MICROBIAL CONVERSION OF CASSAVA STEM (*Mannihot esculenta*) CELLULOSE INTO REDUCING SUGAR BY *Trichoderma reesei* PK_1J_2

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Abstract

Cassava stems can be utilized as an alternative energy source to produce bioethanol due to its cellulose content, polysaccharide compound can not directly used by yeast. Therefore, cellulose need to be hydrolyzed into monomers prior to tranformation into ethanol by *Saccharomyces cerevisiae*. Hydrolysis can be performed by cellulase-producing fungi, such as *Trichoderma reesei* PK_1J_2 with ammonium sulphate used as nitrogen source. The effect of ammonium sulphate addition to the growth and reducing sugar production rate of *Trichoderma reesei* from cassava stems was investigated. Hydrolysis was conducted without pretreatment. Cassava stem was degraded into small pieces with and without addition of ammonium sulphate. The concentration of $(NH_4)_2SO_4$ were 0%; 0,5%; 1% and 1,5%. During cellulose fermentation of cassava stem, analysis were carried on cellulose enzyme activity, dry matter loss, glucosamine content, and reducing sugar. Initial spore concentration was 10^6 spores/g of cassava stem. Addition of ammonium sulphate seem to affect the growth but did not affect of reducing sugar production of *Trichoderma reesei*, therefore hydrolisis continued without ammonium sulphate addition. Highest levels of reducing sugars was obtained after three days of fermentation which was 10,828 g/L.

Keywords: Cassava stem, Trichoderma reesei, cellulose

1. Introductions

Cassava (*Mannihot esculenta*) stem is one of the agricultural waste which has a high cellulose content. However, only 10% of the waste plant stems which is used to be replanted and 90 % is a waste [(1)].

Cassava stems can be utilized as an alternative energy source to produce bioethanol due to its cellulose content. Bioethanol is one of alternative energy sources obtained through fermentation of biomass. Bioethanol can also be produced from cassava, sugar, or corn. However, the demand for these raw materials, which are also food sources, will cause a global food crisis if produced continuously. Therefore, efforts to produce ethanol from other sources such as agricultural waste have been done.

The hydrolysis process of lignocellulose material such as cassava stem can be done by chemical, physical, enzymatic and biological hydrolysis use cellulase-producing fungi or bacteria.

Fungi are a major source of cellulase and hemicellulase. *Trichoderma reesei* fungi have been considered to be the most productive and powerful destroyers of crystalline cellulose[(2)]. *Trichoderma reesei* has the ability to grow well in Solid Substrate Fermentation (SSF), which is a fermentation that occurs in conditions of limited free water. SSF process generally uses natural materials as carbon and energy sources such as cassava, barley, and agricultural industry residues [(3)]. The factors that affect fungi growth are pretreatment processing and the availability of nutrients such as nitrogen, sulfur and other minerals in the fermentation medium. In this research, hydrolysis was conducted without pretreatment. Cassava stem was degraded into small pieces with and without addition of ammonium sulphate. One source of nitrogen that can be utilized by fungi in the fermentation medium is ammonium sulphate ($(NH_4)_2SO_4$). It is an anorganic nitrogen source of nitrogen which is cheap and easy to obtain.

The aims of this research were to analyze the effect of ammonium sulphate addition to the growth and reducing sugar production rate of *Trichoderma reesei* from cassava stems.

2. Materials and Methods

Preparation of the cassava stem

Cassava stem were collected, washed manually using tap water to remove adhering dirt and then they were dried. The dried cassava stem were cut into small size and blended, afterworks the pH, a_w, and cellulose content were analyzed.

Microbiology

Trichoderma reesei PK_1J_2 from Biotechnology Laboratory of Agriculture Technology, Gadjah Mada University were used during the experi-

ments. Spore suspension was obtained by harvesting 7-day-incubated fungi on potato dextrose agar (PDA) slant with 10 mL of 0,05% tween 80 solution. Cassava stem chips was used as a medium for the starter culture. Cassava stem (100 g) was mixed with 100 mL of water and sterilized at 121°C for 30 min. After cooling, 10 mL of spore suspension of *Trichoderma reesei* PK_1J_2 was added for inoculation. Initial spore concentration was 10^6 spore/gram of cassava stem. The culture was incubated at 30±2°C for 7 days.

