0 and temperature 60°C, activated by Na+, Co+2, Cu+2, Mg+2, and Zn+2 ions and the protein content 0.13 µg/mL. The cellulase enzyme activity of B. subtilis A8 on the corncob media have optimum of incubation time 3 days at pH 7.0 and temperature 50°C and activated by Na+ and Co+2, whereas Cu+2, Mg+2, and Zn+2 are inhibitor and the protein content 0.04 µg/mL.

1. Introduction
Agricultural waste handling in Indonesia has not carry out maximally because its quantity too much and contain cellulose that difficult to degrade [1]. Cellulose is a major component of a plant cell wall. Cellulose content of a higher plant cell wall is approximately 35-50% of a plant dry weight [2]. Cellulose waste has to be decrease to avoid environmental pollution, with a way to degrade cellulose polymer to be the simple monomers as glucose [3]. According to [20] cellulase is a enzyme complex that comprise a several enzyme that work gradually or together with any enzyme to degrade cellulose to be a glucose by hydrolizing β-1,4 bond at cellulose. Cellulase (EC 3.2.1.4) hydrolyzes cellulose to be a simple polymer such as glucose.

Fermentation technology is one of strategy that could be carry out in the effort to increase economic value of agricultural waste such as rice bran and corncob (Zea mays) [4, 5]. Rice bran and corncob much more contain cellulose compound that can be used to produce cellulase enzyme that have a high economically value [6-12]. Comercially enzyme production usually use fungi or bacteria [13, 14]. Bacteria that can produce a cellulose enzyme are Pseudomonas, Cellulemonas, Bacillus, Micrococcus, Cellovibrio, and Sporosphytophaga [15]. Cellulase enzymes have a variety of a potential and application in industry field among others in food, animal feed, beer and wine, textile and laundry, pulp and paper, agriculture, research and development, biofuel, pharmaceutical, waste management, and recombinant DNA technology [16- 19].
The research is aimed to characterize a cellulose enzyme of bacteria strain A8 at rice bran and corncob substrates.

2. Materials and Methods

Equipment and materials were used water bath, oven, digital balance, incubator, centrifuge, vortex, shaker incubator, spectrophotometer, measuring pipette, measuring glass, test tube, erlenmeyer, magnetic stirrer, magnetic stirrer bar, micropipette, micropipette tips, test tube rack, petri dish, Nutrient Agar (NA), rice bran, Whatman filter paper strip number 1 with size 6 x 6 mm, corn cob waste (Zea mays), aquadest, yeast extract, bacteriological peptone, Carboxy Methyl Cellulose (CMC), KH$_2$PO$_4$ (Merck), (NH$_4$)$_2$SO$_4$ (Merck), MgSO$_4$.7H$_2$O (Merck), CaCl$_2$ (Merck), NaCl, bacto agar, Rochelle salt (K, Na, tartrat), NaOH, 3,5-dinitrosalicylic acid (DNS), and Bradford reagent.

Microbe used in this research was bacteria strains A8 isolated from shrimp pasta origin Bonang Samarinda, East Kalimantan. Media used in cellulolytic bacteria isolation were selective media contained CMC 1% accordance to [21] composed of 2.0 g of KH$_2$PO$_4$ (Merck), 1.4 g of (NH$_4$)$_2$SO$_4$ (Merck), 0.3 g of MgSO$_4$.7H$_2$O (Merck), 0.1 g of CaCl$_2$ (Merck), 1.0 g of peptone (Difco) and 2.0 g of agar (Bacto).

2.1. Qualitative analysis of cellulase activity
The bacteria were inoculated in selective agar media of CMC 1%. The bacteria was grown in the surface of media and incubated for 72 hours at 37°C. Bacteria that have grown dripped with congo red 0.1% and let it for 30-60 minutes, then washed with NaCl 2%. The formed clear zone around the colony was measured in accordance to [24]. The formed clear zone around a colony was an indication of the existence of cellulase enzyme activity. Value of cellulolytic index was measured by division of diameter of the clear zone devided by the diameter of the colony. Isolate that has high cellulolytic index value is used for further analysis.

