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Bioethanol production from banana's tuber (*Musa paradisiaca*) with hydrolysis using α-amylase and glucoamylase enzyme

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Abstract. *Utami IP, Mahajoeno E, Susilowati A. 2020. Bioethanol production from banana's tuber (*Musa paradisiaca*) with hydrolysis using α-amylase and glucoamylase enzyme. Bioteknologi 17: 76-80.* Energy consumption has increased in lockstep with economic expansion and population development, resulting in the depletion of fossil fuel supplies. Bioethanol is a non-fossil fuel that may be produced from rich biological resources in Indonesia, one of which is the banana tuber (*Musa paradisiaca* L.). The purpose of this study is to find the maximum reducing sugar levels in the banana tuber hydrolysis using a concentration ratio of α-amylase and glucoamylase enzymes, as well as to create the highest amount of ethanol utilizing a variety of baker's yeast concentrations (*Saccharomyces cerevisiae* Meyen ex E.C. Hansen). A Completely Randomized Design (CRD) with two components was used in this study. The first component was the ratio of α-amylase and glucoamylase concentrations, while the second element was the concentration of baker's yeast. The banana tuber was hydrolyzed to a maximum of 0.2 grams using α-amylase and glucoamylase at various concentration ratios (0:0; 100:0; 75:25; 50:50, 25: 75; 0: 25), followed by fermentation with baker's yeast (7.5 mg, 10 mg and 12.5 mg). The amount of reducing sugar created during hydrolysis was determined using the DNS method, whereas the amount of ethanol produced was determined using AOAC tables. ANOVA evaluated the data, and significant differences were found using Duncan's Multiple Range Test (DMRT) at a 95% confidence level. The results indicated that a 75% α-amylase ratio to 25% glucoamylase resulted in 26.17 mg/mL maximum sugar reduction. The highest ethanol concentrations were obtained by combining 75% α-amylase and 25% glucoamylase with 12.5 mg 7.98% baker's yeast. A 7.5 mg baker's yeast produced substantially more ethanol than 10 mg or 12.5 mg baker's yeast.

Keywords: α-amylase, banana's tuber, bioethanol, glucoamylase, hydrolysis

INTRODUCTION

Fossil energy, particularly oil, is the country's primary energy source and a source of foreign exchange. Energy consumption increases with economic and population expansion (Tambunan 2008). Thus, natural resources capable of producing fossil energy will continue to diminish, as most fossil energy sources are non-renewable, such as oil, gas, and coal. Indonesia has seen a reduction in national oil output over the previous few decades due to depleting oil reserves in natural production wells. Population growth will also increase the need and consumption of fuel oil for transportation facilities and industrial activities (Triwahyuningsih and Rahmat 2006).

Bioethanol is a non-fossil fuel alternative produced by the fermentation of carbohydrates-containing biomass using microorganisms. Bioethanol can be produced in Indonesia using the country's extensive biological resources (Hambali 2007). Therefore, bioethanol development as an alternative energy source has the potential to be used in Indonesia. Furthermore, bioethanol raw materials are plentiful and obtained from various carbohydrates-containing plants (Anonymous 2012).

In 2006, Indonesia produced 4.3 million tons of bananas (*Musa paradisiaca* L.) (Gusmawarni et al. 2009). Large production will also generate a great deal of garbage. One of the wastes is the banana tuber; after the fruit is picked, this banana tuber is rarely reused. The banana tuber comprises 76% carbohydrate, 20% water, and the remainder contains protein and vitamins (Yuanita 2008). The starch content of this enormous banana tuber has the potential to be utilized as a raw material for bioethanol production.

