Bioethanol production using the SSF method (*simultaneous saccharification and fermentation*) of microalgae *anabaena* sp.

N S Permatasari¹, M Zainuri², H P Kusumaningrum¹, I Mishbach², E D Hastuti¹

¹Department of Biology, Faculty of Science and Mathematics, Diponegoro University, Jl. Prof. Soedarto SH. Tembalang Semarang 50275
²Marine laboratory. Faculty of Fisheries and Marine Science, Diponegoro University, Jl. Prof. Soedarto SH. Tembalang Semarang 50275

Corresponding Author: nilasucipermatasari@gmail.com

**Abstract.** Bioethanol is one of the alternative energy sources that answer the current energy crisis problems. The advantage of bioethanol compared to the fuel oil (BBM) is biodegradable. It contains an octane and high oxygen so it is flammable and reducing global warming emissions. Recently, the main ingredient of bioethanol has entered the third generation which is using microalgae. *Anabaena* sp. is one of the microalgae types from cyanobacteria which has the advantage of being used as bioethanol because it has carbohydrate biomass of about 20-30%. This research is aimed to produce bioethanol from microalgae *Anabaena* sp. The bioethanol production method used SSF because the advantage showed the converted polysaccharides in to monosaccharides did not return to polysaccharides. It was caused monosaccharides directly fermented into ethanol. The use of one reactor reduced the cost of the process in the equipment used. This research was conducted in several stages, namely the preparation of raw materials, acid hydrolysis using H₂SO₄ 1 M, and fermentation with the help of Saccharomyces cerevisiae microorganisms anaerobically. The results showed that using the SSF method was able to produce ethanol levels of 7%.

1. **Introduction**

Transportation is one of the main sectors that utilize the most energy sources, around 42% [1]. The main material used to meet energy needs in Indonesia is fossil-based fuels, fossils are one of the energy sources that have limitations that are nonrenewable [2]. Current energy needs are still using oil ingredients and their needs continue to increase, so it is feared that it will affect the resilience and sustainability of energy sources in a sustainable manner in Indonesia.

Bioethanol is one alternative energy source that can answer the problems that are currently happening. It is estimated that in 2050 bioethanol is ranked first in the "biofuel ladder" because of its effectiveness in replacing gasoline [3]. The advantage of using ethanol is that it contains higher oxygen, which is around 35% and lower carbon monoxide emissions which are around 19-25% so it is more environmentally friendly compared to the use of BBM (fuel oil), ethanol octane number is relatively high around 129 so it produces combustion stable one. Bioethanol can reduce carbon dioxide emissions by up to 18% [4]. The main ingredient for bioethanol is now entering the third generation using microalgae. The advantages are high productivity microalgae, which do not contain lignin, does not compete with food and fast growth [5].

One alternative that can be utilized is Cyanobacteria such as *Anabaena* sp., *Spirulina* sp. has been shown to have a large amount of carbohydrate content (20-30% of dry weight) [6]. *Anabaena*
sp. is one of the microalgae that have the potential to be developed as bioethanol because of carbohydrate biomass, especially in the form of glycogen, cellulose, and exopolysaccharides are far more easily converted to monosaccharides when compared to materials containing lignocellulose. In recent years, microalgae from genera such as Anabaena, Chlamydomonas, Chlorella, Porphyrydium, Scenedesmus, Spirogyra and Spirulina, have been used for bioethanol production [7].

This study uses the SSF method, this method is considered effective because polysaccharides that are converted to monosaccharides do not return to polysaccharides, monosaccharides are directly fermented into ethanol [8]. SSF method is very important to be developed because it can shorten the process of making bioethanol.

The fermentation process is influenced by the type of microorganisms used when making bioethanol. S. cerevisiae is one of the most commonly used types of microorganisms. This yeast is tolerant of high ethanol concentrations, able to withstand high temperatures, low pH, stable during the fermentation process and able to produce high ethanol. Bioethanol production can be maximized by developing S. cerevisiae through culture optimization in bioreactors or genetic engineering [2]. This research aims to produce bioethanol from microalgae Anabaena sp. using the SSF method.

2. Material and methods

2.1. Microalgae culture

Anabaena sp. on a laboratory scale carried out indoors (indoor). Starting from the preparation of tools and materials, the provision of Anabaena sp. from InaCC LIPI in walne maintenance media on 250 ml Erlenmeyer. Aerated microalgae cultivation uses aerators with 36 watt TL lighting. Microalgae growth rate calculation is done every day, cell density calculation is done by the direct method using a microscope and hemocytometer.

2.2. Harvesting microalgae

Harvesting microalgae culture that has been completed is done utilizing centrifugation to obtain dry biomass. Centrifuge tubes containing microalgae biomass deposits are dried using an oven at 105° C then the dried biomass is weighed.

