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Biodiesel production from *Pichia kudriavzevii* using Algae *Kappaphycus Alvarezii* as a fermentation substrate

Sulfahri*, Nurfadillah, W L Taufan, M S Aska

Departement of Biologi, Faculty of Mathematics and Natural Sciences, Hasanuddin University, Makassar, Indonesia

*E-mail: sulfahri@unhas.ac.id

Abstract. *Kappaphycus alvarezii* is red algae that can be used as an alternative raw material in biodiesel production, because *K. alvarezii* contains high carbohydrate which is 60%. This study aims to determine the effect of nutrition and fermentation duration of *K. alvarezii* algae to hydrolysis results on cell's biomass. This study used the enzymatic hydrolysis method, fermentation method and sonication method. In this study, hydrolysis method was conducted by using 150 KNU/L of α -amylase enzyme with 0.5%, 1%, 1.5% and 2% of algae concentration. *K. alvarezii* algae is fermented by yeast *Pichia kudriavzevii*. This study, fermentation method used was Gandasil-D® as an inorganic nutrition and Yeast Extract as an organic nutrition. The result of this research shows that *P. kudriavzevii* biomass can produce biodiesel by carbon source from *K. alvarezii* algae on 48th hours. The dominant types of fatty acids are oleic acid which is 39.33%. The result shows that it has high potential to be used as biodiesel raw material.

1. Introduction

The critical problems for the development of the industrial world today have happened. One of the critical problems that took people's attention is energy crisis. The energy crisis is getting worse with doing continuous exploitation and it caused the fossil fuels being nonrenewable energy [1]. Besides that, the alternative that government took was the using of food needs that contain glucose, such as corn, potato and breadfruit to produce fuel oil. However, it will cause the needs of those food needs will increase and then it caused another problem [2].

Biodiesel is one of alternatives that can be used to overcome the energy crisis, because biodiesel comes from renewable and environmentally friendly raw materials such as the using of marine algae. *Kappaphycus alvarezii* is one of the raw material to produce biodiesel which is one of the largest marine algae produced in the world, including Indonesia [3-5]. These marine algae contain polysaccharide in the form of kappa-carrageenan. Kappa-carrageenan is a hydrocolloid used as food additive, acting as gel, emulsifying, thickening and stabilizing agent in pharmaceutical, food industry, pharmaceutical, cosmetic and others which are predicted to increase in the future [6-7]. The major components of carrageenan are D-galactose-4-sulphate and 3,6-anhydro-D-galactose-2-sulfate which are potential for fermentation [8].

Biodiesel production, lipids and fatty acids of natural sources have to be extracted from dry biomass of them like algae biomass. Extraction methods such as ultrasound and microwave assisted were also used for oil extraction from natural sources. Biodiesel is a mixture of fatty acid alkyl esters obtained by transesterification (ester exchange reaction) of vegetable oils or animal fats [9]. Biodiesel production can be produced by sonication method. Other than that, enzymatic hydrolysis and fermentation methods have the potential to increase biodiesel production from the amount of cell's biomass obtained [8,10]. Based on another research using the sonication method can produce biodiesel but on that research use phytoplankton. The use of ultrasonic waves has proven to accelerate the reaction, reducing the amount of catalyst used and reducing the ratio of oil to alcohol use than the reaction without the help of ultrasonic waves [1,11].



Marine algae can be solved by enzymatic hydrolysis and fermentation method [12-13]. Enzymatic hydrolysis process can use some enzymes such as cellulase to hydrolyze cellulose, while for hemicellulose hydrolysis can use enzymes such as glucuronidase, xylanase, β -xylosidase, galactomannanase and glucomannanase. Meanwhile, kappa-carrageenan can be hydrolyzed by using alpha-amylase enzyme [14-15]. Kappa-carrageenan is a hydrocolloid composed of D-galactose-4-sulphate and 3,6-anhydro-D-galactose-2-sulphate which is potential for fermentation to obtain cell's biomass from the fermentation process [6, 16].

