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Ethanol Production from Algae Spirogyra with Fermentation by *Zymomonas mobilis* and *Saccharomyces cerevisiae*

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ABSTRACT

This study investigated bioethanol production by means of fermentation using *Zymomonas mobilis* and *Saccharomyces cerevisiae*. The algae Spirogyra was applied for bioethanol production with the addition of the α-amylase enzyme for various concentrations. Results for 96 hours fermentation process revealed that *Z. mobilis* produced more bioethanol and faster than *S. cerevisiae*.

KEY WORDS: algae Spirogyra, ethanol, α-amylase enzyme.

INTRODUCTION

Indonesia has many sources of freshwater such as rivers and lakes, where there are many kinds of aquatic life such as algae. Algae have chlorophyll and consist of one or more cells and form colonies. Algae contain organic materials such as polysaccharides, hormones, vitamins, minerals and bioactive compounds. One of the genus namely Spirogyra is that according to Becker (2006) has a carbohydrate content of up to 64%, or twice the carbohydrate content of cassava. Genus Spirogyra is a group of green algae in the order Zygnematales. Spirogyra has eukaryotic cells and consists of cells that form long strands like yarn. Each cell has a spiral-shaped chloroplast ribbon with the cell nucleus. Vegetative propagation and generative development are each lasting a fragmentation and conjugation (Tze, 1998), which reproduce very fast.

So far, the use of algae as a commodity trading or industrial raw materials is still small compared with the diversity of algae species found in Indonesia. While the chemical components contained in the algae is very useful for raw material for bioethanol. Theoretically, bioethanol production from algae can be a solution for alternative energy sources. Therefore, research is needed to obtain the potential of bioethanol from Spyrogira by means of fermentation process. Fermentation is the decomposition of organic compounds into simpler compounds with the help of microorganisms that produce energy (Hogg, 2005). Most commercial-scale ethanol fermentation is by yeast, one of S. cerevisiae that produced ethanol (Hutkins, 2006). *S. cerevisiae* is also known as baker yeast or Brewer yeast that is able to change nearly 90% of glucose to ethanol (Elvri, 2006). *S.cerevisiae* can use glucose,fructose, maltose, and maltotriosa (Hutkins, 2006). Meanwhile, to change the organic compounds into simpler substances that can be used in the fermentation process required enzymes. Enzymes are molecules composed of a series of biopolymers of amino acids in the composition and structure of a regular chain and fixed. Enzymes has important role in transforming complex carbohydrates are amylase and cellulase.

Starch is a substance that must first be converted into simpler molecules that can be absorbed by the cells. Amylase has the ability to break down the molecules of starchand glycogen. Molecular starch which is a polymer of 1.4 bond -α-glycosides will be split by the enzyme α-amylase on α -1.4 bond yield glucose, maltose, and dextrin (Manoj, 2005). Ethanol fermentation can be accelerated by the addition of α-amylase enzyme in the right amount.

Therefore, this study aimed to determine the amount of α-amylase enzyme effective in the fermentation of carbohydrate extract of Spirogyra into ethanol and comparing the levels of ethanol produced from fermentation of Spirogyra by *Z. mobilis* and *S. cerevisiae*.

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MATERIALS AND METHODS

Extracts of Spirogyra

Spirogyra samples washed with water to clean the dirt, then dried for three days in the sun. Spirogyra which had been dried weighed and added aquadest with compared to aquadest in the propotion of Spirogyra (5: 1), mashed with a blender, put into 250 ml erlenmeyer (Zhang *et al*., 2010). Spirogyra extract sterilized in autoclave 1,5 atm 121°C. Further Spirogyra extracts will be used to process the growth curve of *S. cerevisiae* and *Z. mobilis*, and substrate of fermentation.

Stock Cultures and Work Culture

S. cerevisiae was cultured in test tubes containing Sabouraud Dextrosa medium to tilt and incubated at 30° C for 24 hours. *Z. mobilis* was grown at 30°C for 24 h by regularly streaking on the agar plates of the rich medium (RM) containing (per liter): glucose, 20 g; yeast extract, 10 g; (NH4)2SO4, 1 g; K2HPO4, 1 g; MgSO4·7H2O, 0.5 g (Struch *et al*., 1991).

