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# Biobutanol Production from Microalgae *Nannochloropsis sp.* Biomasses by *Clostridium Acetobutylicum* Fermentation

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**ABSTRACT -** Biobutanol is an example of alternative energy sources to replace liquid fuel with the carbon-neutral characteristic. It has more benefits to the environment compared to the fossil fuel. Biobutanol is synthesized through fermentation of microalgae cells wall or other organism parts as the carbon sources. The aim of this study is to determine the ability of *Clostridium acetobutylicum* bacteria in the fermentation of *Nannochloropsis* sp. to produce biobutanol. Fermentation of *Nannochloropsis* sp. for biobutanol production was used as an initial treatment before lipid extraction. Fermentation was performed with *C. acetobutylicum* bacteria for 96 hours. The result showed that *C. acetobutylicum* was able to produce 2.61% v/v butanol. This process used *Nannochloropsis* sp. microalgae hydrolysates and biomass of viscozyme hydrolysis yield. The process of hydrolysis with cellulose and viscozyme can produce simple sugars, with the highest obtained yield of 1738.38 ppm from hydrolysis using viscozyme.

**Keywords**: biobutanol, fermentation, Clostridium acetobutylicum, Nannochloropsis. sp



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## INTRODUCTION

Energy is one of the major human needs. Currently energy sources are slowly depleting. This is due to the non-renewable petroleum fuel which is commonly used in the world. The process of producing biofuel from microalgae is a long process (Chisti, 2007). It goes through several stages that inflate the production cost and energy use in the process. Hence, the research in this area is one way to solve the problems where the stages that are passed can be improved or can produce other products which have economic value. Thus, it can reduce the high production cost and energy use in the conversion of microalgae into biofuels.

The developed countries are currently focusing on the development and application of biobutanol as a more promising biofuel compared to bioethanol (Kussuryani & Rani, 2015). Research and development of biobutanol as an alternative energy substitute for gasoline in Indonesia has not been carried out intensively unlike bioethanol. It is necessary to have continuous and comprehensive research on the production of bio butanol using fermentation technology as an alternative energy source to be able to answer all the challenges mentioned above.

Several types of microalgae contain high carbohydrates level in their cell walls (in the form of cellulose and other polysaccharides) and plastids

(especially in the form of starch). These carbohydrates have the potential to be used as carbon sources in the fermentation process (Chen, *et al.*, 2013). Polysaccharides in the cell wall are a high carbohydrate content part. Some types of microalgae produce massive amount of carbohydrates that can be used as a fermentation substrate for bio butanol production. The composition and type of carbohydrates contained in microalgae are quite varied and depend on the type of microalgae. The total carbohydrate content in microalgae ranges from 16-69% of the dry weight (Wang, *et al.*, 2017).

In this study, a seawater microalgae called *Nannochloropsis* sp. was fermented to produce biobutanol. The purpose of this study is to determine the ability of *C. acetobutylicum* bacteria in fermenting *Nannochloropsis* sp. to produce biobutanol. Also the effect on the acquisition of microalgae lipids which are expected to reduce production costs in the process of biofuel production for biodiesel production. Hopefully, biofuel and biobutanol based on microalgae can be used as an alternative solution not only in meeting the oil demand but also encouraging the development and utilization of renewable energy.

## METHODOLOGY

### Microalgae Preparation

*Nannochloropsis* sp. was prepared in the slurry form, with 70-80% of water content. Then, it was treated by adding 0.5M hydrochloric acid before the addition of enzymes. Two kinds of enzymes were used in this step, namely cellulase and viscozyme from Sigma-Aldrich. The treatment with cellulase was performed with time variation of 6, 24, 48, 72, and 96 hours, pH variation of 4.00, 4.50, 5.00, 5.50, 6.00, and 6.50 and temperatures of 40, 45, 50, 55, 60, and 65°C. Meanwhile the treatment with viscozyme was also subjected to variation in the treatment time of 6, 24, 48, 72, and 96 hours, pH of 3.00, 3.50, 4.00, 4.50, 5.00, and 5.50 and temperatures of 30, 35, 40, 45, 50, and 55°C. Afterward each treated enzymes were centrifuged at 6000 rpm for 20 minutes. The supernatant was collected to measure the total sugar content by the phenol-sulfuric acid method and the absorbance was observed with a spectrometer at $\lambda$ = 490 nm.

