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Bioethanol production from algae *Spirogyra peipingensis* using *Saccharomyces cerevisiae*, *Pichia kudriavzevii* and *Kluyveromyces thermotolerans*

Sulfahri¹, D R Husain¹, Kasbawati¹, A C M Tassakka², Nurfadilah¹, D P Wulandari¹ and W L Taufan¹

¹Faculty of Mathematics and Natural Sciences, Hasanuddin University, Makassar, Indonesia

²Faculty of Marine Science and Fisheries, Hasanuddin University, Makassar, Indonesia

Email: sulfahri@unhas.ac.id

Abstract. *Spirogyra peipingensis* algae is a microscopic green algae which is one of an alternative raw material to producing bioethanol because it has a high carbohydrate about 64%. This study aims to discover the growth of various types of yeast in *S. peipingensis* algae medium in producing bioethanol. This study uses hydrolysis methods and fermentation methods. In this study, the hydrolysis method using α -amylase enzyme as much as 150 KNU/L. The type of yeast used was *Saccharomyces cerevisiae*, *Pichia kudriavzevii* and *Kluyveromyces thermotolerans*. The result shown that the optimal yeast growth in producing ethanol is *S. cerevisiae* was 0,49 g ethanol/ g sugar at 10 hours fermentation duration. Based in the result shown that *S. peipingensis* algae can be used as raw material for producing bioethanol by using *Saccharomyces cerevisiae*.

1. Introduction

Global warming and climate change are issues that are currently being discussed at this time. One of the causes of global warming and climate change is air pollution caused by transportation and industry [1]. The development of land transportation such as cars and motorbikes using petroleum fuels accounts for extraordinary air pollution [2-4].

Air pollution mainly comes from NO₂, which is 80-92% produced by motorized vehicles. The high average concentration of NO₂ has a tendency that is directly proportional with motorized vehicles quantity, industries and households. Therefore, efforts are needed to overcome this problem to replace the fuel with environmentally friendly raw materials, such as bioethanol [5-7].

In addition to air pollution issue, another one is the increasing of fuel damaging. Therefore, it is necessary to do intensive diversification of fuel. One of the diversification efforts is to produce alternative fuels that can be renewed, such as bioethanol from vegetable oils, microalgae, or seaweed where the basic ingredients are widely available in Indonesia and have not been utilized efficiently.

Bioethanol production from algae is economically profitable and environmentally friendly. Algae is one of the potential raw materials to producing bioethanol which can be applied [8-9]. One of the potential algae is *Spirogyra*. The carbohydrate content of *Spirogyra* algae can reach 64% [10-12].

Spirogyra algae can be made into bioethanol by two stages process. They are hydrolysis process and the fermentation process. The fermentation process is influenced by several factors, including nutrition and duration of fermentation. Nutrition is a very important factor for the microbial agents of fermentation, besides requiring a carbon source. It also requires a source of nitrogen, phosphorus, potassium and mineral elements. Nutrition is available on fermentation medium which functions for the growth and division of microbial fermentation of ethanol [13-14].

S. cerevisiae is a type of yeast commonly used in bioethanol fermentation. *S. cerevisiae* used in bioethanol production industry has several advantages, they are high bioethanol production, high tolerance for ethanol and high inhibitor tolerance, but cannot utilize xylose, which is the dominant pentose sugar in lignocellulose biomass hydrolyzate for growth and bioethanol production [15].

On the other hand *K. thermotolerant* has a faster fermentation time and higher ethanol production than *S. cerevisiae*. Besides, *P. kudriavzevii* is known to be able to live in extreme conditions compared to *S. cerevisiae*, and can also be used as a mixed culture starter with other microbes which are tolerant to ethanol [16]. The success of ethanol fermentation is determined by the microorganisms involved in fermentation [17]. Based on this description, it is necessary to do research on fermentation of *S. peipingensis* algae into bioethanol by using the *Saccharomyces cerevisiae* InaCC Y655, *Pichia kudriavzevii* InaCC Y18, *Kluyveromyces thermotolerans* InaCC Y102.

2. Materials and Method

2.1. Algae and Yeast Strains

Algae *S. peipingensis* obtained from Tanjanea wetland, Makassar at LS coordinates: 05 ° 12'65,229 "and BT: 119 ° 49'18,695". Yeast *Saccharomyces cerevisiae* InaCC Y655, *Pichia kudriavzevii* InaCC Y18, *Kluyveromyces thermotolerant* InaCC Y102 was obtained from LIPI (Indonesian Institute of Sciences).