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\text{Cellulolytic index} = \frac{\text{clear zone diameter}}{\text{colony diameter}} \quad (1)
\]

2.2. Analysis of phylogenetic bacteria A8.
Sequence of 16S rDNA bacteria A8 applied for Basic Local Alignment Search Tool (BLAST) on GenBank NCBI dataLibrary. Phylogenetic analyses of sequence 16S rDNA bacteria A8 were carried out using a Molecular Evolutionary Genetic Analysis (MEGA) programme version 5.2 with Multiple Sequence Comparison by Log-Expectation (MUSCLE) [25]. A phylogenetic tree construction based on genetic distance with a neighbor joining (NJ) method [26]. The strength of phylogenetic tree was tested using bootstrap method [27] with 1000 replication.

2.3. Substrate Preparation
About 100 g of rice bran and 100 g of corncob were dessicated at temperature 70°C in oven for 3 days. Then each substrate were refined using a blender and filtered with a filter 65 mesh. A filter paper were cut into size 6 x 6 mm as positive control. Media for cellulase enzyme production were Nutrient Agar Broth in three erlenmeyers each contain 100 mL. Each medium added with rice bran, corncob and filter paper with a concentration 1%. They were sterilized in an autoclave for 15 minutes at temperature 121°C, cooled and then inoculated with bacteria A8. The media containing bacteria shaken in a shaker incubator with a velocity 120 rpm at temperature 37°C for 5 days. After fermentation, supernatant (crude cellulase enzyme) were obtained by centrifugated on velocity 8000 rpm for 10 minutes at temperature 4°C for separating a biomass with supernatant. Supernatant were collected then saved in a freezer (-10°C) as a crude enzyme and used in further analyses for the cellulase activity.
2.4. Quantitative analysis of cellulase activity
In order to measure cellulase activity quantitatively, the reaction was prepared in accordance to [28]. The cellulase activity was analyzed with spectrophotometer at $\lambda$ 540 nm every 24 h as in accordance to [22]. Cellulase enzyme activity was tested according to DNS method [23]. It was tested by measuring the quantity of reducing sugar produced by enzyme hydrolysis activity in CMC 1% substrate. An equal amount of supernatant (enzyme) (i.e. 0.125 mL) was added into 0.125 mL of substrate (i.e. phosphate buffer solution with pH 7.0 containing CMC 1%). The reaction was incubated at 40°C for 30 minutes. To stop the reaction, 0.25 mL of 3,5 dinitro salisilat (DNS) acid was added into solution. Then it was boiled for 5 minutes and cooled under flowing water. Aquadest 2.5 mL was added to the solution. The mixed solution was measured with spectrophotometer at $\lambda$ 540 nm. Calculation of cellulase activity was carried out with a formula:

$$\text{Cellulase activity} = \frac{\text{glucose content x dilution factor x 1000}}{(\text{glucose molecular weight x incubation time})}$$

(2)

2.5. Characterisation of cellulase enzyme
Characterisation of cellulase enzyme in this study was assessed by determining the optimum conditions for enzyme production, pH, temperature and metal ion addition. The effect of pH on enzyme activity was carried out by reacting enzyme solution with CMC 1% incubated in acetic buffer 0.05 N at various pH. The tested pH were 5.0, 6.0, 7.0, 8.0, and 9.0. The effect of temperature in enzyme activity was carried out by reacting enzyme solution with CMC 1% in buffer solution at optimum pH (based on the previous analysis) at various temperature, i.e. 35, 40, 45, 50, 60 and 70°C. The effect of metal ion with final concentration 1 mM was carried out by reacting enzyme solution with addition of various metal at the obtained optimum conditions pH, and temperature from the previous analysis. The tested ions were Na+, Co2+, Cu2+, Mg2+ and Zn2+ which were available in the form of NaCl, CoCl2, CuCl2, MgCl2 and ZnCl2 respectively. The samples were also compared with reacting enzyme without metal addition (control).

2.6. Quantitative analysis of total protein content
Analysis of total protein content was based on Bradford test. The method was used for measure total protein concentration on solution by colorimetric. On the Bradford test involves Coomassie Brilliant Blue (CBB) that will be bind with a protein in a solution [52]. The sample (protein solution) 20 µL was pipetted into test tubes. One milliliter of protein reagent (Bradford reagent) was added to the test tube, mixed and incubated for 5 minutes at room temperature. For blanks, protein samples were replaced with 20 µL NaCL 0,15N. Absorbance of all samples were measured at 595 nm.