### Activation and Cultivation of Bacteria Culture

The culture of *C. acetobutylicum* FNCC 0085 was obtained from Biotechnology Study Center, Gadjah Mada University, Yogyakarta. *C. acetobutylicum* bacterial culture was heat shocked at 80°C for 10 minutes (Servinsky, *et al.*, 2010). Then it was activated in the new liquid media, namely Reinforced Clostridial Medium (RCM) from Merck®. The first activation was carried out on Cooked Meat Medium (Oxoid) and cultivated on RCM (Merck®). Activation using Cooked Meat Medium was done to activate dormant bacteria due to prolonged preservation. After the activation, determination of *C. Acetobutylicum* bacteria growth curve was performed using RCM, Trypton Glucose Yeast (TGY), and Basal medium. Observation of the growth curve is carried out for 96 hours.

### Fermentation Process

A batch system of 1 L container with the addition of 10% culture of *C. acetobutylicum* was utilized for the fermentation process. Fermentation lasts for 96 hours at 35°C in the anaerobic chamber. Fermentation started with the activation of *C. acetobutylicum* in RCM media for 24 hours at 35°C. The 24-hour-old bacterial culture was then transferred into TGY media for 6 hours at 35°C. The activated bacteria were ready to use for fermentation of microalgae biomass. Afterward it was added into a solution containing microalgae which then supplemented with P2 nutrients that consist of buffers, minerals, vitamins and yeast extract (Kussuryani & Rani, 2015).

### Analysis of Butanol Content

The analysis was carried out by extracting each fermentation solution with Dichloromethane (DCM) 1:1, the subjected to shook and separation by a separating funnel. Analysis of acetone, butanol and ethanol contents was fulfilled by using gas chromatography (GC). The extraction yields were then injected directly into the GC device. The standard solutions of butanol, ethanol, and acetone from Merck® were used as references for GC analysis.

## RESULTS AND DISCUSSION

### Microalgae Hydrolysis

Microalgae hydrolysis can be done by using acids and enzymes. Hydrolysis aims to break the hemicellulose bonds and the structure of cellulose of lignocellulose from microalgae into simple sugar compounds. It also eliminates the existing lignin content (Chen, *et al.*, 2013). Although the use of acid

as a pretreatment is a fast and inexpensive process, it cannot provide maximum hydrolysis results. The use of acid can produce an inhibitor which influencing the formation of simple sugars and, obstructing the fermentation process in the next stage. Therefore, the enzyme is used to carry out the hydrolysis process in this research.

Two types of enzymes were used in the treatment of microalgae biomass, namely cellulase and viscozyme. The contact time between the enzymes and microalgae biomass showed similar results. The highest production of simple sugar was given by 24 hours of treatment time from both enzymes. Viscozyme exhibited the highest sugar production of 1713.08 ppm. It was higher than the cellulase which only produced 224.27 ppm of sugar (Figure 1). The sugar quantity produced from hydrolysis after 24 hours of contact time has no significant addition. The possible explanation for it is because the majority of substrates has been reduced when there is no addition of the substrates. The use of enzymes is related to the appropriate substrate because enzymes have certain specificities to certain substrates so that the desired product can be produced. Therefore, there no more quantity of total sugar added in the solution after passing the 24 hours contact time.

The use of cellulase might only affect the cellulose contained in *Nannochloropsis* sp., but does not significantly affect hemicellulose. Thus the quantity of total sugar produced cannot be maximized. Carbohydrates in microalgae itself, besides cellulose there is also consists of hemicellulose.

The quantity of simple sugars obtained using Viscozyme showed high results. It was possible because viscozyme contains several enzymes such as cellulase, xylanase, arabanase, hemicellulase, and beta-glucanase. Thus viscozyme has a broad spectrum of substrates available. The use of viscozyme not only break down cellulose but also hemicellulose contained in *Nannochloropsis* sp. Hemicellulose is a carbohydrate complex structure consisting of different polymers such as pentose (xylose and arabinose), hexose (mannose, glucose, and galactose) and sugar acids (Hendriks & Zeeman, 2009). Hence viscozyme is more capable of breaking hemicellulose compared to cellulase.