Measurement of Growth

Z. mobilis was inoculated into a 50 ml erlenmeyer containing 5 ml of sterile extract of Spirogyra which had governed the pH to 4 by adding 30% HCl solution (Zhang *et al*., 2010). Then it was incubated in a rotary shaker with agitation speed of 15 rpm at a temperature of 30 °C for 24 hours (Activation I). A total of 1 ml of activation inoculated again into 50 ml erlenmeyer containing 9 ml of Spirogyra extract, incubated in a rotary shaker with agitation speed of 15 rpm at a temperature of 30 °C for 24 hours (Activation II). A total of 5 ml of activation inoculated again into 100 ml erlenmeyer containing 50 ml of extract Spirogyra, were incubated in rotary shaker with agitation speed of 15 rpm at a temperature of 30 ° C for 24 hours is referred to as fermentation cultures (Cazetta *et al*., 2007; Zhang *et al*., 2010).

Performed dilutions were from 10-1 to 10-9. One ml of culture medium were taken and put into a test tube containing 9 ml of sterile distilled water. Test tube contains the mixture with a vortex mixer, pipet as much as 1 ml and put into a test tube next. The treatment is repeated until retailing to 10-9. The curve of growth was made by measuring the absorbance of cultures of *Z. mobilis* on Spirogyra extract.

Z. mobilis was measured at a wavelength of 600 nm at intervals of once every one hour during 24 hours. Graph the growth curve of absorbance values and the fermentation time (Obire, 2005). The same step done for *S. cerevisiae*.

Starter Preparation of Z.mobilis

Z. mobilis was inoculated into 50 ml erlenmeyer containing 5 ml of sterile Spirogyra extract that has been set pH to 4 by adding 30% HCl solution, then incubated in a rotary shaker with agitation speed of 15 rpm at 30 °C for 24 hours (Activation I). A total of 1 ml of reactivation I and inoculated into 50 ml erlenmeyer containing 9 ml of Spirogyra extract, incubated in a rotary shaker with agitation speed of 15 rpm at a temperature of 30 ° C for 24 hours (Activation II). A total of 5 ml of re-activation II and inoculated into 100 ml erlenmeyer containing 50 ml of extract of Spirogyra, were incubated in rotary shaker with agitation speed of 15 rpm at a temperature of 30 °C and incubated until the hour in which log phase of *Z. mobilis* occur (in accordance with the growth curve) (Activation III) (Cazetta *et al*., 2007; Zhang *et al*., 2010).

Starter Preparation of *S.cerevisiae*

S. cerevisiae was inoculated into 50 ml erlenmeyer containing 5 ml of sterile Spirogyra extract that has been set pH to 4.5 by adding 30% HCl solution (Elevri, 2006), then incubated in a rotary shaker with agitation speed of 15 rpm at 30 ° C for 24 hours (Activation I). A total of 1 ml of reactivation I and inoculated into 50 ml erlenmeyer containing 9 ml of Spirogyra extract, incubated in a rotary shaker with agitation speed of 15 rpm at a temperature of 30 ° C for 24 hours (Activation II). A total of 5 ml of re-activation II and inoculated into 100 ml erlenmeyer containing 50 ml of extract of Spirogyra, were incubated in rotary shaker with agitation speed of 15 rpm at a temperature of 30 ° C and incubated until the hour in which log phase of *S.cerevisiae* occur (in accordance with the growth curve) (Activation III) (Wignyanto, 2001).

Hydrolysis Process

Spirogyra extract as much as 50 ml incorporated into the erlenmeyer. Erlenmeyer was heated on a hot plate for 2 hours in with the temperature 100°C, stirring occasionally open funnel-flops (Mosier *et al*., 2006). Cool for two hours until the temperature reaches ± 40 °C. The addition of the α-amylase enzyme with each concentration of 0 grams/50 ml; 0,03 grams/50 ml; 0.06 grams/50 ml; and 0.09 grams/50 ml. Incubated at room temperature for 60 minutes (Bascar *et al*., 2008; Sulfahri *et al*., 2010).