Converting starch into bioethanol consists of two stages: hydrolysis and fermentation. The first stage, hydrolysis, involves the conversion of starch to glucose. It can be accomplished biochemically using the α-amylase enzyme or chemically using an acid/base solution. α-amylase enzyme is an endoamylase enzyme, which means that it can degrade starch randomly from the center or inside the glucose molecule. The optimal temperature range for the enzyme α-amylase is between 4.5 and 70°C., and it is active at a pH of 5.2 to 5.6. The glucoamylase enzyme is an exoamylase enzyme, which means that it can convert the starch chain to glucose molecules in the non-reducing region of the molecule. The optimal pH range for the glucoamylase enzyme is between 4.5 and 5.0, but this value varies according to the source of the enzyme. Therefore, the optimal temperature ranges between 40 and 50°C. Enzymatic hydrolysis of starch with α-amylase enzyme rapidly breaks α-1,4 glycosidic bonds in gelatinized starch solutions. In contrast, although slower, glucoamylase

enzymes can break α-1,4 glycosidic and α-1,6 glycosidic bonds in starch molecules. The second stage, fermentation, involves the conversion of the produced glucose to ethanol and carbon dioxide by the yeast *Saccharomyces cerevisiae* Meyen ex E.C. Hansen (Jumari et al. 2009). To generate ethanol, Baker's yeast can be used in place of *S. cerevisiae* isolates during the fermentation process. It is due to *S. cerevisiae* isolates in fermenting yeast and baker's yeast. Baker's yeast is widely available and does not require special preparation (Reed and Nagodawithana 1991).

According to Komarayati et al. (2011), the greatest ethanol content for sago starch was obtained using a combination of 0.18 mL α-amylase enzyme; 0.15 mL glucoamylase enzyme; 0.48 grams baker's yeast, and three days of fermentation. The highest ethanol content for sago pith was 44.84% when 0.13 mL of α-amylase enzyme; 0.11 mL of glucoamylase enzyme; and 0.33 grams of yeast were combined, while the highest ethanol content for fiber was 8.26% when 0.13 mL of -amylase enzyme; 0.11 mL of glucoamylase enzyme; and 0.33 grams of yeast were combined. Mucaramah (2012) used rice bran substrate hydrolyzed with 0.09 mL of the glucoamylase enzyme and fermented with 2 mg of baker's yeast to achieve the highest ethanol concentration of 6.80%. Risnoyatiningsih (2011) did research on 50 grams of yellow sweet potato hydrolyzed for five days using 0.1 gram α-amylase enzyme, resulting in a glucose level of 5.64%. The substrate to enzyme ratio is 1:0.002. Rahmi et al. (2007) conducted a study employing sorghum substrate that had been hydrolyzed using α-amylase enzyme at a concentration of 0.2%, resulting in an 18 mg/mL reducing sugar content and an 8.22% ethanol content. Hasanah et al. (2012) conducted a study using 5 grams of banana tuber substrate hydrolyzed with 0.09 mL of the glucoamylase enzyme and fermented for three days with 50 mg of tape yeast. The resulting ethanol level was 3.12%.

The objectives of this study were (i) to compare the concentrations of α-amylase and glucoamylase enzymes that produced the maximum concentrations of reducing sugars during the banana tuber hydrolysis process and (ii) to compare the concentrations of α-amylase and glucoamylase enzymes required to create the maximum quantities of bioethanol using baker's yeast.

MATERIALS AND METHODS

Preparation of tools and materials

All tools and media used were sterilized by autoclaving at 121ºC for 15 minutes.

Preparation of *Dinitrosalycilic acid* (DNS) Reagent

Ten grams of *dinitrosalycilic acid* (DNS), 2 g phenols, 0.5 g Na-sulphite or Na-bisulphite, and 10 g NaOH were dissolved in 300 mL H_2O. Further, the solution was diluted into 1 L. The solution was stored in the refrigerator.