Another method that can be used for biodiesel production is fermentation. One of the potential microbes in the fermentation process is *P. kudriavzevii*. This Yeast has a morphology that is round, elliptical or elongated [17]. Meanwhile, previous research showed that the content of *P. kudriavzevii* is 29.3% palmitate, 8.89% stearate and oleic acid 41.9%. This yeast can be combined into ethanol-tolerant [3, 14]. Therefore, this study aims to determine the effect of fermentation nutrition and fermentation duration of algae *K. alvarezii* for cell's biomass. Thus, the biomass obtained will be tested for its fatty acid content to produce biodiesel.

2. Materials and Methods

2.1. *Kappaphycus alvarezii*

This study used the marine algae of *K. alvarezii* obtained from coastal waters of Punaga village, Mangarabombang District, Takalar regency. Algae obtained were then examined to identify those that to *K. alvarezii* type.

2.2. Pretreatment Process

Algae biomass were washed and soaked in clean water for 2-3 hours. Then dried under the sun. Algae *K. alvarezii* that has been dried were mashed using a hummer mill and sifted using a 40 mesh sieve. The resulting algae flour was weighed according to a combination of concentrations are 0.5%; 1%; 1.5% and 2%, then dissolved with 50 mL distilled water.

2.3. Hydrolysis Process

K. alvarezii algae solution with concentration variation of 0.5%, 1.0%, 1.5%, and 2.0%. Then, the algae solution was heated using a hot plate at 100 °C for 90 minutes. Thereafter, an enzyme α amylase of 80 KNU/L was added. The added solution of the incubated apparatus for 24 hours, 48 hours and 72 hours. Once hydrolyzed, the algae solution is filtered to separate the natan and its supernatant. The supernatant was then centrifuged at 9,000 rpm for 10 min. Supernatant of centrifugation was taken and measured for its sugar content.

2.4. Fermentation Process

K. alvarezii solution was prepared according to the optimal concentration obtained at the hydrolysis process, which is 0.5% with the addition of 150 KNU/L enzyme α -amylase and incubated for 24 hours. Then, Gandasil-D® as an inorganic nutrient and Yeast Extract as an organic nutrition (0.5 g/L and 1.0 g/L) was added as nutrients of fermentation. After that, a starter *P. kudriavzevi* was activated with 5% concentration into the fermentor bottle. The fermentation process used various fermentation durations (0 hours, 24 hours, 48 hours and 72 hours). After the incubation period of 0 hours, 24 hours, 48 hours and 72 hours, measurements of sugar levels, ethanol content and cell's biomass of *P. kudriavzevii*.

2.5. Measurement of Cell's Biomass

The cell's biomass of *P. kudriavzevii* was measured using the method of dry cell weight (DCW = dry cell weight). The dry weight of the cell was carried out by first centrifuging the fermentation medium sample by 50 mL at 9,000 rpm for 10 min. After centrifugation, supernatant and pellet cells were obtained. The supernatant was removed by pipette. After that, the resulting pellets were then washed by adding aquadest on the cell pellets and centrifuged at 9,000 rpm for 5 min. The pellet was

suspended with aquadest and vacuumed with a pipette and then transferred into a filter paper with a pore size of 0.47 μm which had previously been diovened to a constant weight (W1). The filter paper containing pellets was dried in the oven at 80°C for 24 hours and weighed (W2). The DCW result was difference between the weight of the final filter paper and the weight of the initial filter paper (W2-W1) expressed measured by the dry weight of the cell with gram/liter (g/L) unit.

2.6. Biodiesel Production

The extracted *Pichia kudriavzevii* oil was fed into a heated erlenmeyer in an ultrasonic cleaner device operated at a frequency of 20 kHz, at 60°C., and then mixed with a solution made of methanol (lipid molar ratio: methanol = 1: 12) and a KOH catalyst (9% by weight of oil) which had been stirred for 15 minutes. The time for the transesterification process was about 90 minutes. While the reaction took place, the heating temperature should be maintained. Furthermore, the transesterification results were left for 3-4 days to form two phases. Then separated, and followed by the addition of anhydrous Na₂SO₄ to the methyl ester to draw residual water in the solution. The next stage was separated between Na₂SO₄ and biodiesel using centrifuge. The supernatant in the form of methyl ester (biodiesel) was taken and then heated in an oven at 70°C. Therefore, pure biodiesel was then analyzed for physical and chemical properties to determine the quality of biodiesel by using UV-Vis spectrophotometry to determine the final concentration of glycerol after sonication.

