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Optimization of The Bioconversion of *Spirogyra Hyalina* Hydrolysates to Become Ethanol Using *Zymomonas Mobilis*

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ABSTRACT

This study aims to determine the effect of different types of gases, initial pH, and the fermentation duration to cell biomass, pH, reducing sugar concentration, and ethanol concentration that was produced from fermentation of the *Spirogyra hyalina* hydrolysates to become ethanol using *Zymomonas mobilis*. Fermentation performed under anaerobic conditions with the variations of nitrogen gas and hydrogen gas in the space fermenter. The results showed that different types of gases, pH, and the fermentation duration give an effect on the cell biomass, pH, reducing sugar concentration, and ethanol concentration that was produced from fermentation of *Spirogyra hyalina* hydrolysates using *Zymomonas mobilis*. The highest levels of ethanol and biomass achieved by *Zymomonas mobilis* in the presence of hydrogen gas. It means that the hydrogen gas that was injected into the fermenter space can act as a reducing agent for the formation of NADH. NADH in the cell metabolism of *Zymomonas mobilis* functioning for the formation of ethanol.

Keywords: *Spirogyra hyalina*, Fermentation, Ethanol, *Zymomonas mobilis*, nitrogen, hydrogen

INTRODUCTION

One of the algae with the potential to be developed as the raw material of ethanol is the algae *Spirogyra*. Fermentation of algae *Spirogyra* by using bacterium *Zymomonas mobilis* more effective in anaerobic conditions with the addition of nitrogen gas [15]. The addition of nitrogen gas is meant by passing nitrogen into the jar fermentor. It is intended to remove all the Oxygen in the bottle and replace it with nitrogen. Meanwhile, anaerobic conditioning using the Hungate technique with the addition of nitrogen gas, can also be done by using carbon dioxide and hydrogen [4].

The addition of nitrogen gas in the fermentation of *Spirogyra* can produce ethanol at 11.36% (v/v) [15]. Whereas nitrogen in the process can not act as a reducing agent. Meanwhile, the addition of hydrogen gas having a function for anaerobic conditioning, also has a function as a reducing agent for the formation of NADH. This is because reduction potential of hydrogen is at -0.4 volts vs. NHE, while the reduction potential of NADH is at -0.32 volts vs. NHE. Reduction potential difference allows the hydrogen acts as a reducing agent for the formation of NADH [10]. NADH in the cell metabolism of *Zymomonas mobilis* function in the formation of pyruvic acid and ethanol [16].

The other factors that influence to the effectiveness of the fermentation are pH and fermentation duration. The pH of the fermentation is important for microbial growth, because only certain enzymes will break down the substrate in accordance with a specific pH [6]. Therefore, pH regulation is very important in the fermentation process [7].

This study aims to determine the effect of different types of gases, initial pH, and the duration of fermentation to cell biomass, pH, reducing sugar concentration, and ethanol concentration produced from fermentation of the algae *Spirogyra hyalina* wich has been hydrolized using *Zymomonas mobilis*.

MATERIALS AND METHODS

Pretreatement and Hydrolysis Procces of *Spirgyra hyalina*

Spirgyra hyalina was collected from a pond located within the campus of Sepuluh Nopember Institute of Technology, Surabaya, Indonesia. *Spirogyra hyalina* obtained and identified under a microscope using a *Sedgewick Rafter Cell* to ensure that the algae are *Spirogyra hyalina*. *Spirogyra hyalina* which has been identified then dried in oven with a temperature of 80°C for 24 hours. *Spirogyra hyalina* which has been dried than blender until crushed and sieved to 40 mesh size sieve. *Spirogyra hyalina* which passes 40 mesh sieve was weighed as much as 62.5 grams of distilled water and added as much as 1 liter, in order to obtain water and *Spirogyra hyalina* ratio is 15 to 1, then stirred [19].

Spirogyra hyalina which has been through a pretreatment process put in Erlenmeyer and heated on a hot plate. Heating process lasts for ± 2 hours with a heating temperature of ±100°C and then cooled until the temperature reaches ±45°C [19], and α-amylase enzyme is added (Liquozyme Supra, Novozymes, Denmark) as

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much as 8.1 KNU (Kilo Novo Unit), and incubated for 80 minutes [14]. Once hydrolyzed, hydrolysates filtered using filter paper to be taken supernatant. Supernatant was then centrifuged at 9000 rpm for 15 minutes. Centrifugation the supernatant was sterilized, and will be use for the substrate of fermentation.