Standard curve setup

To begin, the researchers created a typical glucose solution (10 mL anhydrous glucose in 100 mL). The solution was diluted five times to create glucose solutions with concentrations of 2, 4, 6, 8, and 10 (mg/100 mL). Six sterile test tubes have been prepared. Each was 0.5 mL with a typical glucose solution. One tube was loaded with 1 mL of pure water as a blank. Each solution was placed in a test tube, followed by the addition of 3 mL of DNS reagent. Five minutes were spent heating the solution in boiling water and then cooling it to room temperature. After adding 1 mL of potassium sodium tartrate, the absorbance was measured at a wavelength of 550 nm (Miller 1959).

Making banana tuber pulp

100 g cleaned banana tuber was blended with 400 mL distilled water and then poured into a 500 mL Erlenmeyer. After adjusting the pH to 5.5 with 0.1% HCl, aquadest was added until the volume reached 500 mL.

Hydrolysis process

In the hydrolysis of banana tuber pulp, α-amylase enzyme was added. It was heated at approximately 55ºC for 2 hours (Retno et al. 2009), cooled to 45ºC, adjusted to pH 4.5, and added with glucoamylase enzyme for one hour (Mucaramah 2012).

Measurement of reducing sugar level

After hydrolyzed, the reducing sugar concentration was measured based on the DNS method. Glucose solution was used as the standard solution (Miller 1959).

Fermentation process

The hydrolyzed banana tuber pulp was combined with 7.5, 10, and 12.5 mg of baker's yeast (Jayanti 2011). The Erlenmeyer was then sealed with a cork stopper and left at room temperature (28-30ºC) for three days. The ethanol concentration was determined following incubation.

Ethanol level measurement

We created a distillation tube and a 250 mL volumetric flask. Then, 50 mL of liquid sample from fermented banana tuber starch mixed with 100 mL of distilled water was combined with 100 mL of distilled water and distilled until 50 mL of distillate was obtained.

The pycnometer is calibrated during distillation. The pycnometer was filled and closed with distilled water. Weighing the pycnometer and distilled water yielded the value C. After emptying the pycnometer, it was dried in an oven. Weighing the dry pycnometer yielded the value B. We estimated the weight of distilled water (W) using the C-B method.

Transferring the distillate to a dry beaker was performed. It was agitated to ensure homogeneity before filling the pycnometer with the distillate. The dry pycnometer is filled with distillate and then dried and weighed. The obtained results yielded the value A.

A-B = L is the weight of the distillate. The distillate's weight (L) is determined by its "specific gravity," or spg = L/W. The ethanol percentage was estimated after determining the spg value using the AOAC (Association of Official Analytical Chemists) chart (Horwits and Franklin 1975).

Data analysis

The analysis was conducted using the statistical technique known as analysis of variance (ANOVA). However, there was a significant difference in this research. Thus, it was continued with the DMRT test at a 5% significance level.

RESULTS AND DISCUSSION

Hydrolysis of banana tuber starch

Hydrolysis of starch is when starch molecules are broken down into simpler constituents, such as dextrin, isomaltose, maltose, and glucose (Rindit et al. 1998 in Purba 2009). According to Musanif (2008), starch hydrolysis is when starch polymer chains are broken down into dextrose or monosaccharide units, specifically glucose ($C_6H1_2O_6$). The enzymatic approach is used to hydrolyze banana tuber starch because enzyme hydrolysis results in a higher conversion rate than acid hydrolysis. Additionally, because the nature of the enzyme catalyst is so specialized, it can preserve the flavor and aroma of the basic ingredients (Winarno 1995).