Viscozyme has a pH range of 3.3 to 5.5 to work optimally. Meanwhile, cellulase has an optimal pH range at 4.5 to 6.0 (Sigma-Aldrich). In this treatment, both cellulase and viscozyme were in their respective optimal pH range to hydrolyze *Nannochloropsis* sp. The obtained results showed that the best pH for cellulase enzyme was at pH 5.00 with the obtained simple sugar of 466.47 ppm. On the other hand, the pH treatment using viscozyme gave the best results at pH 3.50 with 1319.35 ppm of produced total sugar

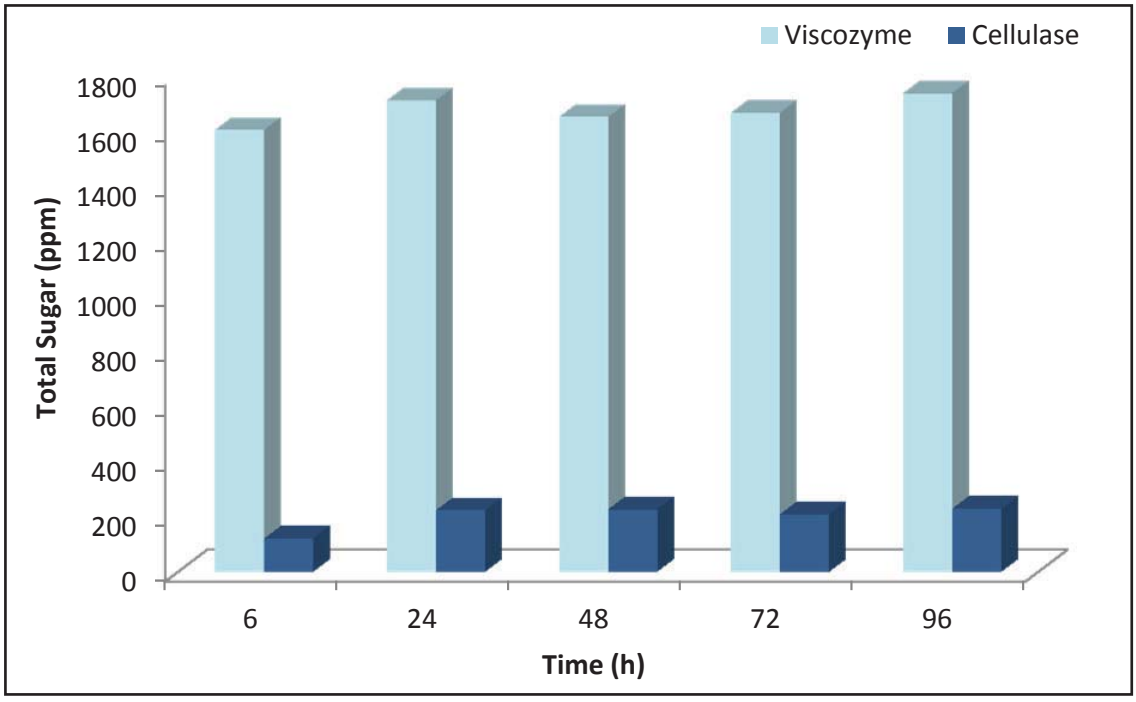


Figure 1
Sugar produced after the hydrolysis process with cellulase and viscozyme with different contact times.

(Figure 2). The enzymatic efficiency is strongly influenced by the pH value at which the enzyme works. Enzyme denaturation might occur when the pH value is too high or too low. Enzymes that are denatured will lose their function as a catalyst. The optimal pH range for cellulase and viscozyme provide the most optimal pH at which the enzyme work to break down carbohydrates in *Nannochloropsis* sp. into simple sugars.

In the determination of the optimum pH for viscozyme the relationship between pH and enzyme activity can be observed. At a certain pH level, enzymes catalyze the reaction at the fastest rate compared to the other pH levels. Viscozyme which consists of several types of enzymes can have their own optimum pH level, so it can be seen in the obtained results that there are 2 pH points at which produced the highest total sugar quantity, pH 3.5 and 5.5 (Figure 3). It showed that there were some enzymes that worked optimally at pH 3.5 while some other at pH 5.5.

The use of enzymes is strongly influenced by temperature in operation. When the temperature is too low, the enzyme becomes inactive. As the temperature rises, enzyme activity is increased. When the temperature keep rising even higher, at some point the denaturation process will begin and the enzyme will lose its function due to the structural damage. The determination of the optimum catalytic temperature of cellulose was at 55°C to get the highest total sugar quantity of 537.65 ppm. When using viscozyme, the highest quantity of sugar obtained at an optimum temperature of 45°C was 1162.57 ppm (Figure 3).

