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by Emma Moko

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1 Enzyme powder amylase complex from *Aspergillus awamori* KT-11 for hydrolysis of cassava (*Manihot esculenta*)

U Perwitasari¹, R Melliawati¹, R O Palit², J Ngangi², E Moko², and Yopi³

¹Research Center for Biotechnology, Indonesian Institute of Sciences, Jln. Raya Bogor Km 46, Cibinong, West Java 16911, Indonesia

²State University of Manado, JalanTondano, Koya, Tondano Selatan, Tondano Sel., Minahasa, North Sulawesi 95618, Indonesia

³Center for Research and Development of Human Resources, National Standardization Agency of Indonesia, Gedung I BPPT Jl. M.H. Thamrin No.8, Kebon Sirih, Center Jakarta 10340, Indonesia

Abstract. Glucoamylase is the common enzyme to produce liquid sugar from starch. This enzyme breaks the starch chain randomly. Enzymes that are commonly used are liquid enzymes. Liquid enzymes stored at room temperature are easily damaged. The aim of the present study is to check the ability of the glucoamylase powder enzyme from *Aspergillus awamori* KT-11 in hydrolyzing starch-based biomass for producing liquid sugar. The produced liquid sugar was analyzed its concentration of reducing sugar, glucose concentration, sweetness level, and micrograph as a result of hydrolysis of biomass by enzyme glucoamylase. Variations concentration of the glucoamylase enzyme with cassava juice substrate was optimized to produce a high concentration of liquid sugar. The optimal time on hydrolysing 30% cassava with the enzyme is at 48 hours with enzyme concentration of 10.0469 U and substrate concentration of 30%. The sweetness level obtained on the refractometer is 4.2 % Brix. Approximately, 4000 ppm of glucose can be produced after 48 hours of reaction.

1. Introduction

The human population in the world is increasing. The high population will affect sugar consumption. Therefore, the increase of sugar production is required. Generally, sugar is divided into 3 main parts namely white, brown, and liquid sugar. Liquid sugar is better known as glucose syrup or fructose syrup. Liquid sugar is used in various industrial sectors such as food industry, pharmaceuticals, agro-industries, chemicals. The liquid sugar has a high level of sweetness. In addition, the characteristics of liquid sugar are colorless and odorless.

The liquid sugar can be produced from starch hydrolysis. Cassava (*Manihotes culenta*) is a higher source of starch than potato (6.5 until 24.8%) [1], sweet potatoes (5 until 25%) [2], rice, and corn. Cassava starch have been known as a source for liquid sugar production [3]. The Indonesia Ministry of Agriculture noted that cassava produced in Indonesia contains around 60% of water, 25-35% starch, proteins, fibre, calcium, phosphate, and minerals.

The method of hydrolysis is conducted in several ways such as acid hydrolysis, enzymatic hydrolysis and a combination of them. Acidic hydrolysis usually uses strong acids like HCl [4]. Strong acids will break the starch chain randomly, whereas enzymatic hydrolysis will break the starch chain specifically at certain branches. In this case, enzymatic hydrolysis using alpha amylase will break starch on the α -1,4 glycoside bond [5], and glucoamylase will break starch on the α -1,4 glycosidic bond and β -1,4 glycosidic bond[6].

Aspergillus awamori KT-11 is a mold capable of producing amylase group enzymes [7]. Perwitasari has produced glucoamylase powder enzyme from *A. awamori*KT-11 from cassava peel [8]. However the ability of the enzyme powder glucoamylase to produce liquid sugar have not been reported. The aim of the present study is to produce liquid sugar by using enzyme powder from *A. awamori* KT-11 expression of the gene of interest to useful levels may depend upon the generation of a recombinant strain that contains multiple copies integrated at the AOX1 locus [18]. In this study,



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stability assay was performed by expression analysis for every 30th generation. The result showed that there was no significant difference of protein profiles among the generations (Figure 5). AUC determination using ImageJ software confirmed that no decreasing and the expression were stable until 90th generations. Further analysis may still needed to state the expression stability such as multicopy integration analysis by southern blot.