2.3. Preparation of S. cerevisiae

S. cerevisiae was grown on a PDA medium and incubated for 3 days at 25-27°C.

2.4. Making bioethanol using the SSF method

Simultaneous Saccharification and Fermentation (SSF) processes were used in this study. 5% (w/v) of cultivated yeast cells, S. cerevisiae were added in each reagent bottles containing yeast nutrients, 1 g of yeast extract, 0.4 g of potassium dihydrogen phosphate (KH₂PO₄) and 0.2 g of ammonium chloride (NH₄Cl) for every 100 ml of hydrolysate [9].

2.5. Reducing sugar concentration

Measurement of reducing sugars using 3,5-dinitrosalicylic acid (DNS) method. 1 ml of the sample plus 3 ml of reagent DNS, then mixed and heated on a temperature of 100°C for 10 minutes. Sugar measurement reduction is done by spectrophotometer UV-Vis with a wavelength of 540 nm. Determination of reducing sugar levels based on the glucose standard curve [10].

2.6. S. cerevisiae growth

Analysis of the number of microorganisms is done by taking absorbance data with a UV-Vis spectrophotometer (λ=600 nm).
2.7. **pH measurement**
The pH measurement is done by dipping the pH indicator probe into the sample bottle so that a pH value will appear.

2.8. **Measurement of ethanol content**
Determination of ethanol content using an alcohol meter.

3. **Result and discussion**
In this research, bioethanol production uses biomass from *Anabaena* sp microalgae by the SSF method. The process of producing bioethanol from biomass through several stages, namely the preparation of raw materials, hydrolysis and fermentation. The hydrolysis process aims to convert carbohydrates into monosaccharide forms so that they are easier to ferment by *S. cerevisiae*. In this study, hydrolysis using sulfuric acid because in research conducted by [11] using sulfuric acid was more effective than other acids and the most optimal yield was 26.08% using sulfuric acid.

The sulfuric acid concentration used is 1% due to reducing the negative impact on the environment, and based on research conducted by [10] the ethanol content of the biomass hydrolyzed using sulfuric acid was reached at a maximum concentration of 1% sulfuric acid which was 34.5%. The biomass hydrolyzed by using sulfuric acid samples turns blackish green, this is because sulfuric acid as a dehydration agent can absorb water content (H and O) in a material that contains carbohydrates, the addition of concentrated sulfuric acid will cause dehydrated carbohydrates [10]. The performance of the process of the SSF method can be seen from several parameters including pH, the number of microbes, reducing sugars and ethanol content.

![Figure 1](image.jpg)

**Figure 1.** The addition of concentrated sulfuric acid cause dehydrated carbohydrates that transform the color into blackish green

3.1. **pH During fermentation**

pH is an important factor in the fermentation process, the initial pH value is 4.5 because *S. cerevisiae* is preferred to grow acidic acid ie pH around 4-4.5 [9]. During the pH fermentation medium in the range of 4.5-4.9, optimal pH conditions can affect the growth of *S. cerevisiae* can grow well to produce high levels of ethanol [12]. At 96 hours of fermentation, there was a decrease in pH caused by the formation of CO and other organic acids. At the time of fermentation 0 - 72 hours an increase in pH caused by the use of yeast extract that increases efficiency can increase pH [13]. Meanwhile, according to [4] pH changes can occur due to the presence of organic acids such
as acetic acid, pyruvic acid and lactic acid that are formed during the fermentation process, while other acidic acids contain acids that contain little for the change in pH of fermentation. Fermentation media become more acidic because of the ammonia present in yeast cells as a source of nitrogen [4].

![Figure 2](image_url)

**Figure 2.** The change of pH at the fermentation process at the time of 0 to 96 hours

3.2. **Number of microorganisms during fermentation**

Fermentation is one of the processes to convert into bioethanol which takes place anaerobically. In this study using *S. cerevisiae* because it can produce higher levels of ethanol. Based on the results of the study, at 0-24 hours the log phase was removed which transferred the number of microorganisms. Factors that can affect the length of the log phase are calculated the number and influence of the medium used, the growing environment and the number of inoculums where a high initial number of cells will increase the log phase. At the time of 24 hours to 96 hours a decrease in the number of microorganisms, this can cause the number of nutrients present in this medium to decrease because it is used for growth.