The Fermentation Process and Measurement of Ethanol Concentration

Starter added into 250 ml erlenmeyer containing Spirogyra extract, incubated for 120 hours at room temperature. If still experiencing increased levels of ethanol, the fermentation was continued. The fermentation process was stopped if

ethanol levels have been reduced. Sample measured by picnometer at 20°C. Ethanol Concentration Measured by using specific gravity method.

Data Analysis

Data were analyzed with analysis of variance (ANOVA) to determine the effect of different addition amount of α-amylase enzyme to ethanol content. If H1 is accepted, followed by Tukey test at the level of 95% (α = 0.05) to find out the real difference between the combination treatment of α-amylase enzyme concentration and length of fermentation.

RESULTS AND DISCUSSION

Age determination starter Z. mobilis and S.cerevisiae in fermentation medium

Every microorganism has a specific shape growth curve. It is also seen in Z.mobilis growth curve in Figure 1.

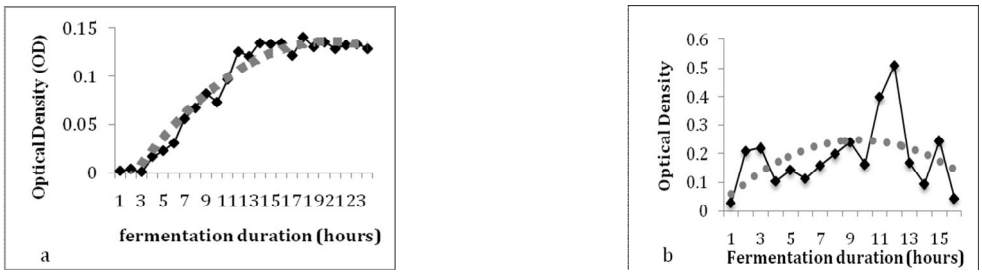

Figure 1. Growth curves of (a) *Z. mobilis* (b) *S.cerevisiae*

A growth curve gives an overview of the environmental factors that affect the growth of a microorganism such as substrate, ambient temperature, pH, and determine the age of starter (Hogg, 2005). Age is a good starter for use as inoculum fermentation medium is along the logarithmic phase, because at this phase of the cell microorganisms have the ability to divide the maximum, the rate of growth and metabolic activity constant. Generally the age of culture used were taken at mid exponential phase. Hogg (2005) explained that in the exponential phase of microorganism cells in stable condition, new cells are formed with a constant speed and optimum microorganism cell divide at the time of doubling time, which is usually achieved in the middle of logarithmic phase. Age starter used as inoculum, determined by calculating the specific growth rate (μ) and time doubling time (tg) with data based on cell number and incubation time on the growth curve (Hogg, 2005). Age starter *Z. mobilis* which is used by the growth curve that is at the 6.5, at μ = 0.592 generations per hour with a time of doubling time the fastest 70 minutes, and age starter of *S. cerevisiae* which is used by the growth curve that is at the 10.5 at μ = 0.887 generations per hour with a time of doubling time the fastest 47 minutes.

Ethanol Fermentation

Based on the results, ethanol content increased in accordance with the addition of α-amylase enzyme concentration and long fermentation time. In general, the results tend to increase fermentation. More and more the addition of α-amylase enzyme concentration, it tends to increase the number of levels of ethanol produced. A decline in the number of bioethanol was found on the addition of 0.09 grams of α-amylase enzyme. So the highest yield was obtained from the addition of 0.09 grams of α-amylase enzyme at 96 hours (Table 1).