2. Materials and methods

2.1 Material

The biomass of cassava used in this study from Cibinong market Bogor. Cassava biomass thinly sliced and dried at a temperature of 60 °C then milled to a fine powder. Biomass is stored at room temperature until it is ready for use. The microorganism used in this research is *A. awamori* KT-11 from Biotechnology Culture Collection. Isolate was incubated in Potato Dextrose Agar for 5 days at room temperature. Subsequently, the isolate was added with 5 mL sterile aquadest. Then, the spores were dissolved using a sterile aquadest.

2.2. Enzyme production

Cassava peel was cleaned from soil and fungi. Then, it was chopped and sterilized it at 121 °C, 1 atm, for 15 minutes. The sterile cassava skin was added 1% spore suspension from *A. awamori* KT-11. The culture was closed using sterile paper. The solid state fermentation was incubated for 5 days at room temperature and stirred once a day. After that, the culture was dried at 60 °C for 2 days. Dry enzymes are milled and stored at room temperature prior to use. In this study the enzymes used were enzymes that had been stored for 9 months at room temperature.

2.3. Enzyme extraction

One gram of dried culture was extracted using 9 mL buffer citrate-phosphate pH 4.8. The extract was agitated for 15 min at 22 °C and 120 rpm. The supernatant was collected from centrifugation the enzyme extract at 10000 g at 4°C for 20 minutes. The enzyme extract is used to hydrolyse the biomass.[8].

2.4. Cassava hydrolysis

The saccharification substrate uses 30% fresh cassava. The parameters of variation were comparison of the number of enzymes with substrate (1:1; 1:2; 1:3), and incubation time (0, 3, 6, 24, 48, and 72 hours). The hydrolysis reaction was carried on 60°C, pH 4.8. The sample was heated into boiling water for 3 minutes to stop the hydrolysis reaction. The sample was centrifuged at 10000 g 4°C for 20 minutes, the supernatant was collected for analysis.

2.5. Sugar analysis

The sweetness level of liquid sugar was measured by refractometer. The qualitative analysis of sugar content from hydrolysis fresh cassava was analysis by Thin Layer Chromatography. Approximately 4 ml sample was spotted on the TLC plate Silica Gel GF-254. Glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, and fructose as standards were also spotted in TLC plate Silica Gel GF-254. Solution of n-butanol: acetic acid: water (2: 1: 1) was used as mobile phase. Reagent sprayer was used DAP reagent (diphenylamine 0.2 g, anilin 0.2 mL, acetone 10 mL, and phosphoric acid 1.5 mL). Subsequently, TLC plate was heated at 120 °C for 15 minutes for visualisation [9].

The quantitative analysis or reducing sugar was conducted by Dinitrosalicylic acid (DNS) method [10]. Approximately 0.5 ml of the sample was added with 0.5 DNS reagents then vortex vigorously. Afterwards the sample is heated for 15 minutes in a water bath at 95 C. The sample was cooled for 15 minutes then measured at a wavelength of 540 nm. Glucose concentration was calculated based on glucose standard.

2.6. SEM analysis

The sample was soaked in caccodylate buffer during 2 hours and agitated in an "ultrasonic cleaner" for 5 minutes at 4 °C. Afterward, it was soaked in 2.5% glutaraldehyde solution for a few hours. Subsequently, it was soaked in 2% tannic acid 6 hours, then washed with caccodylate buffer 4 times, and aquadest once. The water in the sample is removed by several stages of washing with alcohol (alcohol 50% for 5 minutes x 4, alcohol 70% for 20 minutes, alcohol 85% for 20 minutes, alcohol 95% for 20 minutes at room temperature, alcohol absolute 10 minutes x 2). The sample was dried by 50% for 5 minutes x 4 alcohol, alcohol 70% for 20 minutes, alcohol 85% for 20 minutes, alcohol 95% for 20 minutes at room temperature, alcohol absolute 10 minutes x 2.

3. Results and Discussions

The substrate used was characterized its starch content, water content, and pH. The results of the starch content analysis showed that the starch content of cassava in this sample is 59,96% ± 1.86, its relatively close to the starch content from the research conducted by Souto et al. of 60.68% ± 1.86 [11]. Starch in cassava is hydrolyzed by glucoamylase enzymes from *A. awamori* KT-11 to other simple sugars like oligosaccharide or glucose.