According to [14] the number of microorganisms caused by nutrients in the medium has been greatly reduced and is also caused by the results that might inhibit the growth of microorganisms. Increasing and decreasing the number of microorganisms will increase the pH of the media. According to [15] stated that the most optimal fermentation time for the process of making bioethanol with yeast *Saccharomyces cerevisiae* is 3 days. If fermentation is carried out for more than 3 days, the alcohol content can be reduced. Reducing the amount of alcohol caused by alcohol must be another, for example, esters. According to [13] the longer the fermentation time, the number of microbes will increase and will go to the death phase because alcohol is produced more and more nutrients as microbial food is increasing.
3.3. Sugar reduction during fermentation
Reducing sugar is the sugar that can reduce, is the result of complex reserve hydrolysis. The presence of reducing sugars in the bioethanol production medium is one that is not essential for the growth of *S. cerevisiae* because it is made as a source of carbon in the formation of energy [9]. Reducing sugar calculation in this study uses the DNS method. The reaction between reducing sugars and DNS is an oxidation reaction in a sugar aldehyde group and oxidized to a carboxyl group. If there is a reducing sugar then the sample that has been given a DNS license issued yellow will turn reddish-yellow [9].

Measurement of reducing sugars in this study began on day 0 after studying the initial reducing sugars produced. Based on the results of research that has increased reducing sugar, it can be done because *S. cerevisiae* can hydrolyze cellulose into savings so that at 24 hours there is an increase in reducing sugar. If there is a decrease in sugar because these microorganisms use reducing sugars for cell growth and also the formation of ethanol because the reducing sugars in the medium are used as a carbon source for the yeast to synthesize energy through the ethanol fermentation process. Glucose is used as food for the growth of microorganisms and the formation of ethanol as a fermentation product [14]. The resulting cellulase enzymes can loosen and hydrolyze cellulose bonds into bonds, so *S. cerevisiae* is easier to use hydrolysis funds to produce ethanol. Reducing sugar levels that are outside normal limits can affect the osmotic pressure of yeast cells and their metabolic activity capacity so that the resulting product cannot be maximized. At 96 hours fermentation time, bioethanol levels were tested using alcoholmeter, estimation of bioethanol production using microalgae *Anabaena* sp with SSF method was able to produce 7% ethanol content.
Figure 4. The measurement of reducing sugar began on day 0 to 10 hours after studying the initial reducing sugar.

4. Conclusions

Making bioethanol from microalgae Anabaena sp using S. cerevisiae can be done by a method Simultaneous Saccharification Fermentation (SSF). The hydrolysis process uses 1% sulfuric acid. The bioethanol concentration produced was 7% and the fermentation time in this study was 96 hours.

Acknowledgment

This research was funded by Kementrian Riset dan Teknologi Republik Indonesia by Hibah Penelitian Tesis Magister year 2018 which is gratefully acknowledged. We thank our institutions from LPPM Universitas Diponegoro.

References

[1] Yudiartono, Anindhita, Sugiyono, A, Wahid, M A L and Adiarso 2018 *Energi Berkelanjutan untuk Transportasi Darat*. Development 134
[2] Hakim N, Firmansyah M and Adam A 2016 *Mix* 2025 0–8
[3] Azmah S, Abdulla R, Hajar S, Azhar M, Marbawi H, Azlan J and Ravindra P 2016 *Renewable and Sustainable Energy Reviews* 65 756-769
[4] Dmpeipen E J and Dewa R P 2015 *Majalah BIAM* 11(2) 63-75
[5] Eduardo C, Silva D F and Bertucco A 2016 *Process Biochemistry* 51(11) 1833–1842
[6] Nayak B K, Roy S and Das D 2013 *International Journal of Hydrogen Energy* 39(14) 7553–7560
[7] Hernández D, Riaño B, Coca M and García-gonzález M C 2015 Saccharification of carbohydrates in microalgal biomass by physical, chemical and enzymatic pre-treatments as a previous step for bioethanol production 262 939–945
[8] Narra M, James, J P and Balasubramanian V 2015 *Technology* 179 331–338
[9] Phwan C K, Chew K W, Sebayang A H, Ong H C, Ling T C, Malek M A, Show P L 2019 *Biotechnology for Biofuels* 12(1) 1–8
[10] Agustini N W S and Febriani N 2019 *Journal Kimia dan Kemasan* 41(1) 1-10
[11] Jeong T S, ChoiC H, Lee J Y and Oh K K 2012 *Bioresource Technology* 116 435-440
[12] PinakiD, Lhakpa W and Joginder S 2015 *Biosciences Biotechnology Research Asia* 12(1) 87-100
[13] Hernández D, Riaño B, Coca M and García-gonzález M C 2015 *Chemical Engineering Journal* 262 939-945
[14] Adini S, Kusdiyantini E and Budiharjo A 2015 *Bioma: Berkala Ilmiah Biologi* 16(2) 65-75
[15] Amalia, Y, Muria S R and Chairul C 2014 *Jurnal Online Mahasiswa Fakultas Teknik Universitas Riau* 1(1) 1-8