2.2. Algae *S. peipingensis* pretreatment

Algae *S. peipingensis* which was obtained was then identified under a microscope using the *Sedgewick Rafter Cell* to ensure that the algae obtained were *S. peipingensis* algae. The identified *Spirogyra* algae then dried with use the oven with temperature of 80°C for 24 hours. *S. peipingensis* algae the dried one is crushed with a *hammer mill* (Fomac-Miller FCT-2100) at a speed of 12.000 rpm for 5 minutes until it breaks and sifted with sieve size of 40 mesh. *S. peipingensis* algae that pass the 40 mesh sieve weighed with 100 g biomass and added distilled water up to a volume of 900 mL and then stirred [18]. *S. peipingensis* then hydrolyzed by heating, followed by the addition of the α -amylase enzyme.

2.3. Hydrolysis Process

S. peipingensis was put into the Erlenmeyer and heated on a *hot plate*. Heating is done while stirring. The heating process lasts for 2 hours with heating temperature of $\pm 100^\circ\text{C}$ then cooled to a temperature of $\pm 45^\circ\text{C}$ [18] and added α -amylase (Liquozyme Supra, Novozymes, Denmark) enzyme as much as 25 KNU (Kilo Novo Unit), and incubated for 80 minutes [19]. The filtrate was filtered by using filter paper to take the supernatant. The supernatant obtained was then centrifuged at 9.000 rpm during 15 minutes, the supernatant from the centrifugation is taken and sterilized. The supernatant then measured its total sugar level using Glucose refractometer. Supernatant which has known total sugar content will then be used as growth media. *S. cerevisiae* InaCC Y655, *P. kudriavzevii* InaCC Y18, *K. thermotolerant* InaCC Y102 and as a fermentation substrate.

2.4. Making Working Culture and Stock Culture

Isolate of *S. cerevisiae* InaCC Y655, *P. kudriavzevii* InaCC Y18, *K. thermotolerant* InaCC Y102, sub cultured in test tubes containing oblique PDA (Potato Dextrose Agar) medium then incubated at 30°C for 24 hours. One tube was used as work culture and the other one was stored as stock culture.

2.5. Measurement of Growth Curves

S. cerevisiae InaCC Y655, *P. kudriavzevii* InaCC Y18, *K. thermotolerans* InaCC Y102, taken as many as these previously has been cultured in PDA medium (Potato Dextrose Agar) slanted in the test tube and inoculated into 50 mL Erlenmeyer contain 5 mL of substrate *S. peipingensis* sterile that has been arranged pH to 5 with the addition of 0.1 M Na-citrate buffer.

Then incubated inside rotary shaker with agitation speed 15 rpm at 30°C for 24 hours (activation I). One mL activation I (10%) was pipetted and inoculated back into Erlenmeyer 50 mL that contains 9 mL of *S. peipingensis* substrate, incubated in a rotary shaker with agitation speed of 15 rpm at 30°C for 24 hours (activation II). In activation II, 5 ml (10%) (OD 600nm = 0.5) were pipetted and inoculated back into the 100 ml Erlenmeyer contain 45 mL *S. peipingensis* substrate, then incubated inside rotary shaker at agitation speed 15 rpm at 30°C for 24 hours that called as culture fermentation [18, 20]. Measurement the growth curve was done by measuring the growth of fermentation culture. *S. cerevisiae* InaCC Y655, *P. kudriavzevii* InaCC Y18, *K. thermotolerans* InaCC Y 102, which is done by measuring the absorbance (OD) of the spectrophotometer at length 600 nm waves at intervals of 3 hours for 24 hours. A graph of the growth curve is made from the absorbance value and fermentation time.

2.6. Measurement of Cell Biomass and Ethanol Levels

The measurement of Cell Biomass was carried out using the Dry Cell Weight Method [21]. The dry weight of the cell was carried out by the first centrifuging the fermentation medium sample by 50 ml at 9,000 rpm for 10 min. After centrifugation, supernatants and pellet cells are obtained. The supernatant was removed by slowly sucking using a micropipette. After that, the pellets are suspended with distilled water and vacuumed using a micropipette then transferred to a filter paper with a pore size of 0.47 mm which has been optimized to a constant weight (W1). The filters paper containing pellets are dried at the oven at 80°C for 24 hours and weighed (W2). The DCW result is the difference between the weight of the final filter paper and the weight of the initial filter paper (W2-W1) expressed by the dry weight of the cell with a gram/liter (g/L) unit.