Hydrolysis

Cassava stem (100 g) was mixed with 100 mL of water and $(NH_4)_2SO_4$ and sterilized at 121°C for 30 min. After cooling, 1 g of starter of *Trichoderma reesei* PK_1J_2 was added for inoculation. Hydrolysis was conducted using $(NH_4)_2SO_4$ at concentration of 0%; 0,5%; 1% and 1,5%. During cellulose fermentation of cassava stem, analysis parameters were cellulase enzyme activity, dry matter loss, glucosamine content, and reducing sugar. Initial spore concentration was 10^6 spore/gram of cassava stem. The culture was done at 30±2°C for 5 days.

Cellulase enzyme activity [4]

About 1 g of fermented cassava stem was extracted with 15 mL of 0.1% (w/v) Tween 80 for 30 min. After centrifugation at 3000 rpm for 5 min, the supernatant was used for measuring cellulase activity. A 1-mL portion of 0.05 M citric buffer (pH 4.8) was added to a test tube followed by the addition of 0.5 mL of sample properly diluted in the same buffer and a 1 cm x 6 cm strip of Whatman No. 1 filter paper curled around a glass rod. The reaction mixture was incubated at 50°C for 60 min, and then 3 mL of DNS reagent was added to stop the reaction. The suspension was well mixed, and the tubes were transferred to a boiling water bath for exactly 5 min and then cooled in ice. The tube contents were well mixed after 20 mL of distilled water had been added. Finally, the tubes were allowed to stand for at least 20 min to allow the pulp to settle, and the color formed was read in a spectrophotometer at 540 nm. Enzyme blank, reagent blank, and glucose standard solutions were treated in the same way. One international unit of cellulase activity is the amount of enzyme that forms 1 μmol glucose per minute.

Biomass estimation by dry matter loss[5][6]

Dry matter content was determined by measuring the change of weight of ±1.0 g of fermented cassava stem before and after drying at 106 °C for 16 h.

Biomass estimation by glucosamine[7]

The procedure containing a two-step hydrolysis by sulfuric acid followed by one-step nitrous acid depolymerization was applied to all of the reference materials. Sulfuric acid hydrolysis was carried out according to laboratory analytical procedure developed by the National Renewable Energy Laboratory (NREL) for lignocelluloses with some modifications. Samples of 10 mg chitin-/chitosan-containing materials were placed in 15 mL screw cap centrifuge tubes, and 0.3 mL of 72% (v/v) sulfuric acid was added. The suspensions were then mixed every 15 min with a glass bar for 90 min at room temperature. At the end of this step, all solid materials were dissolved in the concentrated sulfuric acid. Shorter mixing times and smaller volumes of sulfuric acid resulted in incomplete dissolution of chitin and lower recovery of this material. Then, 8.4 mL of water was added to each tube, and they were then closed tightly and placed in an autoclave at 121 °C for 1 h for the hydrolysis with the diluted sulfuric acid. At the end of this hydrolysis step, two samples of each 0.5 mL were taken from each tube, while the solutions were still hot around 100°C. It was necessary to take the samples at high temperature, since chitosan procedure for the determination of GlcN in chitin- and chitosan-containing materials.