Table 1. Comparison of ethanol production between *S. cervisiae* and *Z. mobilis*

α-amylase enzyme (gram)	Total Bioethanol produced (%) on time					
	0 Hours		48 Hours		96 Hours	
	Z. mobilis	*S.cerevisiae*	*Z. mobilis*	*S.cerevisiae*	*Z. mobilis*	*S.cerevisiae*
0.00	0.01	0.00	3.19	1.09	5.37	2.11
0.03	0.02	0.03	4.96	2.23	7.10	3.26
0.06	0.00	0.03	5.53	2.92	9.60	4.42
0.09	0.00	0.03	6.06	2.79	9.70	4.09

Carbohydrate is the main substrate that is broken in the process of fermentation while the substrate that can be consumed directly by *Z. mobilis* was in the form disaccharides or monosaccharide (reducing sugar) (Gandjar, 2006). Reducing sugar content of which is owned by algae Spirogyra was 10, 05% (data not shown). According to Hogg (2005), ethanol fermentation of sugar reduction will be effective when at least 10%. Thus, reducing sugar can be used as a

substrate in the fermentation process. *Z. mobilis* can utilize glucose through the glycolysis pathway that converts six-carbon atom becomes redundant three-carbon molecule is pyruvate (Madigan, 2006). Then the molecule of pyruvate is converted into ethanol in anaerobic conditions.

Ethanol from the fermentation product may be affected by the addition of α-amylase enzyme. α-amylase enzyme will cut ties α-1, 4 glycoside with the final product dextrin, maltose and glucose. Enzyme reaction velocity is directly proportional to enzyme concentration, the greater the amount of enzyme the faster the reaction and the more product produced α-amylase enzyme used for starch hydrolysis process that α-glycosidic bond break into glucose monomers. Excess α-amylase enzymes break the bond that is specific to the bond α-1 ,4-glucosidic to produce glucose. While the chemical hydrolysis, using sulfuric acid (H2SO4) or acid chloride (HCl) will break the starch polymer chains at random, and not necessarily to produce glucose (Manoj *et al*., 2005).

In the process of fermentation, reducing sugar is converted into pyruvic acid and pyruvic acid further converted into ethanol. Acetaldehyde acts as a recipient of hydrogen in the fermentation, where the reduction by NADH2 produce ethanol, and NAD are oxidized can then be used again to capture hydrogen (Madigan, 2006).

Analysis of Variance (One-way ANOVA) used Minitab software release 16. Output data showed that P-value = 0. This has been proved that the addition of α-amylase enzyme on the percentage of ethanol produced. Then based on multiple comparison test on treatment without giving the enzyme α-amylase (0 grams) showed significant differences by treatment with α-amylase enzyme 0.03 g, 0.06 g and 0.09 g. Means, without the addition of α-amylase enzymes of ethanol fermentation process that occurs less effective. The enzyme α-amylase hydrolyzes starch to function specifically in the 1, 4-glycoside bond into monosaccharide and disaccharides (Manoj *et al*., 2005).

The Comparison of S. cerevisiae and Z. mobilis in producing bioethanol from Algae Spirogyra

Based on table 1 both *Z. mobilis* and *S. cerevisiae* continued to increase ethanol production because at the time range was still available nutrients in the media needed by both the invader.

From the comparison of variables within 48 hours and 96 hours and the addition of α-amylase enzyme 0.00; 0.03; 0.06; 0.09 *Z. mobilis* was known that can produce more ethanol than *S. cervisiae*. But in the hour-0 *S. cervisiae* producing ethanol was higher than the *Z. mobilis*. Actually, on hour-0 has not occurred the process of fermentation by Z. mobilis and *S. cerevisiae*. The presence of ethanol on hour-0 may occur from the hydrolysis process was conducted at the time of pretreatment.

Z. mobilis highest ethanol production at 0.09 and 96 hours that was equal to 9.70 while the highest at 0.06, S. cerevisiae and the 96 hours that was equal to 4.42. At the time of 96 hours of fermentation, *Z. mobilis* produced more bioethanol than in *S. cerevisiae*. The highest ethanol production of *Z. mobilis* with addition of α-amylase enzyme at 0.09 grams, while *S. cerevisiae* to produce the highest ethanol of α-amylase enzyme as much as 0.06 grams. The results showed that *Z. mobilis* was able to survive at high concentrations compared with *S. cerevisiae*.

CONCLUSIONS

The highest ethanol production of *Z. mobilis* was 9.70 % ethanol (v/v) with addition of α-amylase enzyme at 0.09 grams for 96 hours. The highest ethanol production of *S cerevisiae* was 4.42% ethanol (v/v) with addition of α-amylase enzyme at 0.06 grams for 96 hours.

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