Starter Preparation of *Zymomonas mobilis*

Zymomonas mobilis was inoculated into 50 ml erlenmeyer containing 5 ml of sterile *Spirogyra hyalina* hydrolysates that has been set pH to 4 by adding Buffer Na-citrate, 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 activation I inoculated again into 50 ml erlenmeyer containing 9 ml of *Spirogyra hyalina* hydrolysates, 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 of activation II inoculated again into 100 ml erlenmeyer containing 50 ml of *Spirogyra hyalina* hydrolysates, 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 *Zymomonas mobilis* occur (in accordance with the growth curve) (Activation III) [5] [19].

Fermentation Proccess

Starter inoculum was added to a concentration of 10% (OD_{600nm} = 0.5) into the 100 ml bottle fermenter containing 50 ml of substrate *Spirogyra hyalina*, incubated with a variation of the fermentation duration (0 hours, 24 hours, 48 hours, and 72) at room temperature (±30°C). The fermentation process carried out in anaerobic conditions. Anaerobic condition is done by using the Hungate technique, namely by passing nitrogen gas or hydrogen gas into the fermenter. Fermenter was closed with a rubber stopper cover and then flowed gas (nitrogen or hydrogen) for 2 minutes. After that, carried to the fermentation incubation without agitation [19], with longer appropriate study design. At the time of fermentation has entered a period of incubation 0 hours, 24 hours, 48 hours, 72 hours and then made measurements of *Zymomonas mobilis* biomass, the measurement of pH value, the measurement of reducing sugar concentration, and measured of ethanol consentration [4] [15].

Measurement of *Zymomonas mobilis* Biomass

Zymomonas mobilis cell biomass measurements performed using the method of dry cell weight (DCW). Cell dry weight carried by first centrifugation of 50 ml of fermentation medium at a speed of 9000 rpm for 10 minutes. After centrifugation, the supernatant and the pellet of cells obtained. Supernatant was removed by gently aspirated using a pipette. After that, the pellet was washed by adding 0.1 M phosphate buffer in the cell pellet and centrifuged again at 9000 rpm for 5 minutes. Pellets were suspended in distilled water and then aspirated using a pipette and transferred to the paper filter with a pore size of 0.47 µm which had previously been oven until constant weight (W1). Filter paper which had contained the pellets and then dried in an oven at a temperature of 80°C for 24 hours and weighed (W2). DCW outcome is the difference between the final weight of filter paper and the initial filter paper weight (W2-W1), which is expressed by the cell dry weight with units of grams/liter (g/L).

Measurement of Total Reducing Sugar and Ethanol Concentration

Reducing sugar measurements performed using the method of Luff Schoorl [13]. Measurement of ethanol concentration is done using specific gravity method [9].

RESULTS AND DISCUSSION

***Zymomonas mobilis* biomass**

The success of ethanol fermentation can be seen from the response of microbial growth in the fermentation medium. The cell growth of *Zymomonas mobilis* measurements performed using the method of cell dry weight. Graph of *Zymomonas mobilis* biomass by presence of nitrogen gas and hydrogen gas is presented in Figure 1.

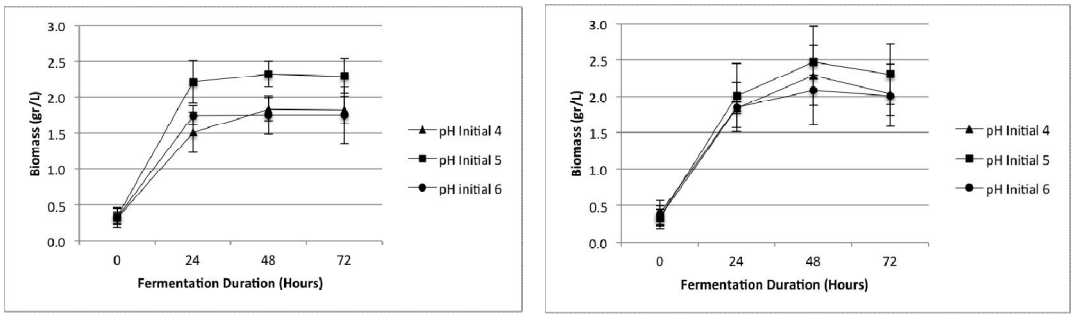

Figure 1. Graphs of *Zymomonas mobilis* Biomass by Presence of Nitrogen Gas (left) and Hydrogen Gas (right)