The hydrolysis of banana tuber starch occurs in three stages: gelatinization, liquefaction by the α-amylase enzyme, and saccharification by the glucoamylase enzyme. The results indicated that the proper ratio of α-amylase and glucoamylase enzymes would result in a high concentration of reducing sugars (Table 1). The function of the α-amylase enzyme in hydrolyzing the α-1,4 glycosidic bond during the liquefaction process, followed by hydrolysis with the glucoamylase enzyme, will cut the α-1,4 glycosidic bond and the α-1,6 glycosidic bond at a lower frequency, allowing the hydrolysis process on starch to run more efficiently. The reducing sugar concentration (y= 0.138x+0.003) was determined using a standard curve (Figure 1). The absorbance value obtained is placed in the formula y= 0.138x+0.003, where x denotes the amount of reducing sugar (appendix 2 p. 44). The maximum reducing sugar levels were obtained by hydrolyzing with 75% α-amylase and 25% glucoamylase enzymes (26.17 mg/mL), while the lowest reducing sugar levels were obtained by hydrolyzing with 100 % glucoamylase (15.067 mg/mL).

In the hydrolysis reaction of banana tuber starch, the inclusion of α-amylase and glucoamylase enzymes acts as a catalyst. With the presence of this enzyme, the reaction will be accelerated, resulting in the production of more products. Reducing sugar content can be generated optimally with a precise hydrolysis procedure and an optimal enzyme dosage. According to Aransiola (2006), increasing the concentration of the enzyme accelerates the enzymatic reaction. To a certain extent, the rate of the enzymatic reaction is directly proportional to the enzyme concentration, indicating that the reaction is in equilibrium. Increases in enzyme concentration had no effect at equilibrium.

Additionally, the inclusion of enzymes can enhance the amount of glucose converted. This rise was attributable to the chance of starch coming into contact with enzymes, increasing proportion to increased starch concentration. It is consistent with the general rule that the rate of an enzymatic reaction increases proportionally to the concentration of the enzyme catalyzing the reaction (Sukandar et al., 2010). It is also supported by Yunianta et al. (2010); the higher the rate of enzyme reaction, the more starch is hydrolyzed, but the enzyme reaction rate decreases when practically all starch is hydrolyzed.

ANOVA analysis revealed a significant difference ($P<0.05$) between the ratio of enzyme concentrations to lowering sugar production. This data demonstrates that the ratio of the enzyme's concentrations considerably affects the amount of reducing sugar generated. The enzymes α-amylase and glucoamylase function as biocatalysts to accelerate the rate of the banana tuber starch hydrolysis reaction.

Without the addition of enzymes (control), hydrolysis produced a reducing sugar concentration of 8.14 mg/mL. It could be because water can also hydrolyze starch into glucose when heated to high temperatures. Additionally, the sterilization procedure at elevated temperatures and pressures will alter the raw components, one of which is starch. When water is added to the starch particles, they swell and lose their link cohesion; specifically, some amylose diffuses out under heat, allowing the starch to be hydrolyzed to release glucose (Winarno 2002).

Table 1. Reducing sugar levels at different enzyme concentrations

Comparison of the concentration of α-amylase and glucoamylase enzymes (0.20 g/500 mL)	**Reducing sugar concentration (mg/mL)**	**Reducing sugar level** (%)
A0	08.14[a]	0.81
A1	20.94[e]	2.09
A2	26.17[f]	2.61
A3	19.40[d]	1.94
A4	17.68[c]	1.77
A5	15.06[b]	1.56

Note: a-f: numbers followed by different letters indicate significant differences in the 5% DMRT test for the addition of α-amylase and glucoamylase enzymes. A0=control; A1=100% α-amylase; A2=75% α-amylase:25% glucoamylase; A3=50% α-amylase:50% glucoamylase; A4=25% α-amylase:75% glucoamylase; A5 = 100% glucoamylase.