3. Results and Discussion

3.1. The growth of yeast in the algae medium

The ethanol fermentation process is influenced by several factors, including the viability of fermented frozen microbial cells. Therefore, yeast as a fermentation agent must be optimized in the medium resulting from *S. peipingensis* algae hydrolysis. This aims to optimize the viability of microbial cells in the fermentation agent so that it is optimal for ethanol fermentation. *S. peipingensis* algae medium is not a common medium for yeast growth. Optimization of microbial growth is an important thing to do because the yeast growth response must be known in the medium to determine the period in which cell activity and conditions are optimal. Each microorganism forms a specific growth curve. The growth of *S. cerevisiae* InaCC Y655 which on the *S. peipingensis* substrate has 2 growth phases, namely the exponential phase and the stationary phase. This is because the starter medium is the same as the fermentation medium. This is supported from the study states that the lag phase does not occur if the medium starter is the same as the fermentation medium. The exponential phase is the cell phase of microorganisms in a stable state, new cells that formed at a constant rate and the microorganism cells divide optimally when doubling time is usually reached in the middle of the exponential phase [17]. Growth of *S. cerevisiae* InaCC Y655, *P. kudriavzevii* InaCC Y18, *K. thermotolerans* InaCC Y102 in the algae *S. peipingensis* medium is shown in Figure 1.

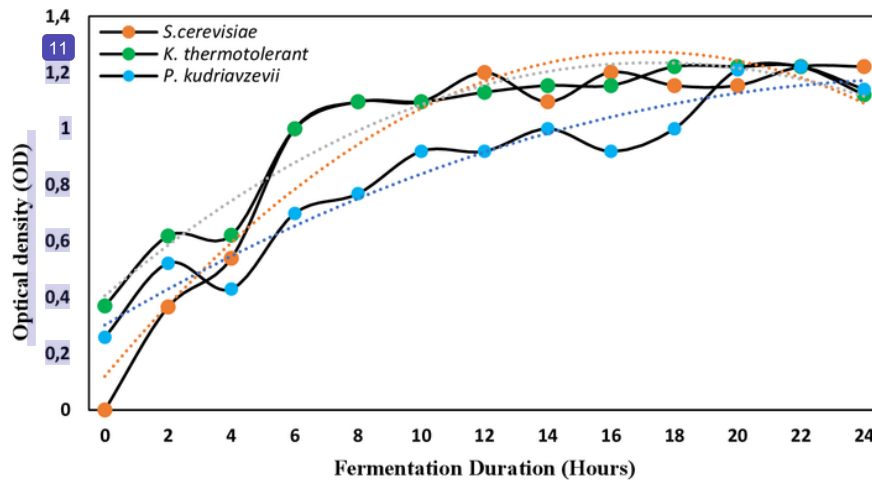


Figure 1. Growth *S. cerevisiae* InaCC Y655, *P. kudriavzevii* InaCC Y18, *K. thermotolerant* InaCC Y102 in *S. peipingensis* Algae Medium

The exponential phase is a multiplication phase of cell numbers, cell activity increases, and is an important phase in the growth of microorganism cells [22]. Log phase of *S. cerevisiae* InaCC Y655 occurs at 0 o'clock to 10 o'clock. After the exponential phase, there is a stationary phase, the phase number of cells tends to stagnate. The results of the growth curve measurements showed that the stationary phase in *S. cerevisiae* InaCC Y655 occurred at the 10th to 24th hour. Based on the calculation of generation time, age starter from *S. cerevisiae* InaCC Y655 is 5 hours. After the exponential phase, there is a stationary phase, ie the phase number of cells tends to stagnate. The measurement results of the growth curve indicate that the stationary phase in *P. kudriavzevii* InaCC Y18 occurs at the 20th to 24th hour. Based on the calculation of generation time, the age of the starter from *P. kudriavzevii* InaCC Y18 is 11 hours.

After the exponential phase, there is a stationary phase, the phase number of cells tends to stagnate. The measurement results indicate that the growth curve of the stationary phase on *K. thermotolerans* InaCC Y102 occurs at the 18th to 24th hour. Based on the calculation of generation time, age starter from *K. thermotolerans* InaCC Y102 is 9 hours. Based on the results of the research that has been obtained, it can be seen in all the above curves that the starter incubation time is 24 hours with an interval of 2 hours, and the optimum age of the starter is obtained at the 5th hour where the exponential phase is the time optimal for use as a starter because in this phase the cell *S. cerevisiae* InaCC Y655, *P. kudriavzevii* InaCC Y18, *K. thermotolerans* InaCC Y102 is actively carrying out an optimal amount of cleavage.

The results showed that the log phase in *S. cerevisiae* InaCC Y655 which is 5 hours, for *P. kudriavzevii* InaCC Y18 which is 11 hours, while *K. thermotolerans* InaCC Y102 which is 9 hours. Based on the study showed that the age of a starter that is well used as a fermentation medium inoculum is along the exponential phase, because in this phase the cells of microorganisms have the maximum ability to divide, the rate of growth and activation of the metabolism is constant [20]. Therefore, the age of culture *S. cerevisiae* InaCC Y655, *P. kudriavzevii* InaCC Y18, *K. thermotolerans* InaCC Y102 is taken in the middle of the exponential phase which is at the 16th hours.