After 24 hours, the biomass of *Zymomonas mobilis* stagnant either on the nitrogen gas and the hydrogen gas. This is because the method of measuring the number of cells using dry cell weight technique is a method that has a weakness can not distinguish between viable cells and non viable cells, so that cells that have died will also be counted. *Zymomonas mobilis* biomass in the presence of nitrogen gas and hydrogen gas at pH 5 after 72-hour is above 2.3 g/L. Biomass was higher when compared with the cell biomass *Zymomonas mobilis* in sucrose medium (200 g/L) enriched with glucose 100 g/L, yeast extract 10 g/L, $(NH_4)_2SO_4$ 2 g/L, KH_2HPO_4 3 g/L, $MgSO_4.H_2O$ 0.3 g/L, peptone 0.5 g/L $FeSO_4$ and 0.2 g/L showed the value of *Zymomonas mobilis* biomass is only 1.65 g / L after incubation for 48 hours [3]. This suggests that although the hydrolysis medium alga *Spirogyra hyalina* is not enriched with elements of N, P, K and treace element, *Zymomonas mobilis* can still live and produce high biomass. Therefore, the medium of *Spirogyra hyalina* hydrolysates suitable for the growth of *Zymomonas mobilis*.

Microbial growth is generally accompanied by the formation of metabolic products. Metabolic products will affect to pH of the fermentation medium. The fermentation process will produce a variety of acidic substances, such as pyruvic acid and acetaldehyde are that will affect to the fermentation media [8]. Ethanol fermentation process is influenced by pH of the medium. Graph pH value of *Zymomonas mobilis* culture on the fermentation medium in the presence of nitrogen gas and hydrogen gas are presented in Figure 2.

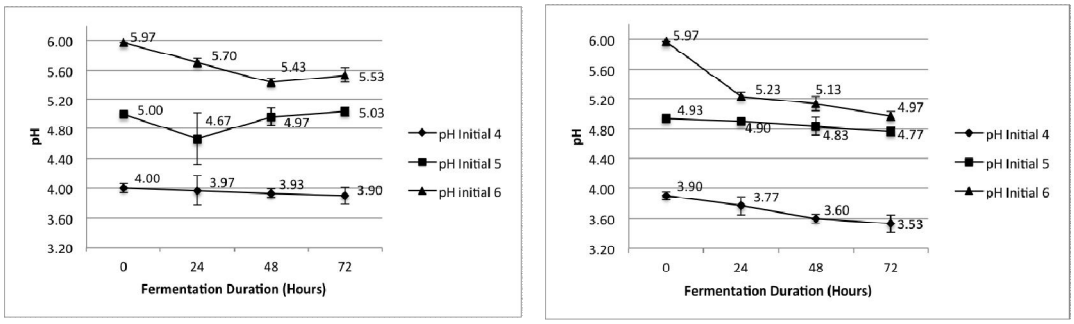

Figure 2. Graph of the culture pH value *Zymomonas mobilis* in fermentation medium with the Presence of Nitrogen Gas (left) and Hydrogen Gas (right)

Decrease in pH in the fermentation medium in the presence of nitrogen gas until the 48 hours and after that to increase. While in the fermentation medium in the presence of hydrogen gas decrease continuously until the pH up to 72 hours. Decrease in pH is an indication of the amount of organic acids formed by the activity of microorganisms [7]. In addition, the decrease in pH caused by the formation of metabolites of ethanol and other products such as organic acids [17].

Reducing Sugar Concentration

Measurement of reducing sugar concentration during the fermentation process is monitored to see the total of reducing sugar that was used by bacteria *Zymomonas mobilis*. Sugar is an important factor for *Zymomonas mobilis* as a source of energy for metabolism, so the sugar give an effect on the levels of ethanol produced. Graph of reducing sugar that was generated by *Zymomonas mobilis* in the presence of nitrogen gas and hydrogen gas are presented in Figure 3.