2.7. Data Analysis

This study used RAL (Complete Random Design) with three replications. The parameters measured were sugar content and cell's biomass. Data were analyzed statistically using Analysis of Variance (Anova) with 95% confidence interval ($\alpha = 0.05$). The analysis was conducted to compare the effect of acid hydrolysis process on sugar content and fermentation process effect in yielding sugar content and cell's biomass from algae *K. alvarezii*. If there is influence then continued with Tukey test at 95% confidence level ($\alpha = 0.05$) to know pair of group the same and different data in each treatment.

3. Result and Discussions

3.1 Biomass Levels in Fermentation Process

3.1.1 Effect of Supplementation on Cell's Biomass.

Fermentation method used Gandasil-D® as an inorganic supplementation and Yeast Extract as an organic supplementation with different concentrations (0.5% and 1.0%).

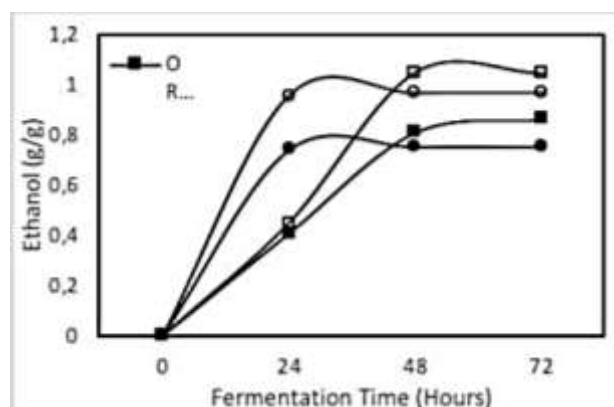


Figure 1. Graph of Cell Biomass during Fermentation Process

Based on the graph of cell Biomass during Fermentation Process with addition of fermentation nutrition 0.5% and 1% in 0 hour of fermentation duration doesn't indicate of biomass cell. In fermentation duration of 24 hours by adding organic fermentation nutrition was (OR 0.5%: 0.11 g/L and OR 1%: 0.12 g/L). while the adding of inorganic nutrition was (AN 0.5%: 0.12g/L and AN 1%:

0.24 g/L). In fermentation duration of 48 hours by adding organic fermentation nutrition was (OR 0.5%: 0.21 g/L and OR 1%: 0.27 g/L). While the adding of inorganic nutrition was (AN 0.5%: 0.02g/L and AN 1%: 0.25 g/L). In fermentation duration of 72 hours by adding organic fermentation nutrition was (OR 0.5%: 0.23 g/L and OR 1%: 0.27 g/L). while the adding of inorganic nutrition was (AN 0.5%: 0.20 g/L and AN 1%: 0.25 g/L).

The results showed that organic and inorganic supplementation in the form of yeast extract and Gandasil-D® did not have a significant effect on the biomass of *P. kudriavzevii* cells. this is because [15], which said that the concentration of organic nitrogen on the fermentation medium was better than 2.5%. Meanwhile yeast extract was organic nitrogen with a nitrogen content of 10.24% and Gandasil-D® had nitrogen content of 20% using the conversion factor of protein to nitrogen. The content of algae *K. alvarezii* which had organic nitrogen 4,6% using the protein to nitrogen conversion factor [3]. No organic and inorganic supplementation was not seen anymore. This was supported by Shen [16], who found that if the fermentation substrate had enough organic nitrogen, supplementation with organic nitrogen did not affect ethanol fermentation.

3.1.2 The Effect of fermentation time on Cell Biomass.

The success of fermentation can be seen based on the microbial growth response on the fermentation medium. In this study, the measurement of yeast cell biomass *P. kudriavzevii* was done by using dry weight method (dry cell weight). Cell biomass measurements were performed during fermentation of 0 hours, 24 hours, 48 hours and 72 hours.