Determination of GlcN and precipitates by cooling to room temperature, which results in a nonhomogeneous solution. After the samples were cooled to room temperature, 0.5 mL of 1 M $NaNO_2$. All tubes were closed tightly, mixed, and left for 6 h at room temperature. They were then opened and left overnight under the hood to complete the depolymerization-deamination reaction and to remove the NO_2 that arose as a byproduct in the reaction mixture of sample. At the end of this step, both chitin and chitosan were converted to anhydromannose, which was quantified by the colorimetric method with minor modifications. Briefly, the excess nitrous acid was inactivated by the addition of 0.5 mL of ammonium sulfamate (12 wt %) to samples A and B and mixing for 4 min. Then, 0.5 mL of 0.5% MBTH (3-methyl-2-benzothiozolone-hydrazone-hydrochloride) was added, and the tubes were left for 1 h without mixing. It was followed by addition of 0.5 mL of 0.5% $FeCl_3$ and mixing. MBTH and $FeCl_3$ create a blue color complex with anhydromannose. After 1 h, the sample was diluted 100 times with water, and the absorbance of solution A was measured at 650 nm against solution B (without $NaNO_2$). A solution of pure GlcN hydrochloride in 2.48% (v/v) sulfuric acid (8.4 mL of water mixed with 0.3

mL of 72% sulfuric acid) was used as standard, and the concentration of GlcN in samples was measured.

Sugar estimation[8]

Total reducing sugar was estimated using dinitrosalicylic acid (DNS) reagent.

3. Result and Discussion

Characteristics of cassava stem

Cassava stems that were used in this study was about 6 months when cassava was harvested. Part of the cassava stems that were used is the middle of the stem to the top. The average composition of cassava stems were 15.28% moisture content, 27.82% cellulose content, pH 5.1 and a_w 0.971. The result indicated that cassava stem could be a good source for bioconversion.

Preparation of *Trichoderma reesei* PK_1J_2 starter

Making starter is important for aclimatization or adaptation to the new medium because *Trichoderma reesei* was grown on PDA which have a complete nutrient composition for the optimal growth previously. At the end of fermentation, in the seventh day, the calculation of the spores number was done by platting on DRBC medium. The number of *Trichoderma reesei* spores reached 10^8 spores/g of cassava stems.

Cellulase enzyme activity at different concentrations of Ammonium Sulfate

Figure 1 shows the cellulase enzyme activity at various concentrations of ammonium sulphate. The cassava stems hydrolysis in the preliminary study resulted a low concentration of reducing sugar. Probably due to the nitrogen content in cassava stems insufficient for fungi growth. Therefore, the addition of a nitrogen source in several variations was used to determine the most appropriate concentration of the nitrogen source for fungal growth. Fermentation was carried out for three days based on the preliminary study which showed that the highest reducing sugar produced after fermentation for three days.

The concentration of ammonium sulphate 1% resulted in the highest cellulase enzyme activity at 0.724 IU/ml. Other studies with corncob substrate produced the highest cellulase enzyme activity at a concentration of 0.5%[9]. Differences substrate will affected the ability of *Trichoderma reesei* to produce cellulase.

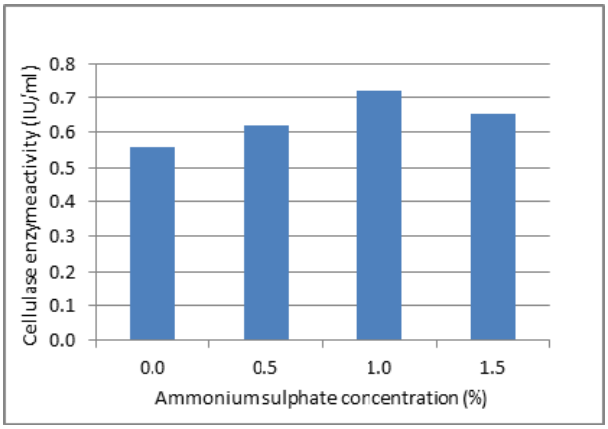

Figure 1. Cellulase enzyme activity at various concentrations of ammonium sulphate

The relationship between biomass growth, cellulase enzyme activity and reducing sugar produced on cassava stems hydrolysis with the addition of ammonium sulphate 1%

The growth of the fungi can be seen from the rate of dry matter loss. The highest increasing of the growth occurred after two days of fermentation. Results are linear with enzyme activity and reducing sugar produced which increased significantly after two days of fermentation. This can be seen in Figure 2.