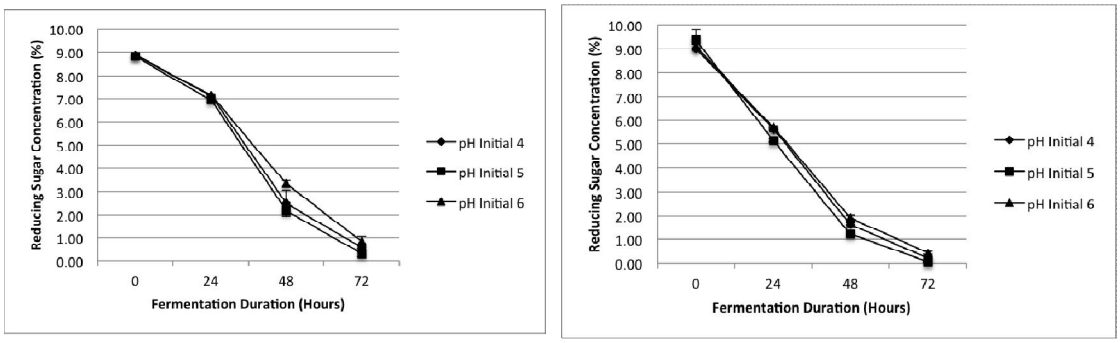

Figure 3. Graph of Reducing Sugar Concentration that was Generated by *Zymomonas mobilis* in the Presence of Nitrogen Gas (left) and Hydrogen Gas (right)

The more reducing sugar that can be utilized by microbial cells caused the higher levels of ethanol produced [18]. Based on Figure 3, the concentration of reducing sugar decrease in line with increasing of

fermentation duration. The mean reducing sugar concentration at the beginning of fermentation (0 h) is reached 9.01% and steadily decreasing with the increasing fermentation duration, where reducing sugar at the end of the fermentation time (72 hours) has expired. Bacterium *Zymomonas mobilis* can use the reducing sugar for 72 hours to convert it into ethanol and carbon dioxide.

Etanol Concentration

Measurement of ethanol concentration was conducted using *Specific Grafity Methods*. Graphs of ethanol that was produced by *Zymomonas mobilis* in the presence of nitrogen gas and hydrogen gas are presented in Figure 8.

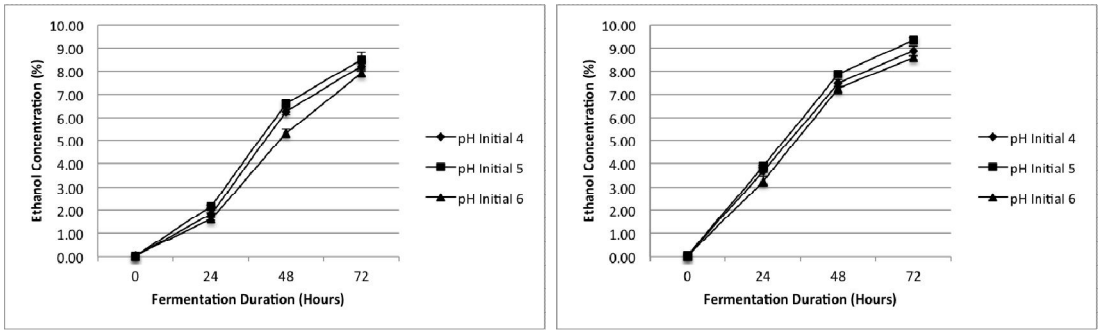

Figure 4. Graph of Ethanol Concentration that was Produced by *Z. mobilis* in the Presence of Nitrogen Gas (left) and Hydrogen Gas (right).

Based on the ethanol concentration that was produced in the presence of nitrogen gas and hydrogen gas showed that the pH 5 give the best results and are significantly different from pH 4 and 6. Ethanol concentration increased with increasing of fermentation duration. The highest ethanol concentration achieved at the 72 hours that reached 9.35% (v/v). Based on the Tukey test at the 95% confidence interval, the most optimum treatment in producing ethanol was produced at pH 5, the type of hydrogen gas and 72 hour of fermentation. Therefore, the initial pH of the medium influence on levels of ethanol produced. The pH of the fermentation medium is important for the growth of microorganism fermentation offender, because of certain enzymes will break down the substrate only in accordance with a specific pH [8]. The initial pH of fermentation medium affects the levels of ethanol produced. The proton give an affect the performance of the enzymes in the *Entner-Doudoroff pathway* and the *Emden Mayerhoff pathway* [12]. Therefore, pH regulation is very important in the fermentation process.