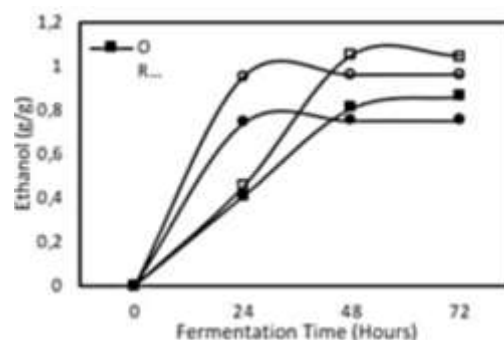


Figure 2. Graph of Cell Biomass during Fermentation Process

Based on the graph of cell Biomass during Fermentation Process with addition of fermentation nutrition 0.5% and 1% in 0 hour of fermentation duration doesn't indicate of biomass cell. In fermentation duration of 24 hours by adding organic fermentation nutrition was (OR 0.5%: 0.11 g/L and OR 1%: 0.12 g/L). While the adding of inorganic nutrition was (AN 0.5%: 0.12g/L and AN 1%: 0.24 g/L). In fermentation duration of 48 hours by adding organic fermentation nutrition was (OR 0.5%: 0.21 g/L and OR 1%: 0.27 g/L). While the adding of inorganic nutrition was (AN 0.5%: 0.02g/L and AN 1%: 0.25 g/L). In fermentation duration of 72 hours by adding organic fermentation nutrition was (OR 0.5%: 0.23 g/L and OR 1%: 0.27 g/L). While the adding of inorganic nutrition was (AN 0.5%: 0.20 g/L and AN 1%: 0.25 g/L).

The biomass content that produced is determined by the ability of yeast to divide carbohydrates and produce ethanol. Microbe as the agent of fermentation is important factor in bioethanol fermentation (Choudhary et al., 2016; Zhang et al., 2018). One of the potential microbes is *Pichia kudriavzevii* because it has faster ethanol production, higher ethanol production and endure toward the extreme condition of environment [20-22].

The results showed that the longer the duration of fermentation, the more cell biomass produced. This means that yeast *P. kudriavzevii* has used carbohydrates from the algae medium to divide. The fermentation time effect on yeast activity because the longer the fermentation then the yeast will be more active breed or split. The result of this study were statistically tested using the ANOVA test at

95% confidence interval that showed the variation of fermentation influences cell biomass. It was concluded that most cell biomass was produced at 48 hours of incubation duration and yielded cell biomass of 0.27 ± 0.003 g/g.

3.2 Biodiesel Production

Biodiesel was produced from *P. kudriavzevii* by isolated the lipid from the yeast by using ultrasonic extraction method. At this stage 96% ethanol solvent was used. Ultrasonic method played an important role to destroy the composition of yeast cell walls. The ethanol function would be more efficient in extracting lipids because they have the same polarity as the material to be extracted. This transesterification serves to synthesize biodiesel from yeast acids.

Table 1. Fatty Acid Content Test Results Of *P. Kudriavzevii* Biomass

Fatty Acid	Content
Palmitic Acid	28.33+0.01%
Palmitoleic Acid	1.29+0.00%
Stearic Acid	9.30+0.02%
Oleic Acid	39.37+0.05%
Linoleic Acid	11.09+0.02%

Based on the test results, it is known that the highest fatty acid produced was oleic acid which is 39.33%. This result was supported by several research which reported that the high oleic acid content is potential to produce biodiesel [22-23]. This suggests that biodiesel from yeast biomass *P. kudriavzevii* has great potential to be used as an alternative to solve government problems in energy crises.

4. Conclusion

The result of this research shows that *P. kudriavzevii* biomass can produce biodiesel by carbon source from *K. alvarezii* algae on 48th hours. The dominant types of fatty acids is oleic acid which is 39.33%, with the result that it has high potential to be used as biodiesel raw material.

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