3. Result and Discussion
Selection of cellulolytic microbe was aimed to select isolates which have a cellulase enzyme. Bacteria that can produce cellulase enzyme will produce a clear zone around colony on media containing CMC (Carboxy Methyl Cellulose) 1% [29]. The CMC in media will be used as a carbon source for support a growth [30, 31]. Bacteria strain A8 was isolated form a shrimp paste of Bonang, Samarinda, East Kalimantan has ability to produce cellulose enzyme indicating by degrading CMC in substrate and forming a clear zone around the colony (Figure 1). According to Sonia and Kusnadi [1] colony that produce cellulase will be detected by the forming of a clear zone with color a young orange till clear after be covered with congo red and washed with NaCl solution. The association of congo red and microcrystalline cellulose or carboxymethyl cellulose generates an intense coloration that fades before the depolymerising activity of cellulases [24].
Figure 1. Bacteria A8 in selective media containing CMC 1%.

The BLAST result of 16S rDNA sequence strain A8 at NCBI have maximum identity 99% to Bacillus subtilis strain BC42 (KF636530). The phylogenetic analyses based on Neighbor Joining method showed that sequence have some clade with type species of B. subtilis DSM10^T (AJ276351) with bootstrap value 75%. Based on data of BLAST result and phylogenetic tree analyses the isolat A8 was identified as B. subtilis (Figure 2).

Figure 2. Phylogenetic tree based on 16S rDNA sequence of Bacillus subtilis A8 using neighbor-joining tree method and Brevibacillus agri DSM 6348^T (AB112716) as outgroup.

Decomposition cellulose by cellulase enzyme have a vital role because many agricultural waste contain cellulose (a major component of a plant cell wall), that could be changed to become glucose products, then glucose could become a raw materials for alcohol production [36]. Indonesia is one of a centre of rice diversity [37] and likewise the rice waste. Fermentation process it has long been used because through fermentation process could enhanced nutrition content and could be decreasing the anti-nutrition content, toxin, and the contamination rate [38]. A promising strategy for waste management by enzymatic hydrolysis of lignocellulosic waste and the resultant of fermentation could be a reducing sugars for production of a desired metabolites or biofuel [39].

Microorganisms have been used to decompose cellulytic waste in agriculture for the past many years [34]. Cellulose and hemicellulose utilized by microbes mainly as a carbon source [12]. Bacteria have a function as a fermentation starter with substrates of rice bran and corncob can produce glucose,
a material for bioethanol. A natural substrate is a good source for a cellulase enzyme production rather than a pure substrate [40]. Corncob and rice straw were waste but after fermented can increase their economic value [41, 42].

Based on the result of researchs of [43, 44] that water content, pH and fermentation time influence cellulase enzyme activity. According to [45] cellulase produced by Bacillus sp. is the highest at 8 hours after incubation period. In this research showed the optimum cellulase activity with substrates of rice bran, corncob and filter paper were at the third day after incubation. This difference caused by a different bacteria species and a different environmental condition.

The effect of pH on enzyme activity showed (Figure 3), enzyme activity on rice bran substrate optimum at pH 5.0 was 15.3 U/mL, and enzyme activity decreased at pH 7.0, pH 8.0, pH 9.0 as much as 15.1 U/mL, 11.8 U/mL and 7.7 U/mL respectively. Enzyme activity on corncob substrate optimum at pH 7.0 was 7.6 U/mL. At pH 5.0 was 7.2 U/mL and at pH 6.0 was 3.6 U/mL, at pH 8.0 and pH 9.0 decreased to about 4.8 U/mL and 0.6 U/mL respectively. Enzyme activity on filtered paper substrate at pH 5.0 was 5.4 U/mL and at pH 6.0 was 7.0 U/mL, optimum at pH 7.0 was 7.6 U/mL, and pH 8.0 and pH 9.0 decreased to 5.5 U/mL and 1.6 U/mL respectively. Acidity (pH) of media influences the growth of microorganisms. Every microorganism have a minimal, maximal, optimal pH for the growth [46]. According to [40] enzyme activity that contained in the cell is not fixed, but depend on the substrate.

![Figure 3. The effect of pH on the cellulase enzyme activity](image)