DISCUSSION

The results showed that average levels of ethanol produced in the presence of hydrogen gas tends to be higher when compared with the presence of nitrogen gas. The highest ethanol concentration in the presence of hydrogen gas is 9.35%. While the highest levels of ethanol in the presence of nitrogen gas only 8.47%. High levels of ethanol in the presence of hydrogen gas is accompanied by the use of sugar is also high. Conversion of reducing sugar in the presence of hydrogen gas is higher than nitrogen.

Higher ethanol yield accompanied with the use of a higher reduction of sugar in the presence of hydrogen gas when compared to nitrogen gas showed that hydrogen gas is likely to act as a reducing agent for the formation of NADH. This is because reduction potentials of hydrogen gas equal to -0.4 volts vs. NHE. While the reduction potential of NADH is at -0.32 volts vs. NHE. Reduction potential difference allows the hydrogen gas acts as a reducing agent for the formation of NADH [10]. NADH in the cell metabolism of *Zymomonas mobilis* has a function in the formation of pyruvic acid and ethanol [16]. Therefore, the hydrogen which act as a reducing agent for the NADH formation can increase levels of ethanol, while the nitrogen is not acting as a reducing agent can not increase the ethanol content. This causes the levels of ethanol produced by the existence of hydrogen gas is higher than nitrogen gas.

This premise is supported by the pH value did not decrease significantly during 72 hours with the addition of nitrogen gas. However, pH decreased significantly during 72 hours with the addition of hydrogen gas, where in the initial pH 6 decreased to 5.07, the initial pH 5 decreased to 4.57, and initial pH 4 decreased to 3.33. If the more levels of ethanol produced, the pyruvic acid produced is also increasing. Pyruvic acid is an acidic substance that can be decreasing the pH, so that the presence of hydrogen gas was decreasing pH more drastically than the presence of nitrogen gas.

Fermentation kinetics parameters calculated in this study on the fermentation of the best treatment results in *Zymomonas mobilis* inoculum that is with hydrogen gas, pH 5 and 72 hours fermentation duration. The μ max value of *Zymomonas mobilis* was 0.0739 generation/hours, so that the resulting double time 9.37 hours. *Zymomonas mobilis* meaning takes 9.37 hour to multiply two times the original amount. Value of Yp/x, Yp/s, Y/s is a very important parameter in the fermentation process. This value is useful for determining the amount of substrate required to produce a certain amount of product. Kinetic information is used to improve the efficiency of fermentation. These results yield the value of Yp/x = 10.234 grams product/gram of biomass, Yp/s = 1.9483 grams product/grams substrate, Y/s = 0.0602 grams biomass/grams substrate.

The value of *Zymomonas mobilis* kinetics in the *Spirogyra hyalina* hydrolysates are convert when compared with ethanol fermentation by using a substrate of Mahula flowers (*Madhuca latifolia*) by *Zymomonas mobilis* MTCC 92 with 96 hours of fermentation duration yield Yp/s 0.473 g product/g substrate, and Y/s 0.033 g biomass/g substrate [2]. Based on these studies can be seen that the kinetics of fermentation using *Zymomonas mobilis* with a pure culture with the substrate of *Spirogyra hyalina* that has been hydrolyzed shows the kinetics of a higher value. This is because the substrates that was used in this study is *Spirogyra hyalina* that has been hydrolyzed were detected as reducing sugar, sucrose which is not included in the reduction of sugar. All monosaccharide (glucose, fructose, galactose) and disaccharides (lactose, maltose), except for sucrose and starch (polysaccharides), including as a reducing sugar [11]. *Zymomonas mobilis* is a anaerobic facultative bacteria and is a Fermentative bacteria that utilize sucrose, glucose and fructose by the *Entner Duondoroff Pathway* following the path to produce ethanol [19]. Therefore, the possibility of substrate used is higher because there is a disaccharide sugar (sucrose) is not detected as a reducing sugar and does not enter into the calculation of Yp/s. The higher the substrate, the lower the Yp/s, so it should be in this study, Yp s lower. The species of *Spirogyra hyalina* contained disaccharide sucrose, and polysaccharides that Xylosmacin [1].

CONCLUSIONS

The highest ethanol concentraion and biomass achieved by *Zymomonas mobilis* at pH 5 in the presence hydrogen gas with 72 hours of fermentation duration. This suggests that the hydrogen gas is injected into the fermenter space can act as a reducing agent for the formation of NADH. NADH in the cell metabolism of *Zymomonas mobilis* has a function in the formation of ethanol.

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