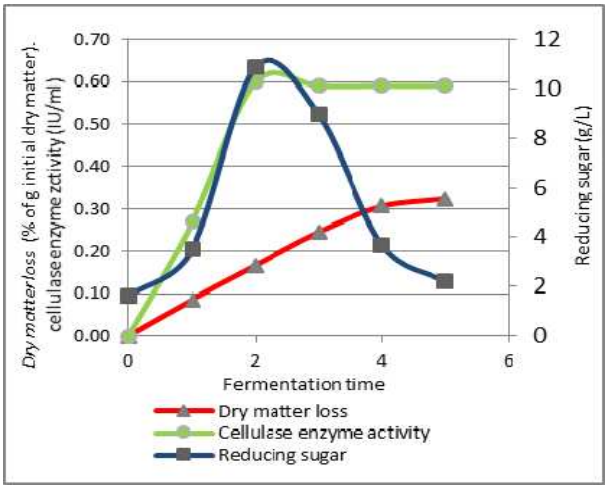

Figure 2. The relationship between biomass growth, cellulase enzyme activity and reducing sugar produced on cassava stems hydrolysis with the addition of ammonium sulphate 1%

The highest of enzyme activity occured after two days of fermentation, from 0.2697 IU/ml increased to 0.6005 IU/ml. The dry matter loss also increased significantly in those days, from 0.0861 up to 0.1667 % of g initial dry matter. The highest

reducing sugar levels after two days of fermentation at 10.9 g/L. If the rate of dry matter loss was associated with metabolic activity based on dry matter degradation, it could be suggested that the highest metabolic activity of *Trichoderma reesei* PK_1J_2 occured in second days of fermentation.

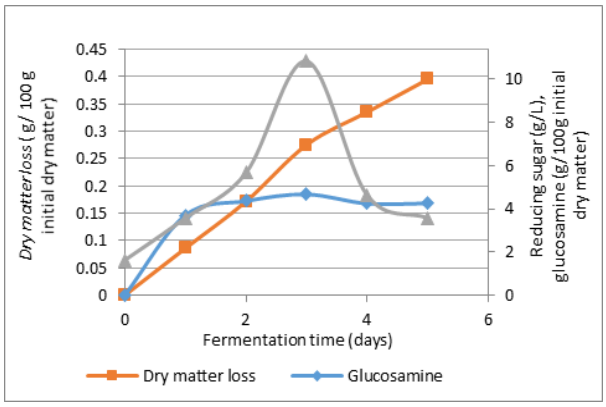

Figure 4. The relationship between biomass growth, glucosamine content and reducing sugar produced on cassava stems hydrolysis without ammonium sulphate.

It showed that there was a possitive correlation between the accumulation of CO_2 production (mmol/g dry matter) and the loss of dry matter (g/g dry matter) in a solid substrate fermentation with *Aspergillus oryzae*[6]. The other research showed that the amount of CO_2 produced during fermentation (72 hours) can be predicted by a major composition change of dry matter as carbon sources[5].

Effect of ammonium sulphate 1 % to the biomass concentration of *Trichoderma reesei* PK_1J_2 and reducing sugar during cassava stems fermentation

The addition of 1% ammonium sulphate showed the highest activity of cellulase enzymes than others. The next stage was to compared the cassava stems hydrolysis, either with the addition or without addition of ammonium sulphate 1%.

Figure 3 showed that the growth of the *Trichoderma reesei* PK_1J_2 with ammonium sulphate 1% had no significant difference in the growth of *Trichoderma reesei* PK_1J_2 without ammonium sulphate. The addition of ammonium sulphate 1% only affected the accelerated of the metabolic rate. Without ammonium sulphate, the highest growth occurred after three days of fermentation, from 0.1797 up to 0.2896 % of g initial dry matter, while the highest growth with addition of ammonium sulphate 1% obtained after two days of fermentation, from 0.0861 up to 0.1667 % of g initial dry matter.