The effect of temperature on enzyme activity (Figure 4). Enzyme activity on rice bran substrate showed at temperature 35°C, 40°C, 45°C and 50°C were 9.1 U/mL, 15.3 U/mL, 12.0 U/mL and 15.9 U/mL respectively, optimum at temperature 60°C was 20.9 U/mL and at temperature 70°C enzyme activity decreased to 4.1 U/mL. Enzyme activity at corncob substrate at temperature 35°C, 40°C and 45°C were 5.0 U/mL, 7.2 U/mL and 7.9 U/mL respectively, optimum temperature at 50°C 10.2 U/mL, and at temperature 60°C and 70°C decreased become to 8.4 U/mL and 3.5 U/mL respectively. Enzyme activity at filter paper substrate at temperature 35°C, 40°C and 45°C were 1.7 U/mL, 7.6 U/mL and 5.2 U/mL respectively, optimum temperature at 50°C was 7.7 U/mL, and at temperature 60°C and 70°C decreased become to 6.6 U/mL and 3.9 U/mL respectively.
At a low temperature or at suboptimal temperature will lead the enzyme have an energy deficiency so that can not do the activity maximally. Increasing temperature until the optimal temperature will increase enzyme activity, after exceed the optimal temperature, then enzyme will have a thermal denaturation and change the enzyme structure, so that enzyme activity decrease [47]. Cellulase is highly sensitive towards temperature and high temperature decrease the growth of microorganisms and enzyme production [39].

The effect of metal ion supplentation on enzyme activity (Figure 5). On rice bran substrate, metal ions of Na+, Co²⁺, Cu²⁺, Mg²⁺ and Zn²⁺ as activator and have an important role, because can increase enzyme activity, that were 39.4 U/mL, 50.8 U/mL, 41.0 U/mL, 38.9 U/mL and 38.2 U/mL respectively, rather than without metal ion supplementation (control) about 37.0 U/mL. On corncob substrate, ions of Na⁺ and Co²⁺ have a role as an activator were 14.7 U/mL and 32.1 U/mL respectively, while ions of Cu²⁺, Mg²⁺ and Zn²⁺ act as an inhibitor because decreasing enzyme activity each 13.0 U/mL, 12.4 U/mL, and 10.5 U/mL respectively rather than without metal ion supplementation (control) was 14.6 U/mL. On the filter paper substrate, ions of Na⁺ and Co²⁺ have a role as an activator each 16.4 U/mL and 21.3 U/mL respectively, while ions of Cu²⁺, Mg²⁺ and Zn²⁺ as an inhibitor, decreasing enzyme activity each 11.5 U/mL, 10.1 U/mL, and 10.8 U/mL respectively rather than without metal ion supplementation (control) was 15.6 U/mL.
Mg$^{2+}$, Mn$^{2+}$, Zn$^{2+}$. The influence of these chemicals on the cellulase activity suggests that some divalent cations may be interacting with basic or acidic amino acid residues of cellulases enzymes [48]. According to [49] numerous monovalent, divalent, and trivalent metal ions such as Na$^+$, K$^+$, Ca$^{2+}$, Mg$^+$, Mn$^+$, Fe$^2$+, Co$^{3+}$, Cu$^{2+}$, Ni$^{2+}$, Zn$^{2+}$, Hg$^{2+}$ and Fe$^{3+}$ served as inhibitor and activator in cellulose characterization.

Cellulase enzymes that are formed will break down the cellulose contained in the carbohydrate substrate into glucose. The glucose formed will be used by microbes for growth. These compounds together with ammonium will form protein, therefore, it is necessary to see the relationship between proteins formed by microbes and the activity of enzymes resulting from hydrolysis [50]. According to [51] that the protein content of fermentation is varied (depending on the raw material used). This research results showed that the soluble protein content of rice bran substrate, corn cobs and filter paper were 0.13 µg/mL, 0.05 µg/mL, 0.04 µg/mL respectively (Figure 6).

![Figure 6. Dissolved protein activity](image)

4. Conclusion

Bacteria strain A8 has a cellulolytic ability. Molecular identification based on 16S DNA sequence, the strain A8 is *B. subtilis*. Quantitative results of analyses showed that the cellulase enzyme activity of *B. subtilis* A8 on the rice bran media have optimum of incubation time 3 days at pH 6.0 and temperature 60°C with the protein content 0.13 µg/mL and activated by Na$^+$, Co$^{2+}$, Cu$^{2+}$, Mg$^{2+}$, and Zn$^{2+}$ ions. The cellulase enzyme activity of *B. subtilis* A8 on the corn cob media have optimum of incubation time 3 days at pH 7.0 and temperature 50°C with the protein content 0.04 µg/mL and activated by Na$^+$ and Co$^{2+}$, whereas Cu$^{2+}$, Mg$^{2+}$, and Zn$^{2+}$ are inhibitor.

5. References

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Acknowledgments
This research was funded by DIPA of thematic of Research Center for Biology, Indonesian Institute of Sciences. Authors acknowledge the support of Resma Maharani and Fitri Nur Indah Sari for laboratory assistance.