The results of the pH analysis showed that pH of cassava was 7. At the time the fresh cassava was milled with buffer pH 4.8 the pH reaction was maintained. The pH measurement needs to be carried out because the activity of the glucoamylase enzyme from *A. awamori* KT-11 is stable in a pH range of 4.8 [7]. The water content of cassava is 59.22%. The result is too high, and it will reduce the concentration of starch in the reaction, therefore, the concentration of sugar produced from hydrolysis will be low.

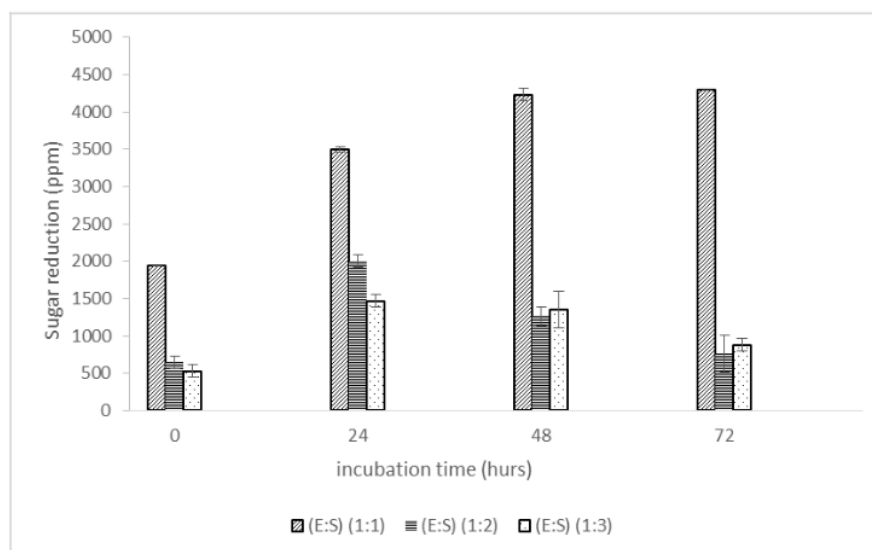


Figure 1. Reduction of sugar from hydrolysis of 30% cassava with variations in the number of enzymes (E= enzyme; S= substrate)

We varied the ratio of glucoamylase enzymes to the substrate (Figure 1). The results obtained with a little substrate, the higher the reducing sugar produced. This is probably because the substrate that is too thick will provide steric obstruction for the activity of the enzyme. Enzyme activity is influenced by substrate concentration. Enzymes with a low K_m (Michaelis constant) will experience saturation if given a lot of substrate [12]. Therefore, in this reaction of enzymes with the least substrate produces the highest reducing sugars.

About 4000 ppm of reducing sugar can be produced after 48 hours of reaction. This process must still be optimized, time incubation, type of substrate, and enzyme addition in reaction. Saccharification process of starch can be improved by adding the pullulanase enzyme [13]. Pullulanase is an enzyme that can hydrolyze α -1,6 glycosidic bonds. Therefore, the enzyme can cut branching in starch [14].

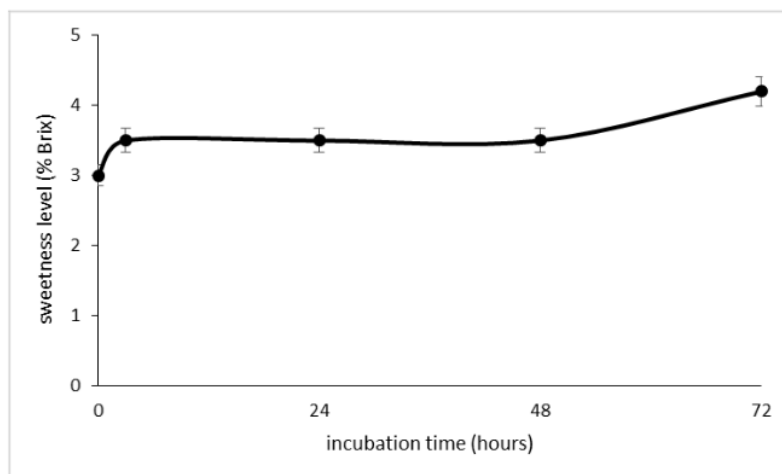


Figure 2. Sweetness level from hydrolysis of 30% cassava

Measuring the sweetness level of sugar produced from the hydrolysis of cassava peel, the highest value was obtained after 72 hours incubation (Figure 2). With glucose concentration 3.68 g/L, the sweetness level obtained on the refractometer is 4.2% Brix indicating that there are 4.2 g of sucrose in 100 g of liquid. High levels of sweetness are likely to be obtained by reacting enzymes with specific substrates such as starch. Souto et al hydrolyzed starch from cassava peels using two specific enzymes capable of producing a sweetness level 12 % Brix[11]. This liquid sugar from hydrolysis cassava has the same level of sweetness as liquid sugar from the extraction of *Hibiscus sabdariffa* L. (11-13% Brix) [15].