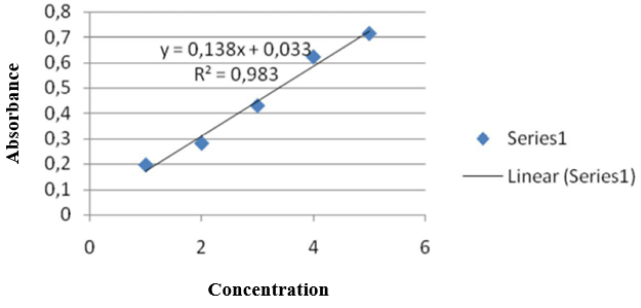

Figure 1. Glucose Standard Curve 10 mg anhydrous glucose/100 mL aquadest

Ethanol content fermented using baker's yeast

Ethanol fermentation is a biological process that converts carbohydrates like glucose, fructose, and sucrose into cellular energy and metabolic waste products like ethanol and carbon dioxide. Fermentation's fundamental premise is to stimulate the activity of microbes to alter the character of raw materials to produce a product. For example, the sugar produced by the hydrolysis of banana tuber starch is transformed into ethanol by *S. cerevisiae* present in baker's yeast. Zimase invertase is produced by *S. cerevisiae*. The invertase enzyme breaks down the remaining polysaccharides (starch) into monosaccharides (glucose) during hydrolysis. In contrast, the zymase enzyme further transforms monosaccharides into alcohol during fermentation (Stark in Underkofler and Hickey 1954). The higher the concentration of dissolved sugar, the more alcohol is produced, as more sugar must be converted to ethanol by yeast (Judoamidjojo et al. 1992).

Three days of ethanol fermentation were used in this study. According to Kusmiyati's (2010) research, the microorganism (*S. cerevisiae*) was most active or in the logarithmic phase at 72 hours. The logarithmic phase is the phase during which the biggest ethanol product is formed. After 72 hours, the bacteria enter a stationary phase. The number of growing microorganisms equals the number of dead microorganisms, ensuring that no more microorganisms convert the substrate to ethanol, so the amount of ethanol created tends to be constant. After a stationary phase, the bacterium enters the death phase. The ethanol produced at 24 and 48 hours was suboptimal due to *S. cerevisiae* being in the lag and exponential phases. The lag phase is when microorganisms adjust to their environment, while the exponential phase is when they begin to increase. As a result, the activity for the synthesis of ethanol products is suboptimal. According to Azizah et al. (2012), *S. cerevisiae* can produce up to 2% alcohol after 72 hours of fermentation.

After fermentation, the fermentation result is distilled to separate the ethanol from other components. Distillation is evaporating and condensing a vapor at a specified pressure and temperature. Distillation is used to purify liquids at their boiling points. It can separate liquids from solids or combinations with different boiling points. The separation will begin with the components with the lowest boiling points. Pure ethanol has a boiling point of 78°C, while water has a boiling point of 100°C under typical conditions. Distillation at a temperature of 70°C because Sari et al. (2008) state that running the distillation process at a temperature of 70-80°C causes the majority of the ethanol to evaporate and pass through the condensation unit, resulting in the production of ethanol.

The results indicated that the hydrolysis treatment with 75% α-amylase enzyme, 25% glucoamylase enzyme, and 12.5 mg baker's yeast resulted in the highest ethanol content of 7.98% (Table 2). Conversely, the hydrolysis treatment with 100 % glucoamylase enzyme and 5 mg baker's yeast resulted in the lowest ethanol content of 2.7%.

The higher the enzyme concentration added, the more sugar is generated, which results in a larger ethanol content produced by yeast fermentation. Roukas (1996) asserts that the amount of ethanol produced is highly dependent on the amount of available substrate. Yeast uses reducing sugar to grow, and during metabolism, it produces ethanol.

According to the statistical analysis (Appendix 4, p. 46), there is a substantial difference between the ratios of the enzymes α-amylase, glucoamylase, and baker's yeast and the ethanol concentration. It demonstrates that the concentration ratios of the amylase and glucoamylase enzymes and baker's yeast substantially affect the sugar content of the ethanol generated. The greatest ethanol level is 7.98% when added by 75% α-amylase enzyme, 25% glucoamylase enzyme, and 12.5 milligrams of baker's yeast. Each addition of enzymes during hydrolysis alters the concentrations of reducing sugars utilized as substrates in ethanol fermentation, whereas baker's yeast ferments glucose to ethanol. Therefore, the more reducing sugars present, the more ethanol is created.