3.2. Bioethanol Production

The results showed that different types of yeast produced different ethanol levels. *S. cerevisiae* InaCC Y655 yeast produced ethanol of 0.49 g/g at 10 hours fermentation duration. *P. kudriavzevii* InaCC Y18 yeast produced ethanol levels of 0.49 g/g at 20 hours fermentation duration. *K. thermotolerans* InaCC Y102 yeast was produced ethanol levels of 0.50 g/g at 18 hours fermentation duration. The results of ANOVA analysis at the 95% confidence interval indicate that the type of yeast does not significantly influence the ethanol content produced. This shows that the type of yeast *S. cerevisiae* InaCC Y655, *P. kudriavzevii* InaCC Y18, *K. thermotolerans* InaCC Y102 tends to produce the same ethanol level (Figure 2).

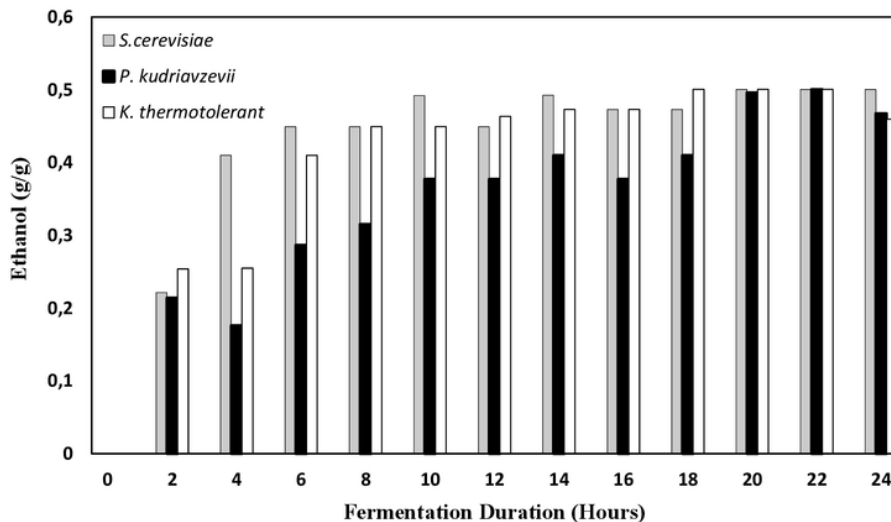


Figure 2. Ethanol production of various types of Yeast using *Spirogyra peipingensis* algae substrate

The ethanol yield obtained in this study using the *S. cerevisiae* InaCC Y655 yeast [15] is higher when compared to other studies. The results of the other study used *S. cerevisiae* produced the ethanol yield value of 0.48 g/g [13]. Whereas in the other study also used yeast *S. cerevisiae*, produced ethanol of 49.44 g/L with a yield value of 0.42 g/g, and *P. kudriavzevii* the ethanol value reached a maximum of 42.60 g/g to obtain a value Y_p/s 0.40 g/g [24].

The result of ANOVA analysis at 95% confidence interval indicate that the duration of fermentation significantly influences the ethanol content produced. *S. cerevisiae* InaCC Y655 produce the highest ethanol at 10 hours fermentation duration, *P. kudriavzevii* InaCC Y18 produce the highest ethanol at 20 hours fermentation duration and *K. thermotolerans* InaCCY102 produce the highest ethanol at 18 hours fermentation duration.

One of the potential yeast criteria in ethanol fermentation is to have a short fermentation time [25]. The result obtained in this study are *S. cerevisiae* InaCC Y655 has a shorter fermentation time (10 hours) if it compared with *P. kudriavzevii* InaCC Y18 (20 hours) and *K. thermotolerans* InaCCY102 (18 hours). Therefore, it can be concluded that *S. cerevisiae* InaCC Y655 is a potential yeast used for ethanol fermentation using *S. peipingensis* algae raw material because it has a shorter fermentation time with high ethanol levels.

4. Conclusion

The amount of enzyme used in the hydrolysis process of *Spirogyra peipingensis* algae affects the sugar content produced. The effective amount of enzyme to produce the highest sugar was 50 g/KNU with a sugar content of 0.45 g sugar/g of *S. Peipingensis* algae dry. The type of yeast used in *S. peipingensis* algae fermentation affects cell biomass and ethanol content produced. The highest and fastest ethanol content was obtained by *S. cerevisiae* with ethanol content of 0.49 g ethanol/g sugar at 10 hours fermentation duration.

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