A. awamori KT-11 is known to have endoamylase and exoamylase [7]. Endoamylase will break the glycosidic bond of starch from the middle part of the chain, therefore, maltooligosaccharides will be generated. Meanwhile, exoamylase will cut the glycosidic bond from the outside chain of starch. Therefore, the sugar produced from the hydrolysis of cassava are varies. After 12 hour, the glucose concentration immediately looks thickened. Spots of sugar from maltotriose begin to form after 48 hour (Figure 3).

The ability of glucoamylase powder enzymes to degrade cassava skin substrate has been carried out investigating. The fiber of cassava peel before the addition of complex enzyme amylase powder still looks intact and straight. The results of the Scanning Electron Microscope (SEM) analysis showed the structure of cassava skin fibers which were destroyed after being hydrolyzed by this enzyme (Figure 4). Figure 4A, shows that the fiber is still smooth, this is because the fiber structure of cassava flour is composed of cellulose microfibrils fiber which is still bound by lignin and

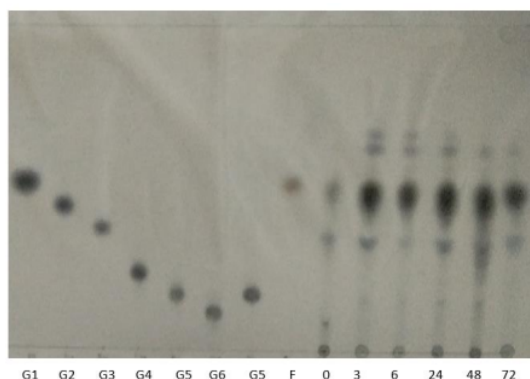


Figure 3. TLC from hydrolysis of 30% cassava

hemicellulose fibers [12]. After hydrolysis (Figure 4B) the structural change occurs because the complex enzymes produced by *A. awamori* KT-11 do hydrolyzes the chemical bonds in cassava skin fibers. Amylase can hydrolyze the starch both in static and dynamic conditions, the thickness of the fiber also does not affect the work of amylase in cutting the glycosidic bonds, besides the addition of amylase will not reduce the weight of the sample [16].

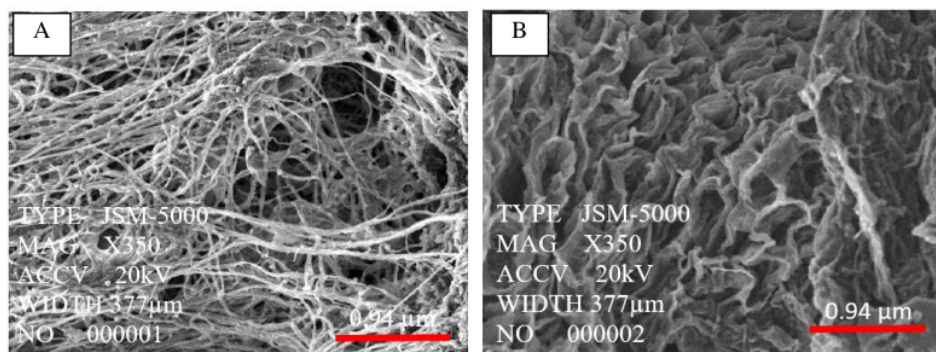


Figure 4. Micrograph cassava (A: before hydrolysis; B: after hydrolysis)

4. Conclusion

Enzyme powder from *Aspergillus awamori* KT-11 stored for 9 months at room temperature still has stable activity. The powder enzyme glucoamylase can be used to hydrolyze 30% dry cassava. The optimum hydrolysis time is at 48 hours with glucose concentration of 98 g/L. The highest sweetness level is 4.2% Brix.

5. Acknowledgment

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