At the end of the fermentation, the dry matter loss in cassava stems with the addition of 1% ammonium sulphate reached 0,3237 % of g initial dry matter, while the cassava stems without ammonium sulphate reached 0,3927 % of g initial dry matter. It was estimated that cassava stems with the addition of ammonium sulphate 1% tend to induced fungal growth activity while without ammonium sulphate tend to stimulated metabolic activity. After two days of fermentation, *Trichoderma reesei* PK_1J_2 had stationary phase so that the rate of dry matter loss also decreased. These results was consistent with studies conducted by Smith[5] who reported that the growth of *Trichoderma reesei* QM9414 on wheat bran had the highest growth after two days of fermentation, from 1-10.1 % of g initial dry matter.

There was no significant difference in the reducing sugar produced between the two treatments. The highest reducing sugar content of cassava stems with the addition of ammonium sulfate 1% occurred after two days of fermentation. It was 10.9 g/L, while the cassava stems without ammonium sulphate obtained the highest reducing sugar 10.828 g /L after three days of fermentation. According to this result, the hydrolysis without ammonium sulphate was used to the next step fermentation.

The relationship between biomass growth, glucosamine content and reducing sugar produced on cassava stems hydrolysis without ammonium sulphate

Figure 4 showed the relationship between dry matter loss, glucosamine content and reducing sugar during the fermentation of cassava stem without ammonium sulfate. The highest dry matter loss was reached 0.2896 g / 100 g initial dry matter in the third day of fermentation. The rate of increasing in dry matter loss was linear with increasing levels of reducing sugar produced. The highest reducing sugar content was obtained on the third day of fermentation in the amount of 10.828 g / L.

Measurements of glucosamine which was contained in the Chitin as fungal cell wall compounds was one of the methods commonly used to measure the rate of growth of the fungus. The results showed the highest levels of glucosamine at 4.67 g / 100 g initial dry matter in the third day of fermentation. It showed that the fungus was in the exponential and then stationary phase after three days fermentation which was

indicated by the relatively similar levels of glucosamine until the end of fermentation. All of the results indicated that fungal metabolic activity was highest on the third day of fermentation.

4. Conclusion

Addition of ammonium sulphate seem to affected to the growth of *Trichoderma reesei* PK_1J_2 but did not affect the reducing sugar production of *Trichoderma reesei* PK_1J_2, therefore hydrolisis continued without ammonium sulphate addition. Highest levels of reducing sugars was obtained after three days of fermentation which was 10,828 g/L.

References

(1)Sumada, K., Tamara, P.E., dan Alqani, F. 2011. Kajian Proses Isolasi α - Selulosa dari Limbah Batang Tanaman *Manihot esculenta crantz* yang Efisien. *Jurnal Teknik Kimia* Vol.5, No.2: 434-438.

(2)Balat, M., Balat, H., and Oz, C. 2008. Progress in Bioethanol Processing. *Progress in Energy and Combustion Science* (34): 551-573.

(3)Shetty, K., Paliyath, G., Pometto, A., and Levin, R. 2006. *Food Biotechnology second Edition.* CRC Press, Taylor and Francis Group, New York.

(4)Ghose, T.K. 1987. Measurement of Cellulase Activity. *International Union of Pure and Applied Chemistry*. Vol 59. No 2. Pp 257-268

(5)Smith, I.P. 1998. *Solid State Fermentation: Modelling Fungal Growth and Activity.* Agro Food Industry Hi-tech, Holland.

(6)Sardjono (2008). The Growth Kinetics of *Aspergillus oryzae* KKB4 on Solid State Culture System and The Activity of Crude Extracellular Enzyme on Reducing Aflatoxin B. *Agritech* 28 (4): 145-149.

(7)Zamani, A., A. Jeihanipour, L. Edebo, C. Niklasson dan M. J. Taherzadeh (2008). Determination of Glucosamine and N-Acetyl Glucosamine in Fungal Cell Walls. *Journal of Agricultural and Food Chemistry* 56: 8314-8318.

(8)Miller, G.L. 1959. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Analytical Chemistry* Vol 31. No 3: 426-428.

(9)Guowei, S., dkk. 2011. Effect of Some Factors on Production of Cellulase by *Trichoderma reesei HY07*. *Procedia Environmental Sciences* 8 (2011): 357-361