The addition of baker's yeast affects the ethanol output. Adding baker's yeast to the ideal limit increases ethanol production because the more yeast present, the more microorganisms capable of converting glucose to ethanol. Admianta et al. (2001) reported that the higher the dose of yeast *S. cerevisiae* used, the higher the bioethanol content. It is because yeast is the primary generator of bioethanol. However, the amount of yeast given must be appropriate, as a tiny amount of yeast used to convert glucose to ethanol will diminish the yeast's ability to ferment. Similarly, excessive yeast use will hinder the fermentation process, resulting in a delayed growth phase (Sari et al. 2008).

Table 2. The ethanol content of fermented banana tuber starch using baker's yeast

baker's yeast (mg)	Comparison of enzymes administration of α-amylase and glucoamylase (g/500 mL)					
	A0	**A1**	**A2**	**A3**	**A4**	**A5**
B1	$0,40^{a}$	$3,70^{de}$	$6,57^{h}$	$3,12^{bc}$	$2,91^{bc}$	$2,70^{b}$
B2	$0,53^{a}$	$4,91^{f}$	$7,27^{i}$	$3,33^{cd}$	$3,19^{bcd}$	$3,12^{bc}$
B3	$0,73^{a}$	$5,58^{g}$	$7,98^{j}$	$3,94^{e}$	$3,40^{cd}$	$3,33^{cd}$

note: a-j: numbers followed by different letters showed significant differences in the 5% DMRT test for the addition of α-amylase and glucoamylase enzymes with baker's yeast to the ethanol content (α=0.05). A0=control; A1=100% α-amylase; A2=75% α-amylase: 25% glucoamylase; A3=50% α-amylase:50% glucoamylase; A4=25% α-amylase:75% glucoamylase; A5= 100% glucoamylase and B1= 5 mg baker's yeast; B2= 7.5 mg baker's yeast; B3 = 12.5 mg of baker's yeast

The maximum level of ethanol generated by banana tuber starch substrate was 7.98% in this investigation. This ethanol level is consistent with the percentage range determined by Wijaya et al. (2012), which indicates that the ethanol content of products created through fermentation ranges between 3 and 10%, depending on the type of product fermented. Therefore, around 8% to 12% bioethanol will render yeast inactive due to an overabundance of ethanol, which is harmful to yeast (Retno et al. 2009).

The lower the overall sugar concentration, the less substrate *S. cerevisiae* can consume. The amount of accessible simple sugars are very limited at low concentrations. Simple carbohydrates such as glucose and fructose are critical throughout *S. cerevisiae*'s early development phases. However, if the substrate concentration is too high, the fermentation period will be prolonged, and more sugar will be left unused (Sari et al. 2008).

According to Schlegel (1994), ethanol, or ethyl alcohol in industry, can be used as a fuel, a solvent, a medicine, a detergent, an oil, a candle, or gasohol. In addition, it is possible to increase the alcohol concentration of alcohol by distilling it in stages.

The cost of producing bioethanol from banana tuber at a concentration of 7.98% in 1L is Rp. 52,000.00 (Appendix 6, p. 49), whereas the cost of unsubsidized gasoline and diesel is Rp. 8,400.00. Depending on the purity of the bioethanol, it can be used in place of fuel oil. Bioethanol with a concentration of 95-99% can be utilized as a premium material (gasoline), whereas bioethanol with a concentration of 40% can be used in place of kerosene.

This study indicates that a 75% α-amylase ratio to 25% glucoamylase produces the highest reducing sugar content of 26.17 mg/mL during the banana tuber hydrolysis process. A ratio of 75% α-amylase to 25% glucoamylase produces the highest ethanol content of 7.98% compared to a ratio of 75% α-amylase to 12.5 mg baker's yeast.

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