Microalgae are known to contain total carbohydrates ranging from 16 to 69% of their dry weight depending on the species of the microalgae (Wang, *et al.*, 2017). The ability of enzymes to degrade carbohydrates from microalgae varies greatly because it is strongly influenced by the type of enzyme used, the source of raw materials as the substrate, the type and presence of initial treatment, the optimal condition of the enzyme itself and the presence of other inhibitors (Guo, *et al.*, 2018; Juárez, *et al.*, 2016; Maffei, *et al.*, 2018).

The enzyme combination that catalyzes the hydrolytic division of β-1,4-glycosidic bond in cellulose is consisted of at least three classes of enzymes namely endoglucanase, exoglucanase or cellobiohydrolase and β-glucosidase (Juturu & Wu, 2014) endoglucanase (E.C. 3.2.1.4. Viscozyme contains arabanase, cellulase, β-glucanase, hemicellulase and xylanase. Arabinose and hemicellulase hydrolyzes araban and hemicellulose, respectively. β-Glucanase is endo-glycosidase. Xylanase degrades linear polysaccharides β-1,4-

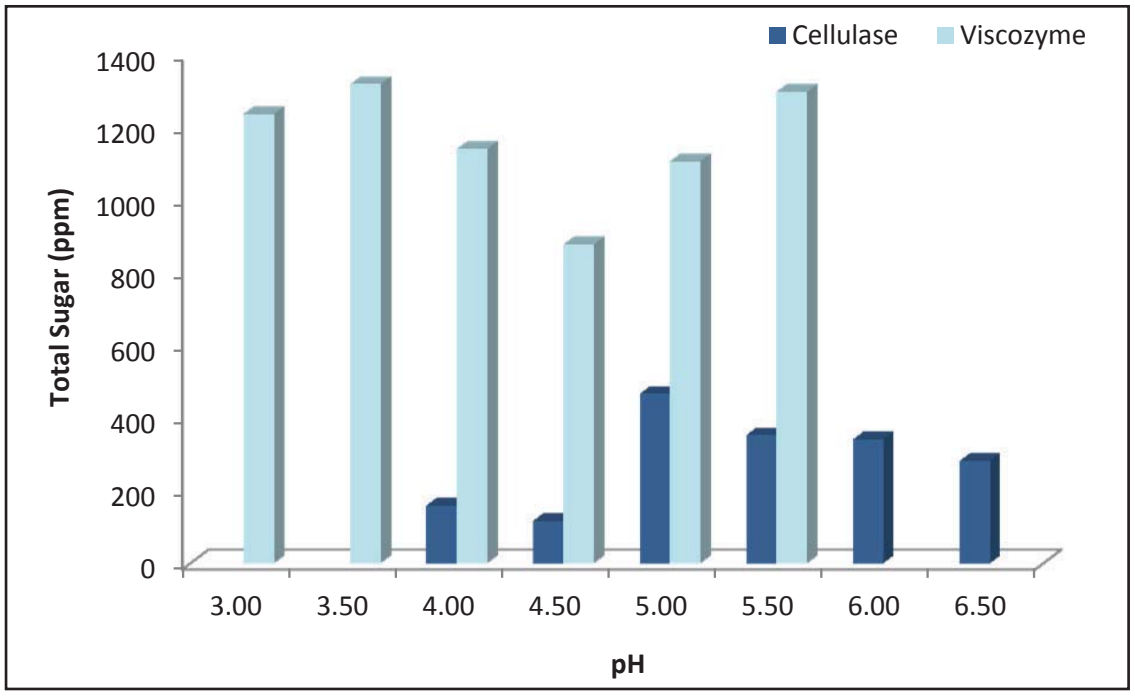


Figure 2
Sugar produced after the hydrolysis process with cellulase and viscozyme with different pH treatments.

xylan to xylose (Lee, *et al.*, 2013). As shown in the experiment, viscozyme showed a relatively better hydrolysis activity than cellulase.

**Biobutanol Production**

Activation and cultivation of *C. acetobutylicum* as a producer of biobutanol has been carried out with several variations of growth media (Kussuryani & Rani, 2015). Activation was done by giving heat shocked to break the bacterial spores that have been formed so that the bacteria can grow. Through the cultivation of *C. acetobutylicum* on RCM media, we obtained data on bacterial growth based on absorbance at a wavelength of 635 nm. The higher

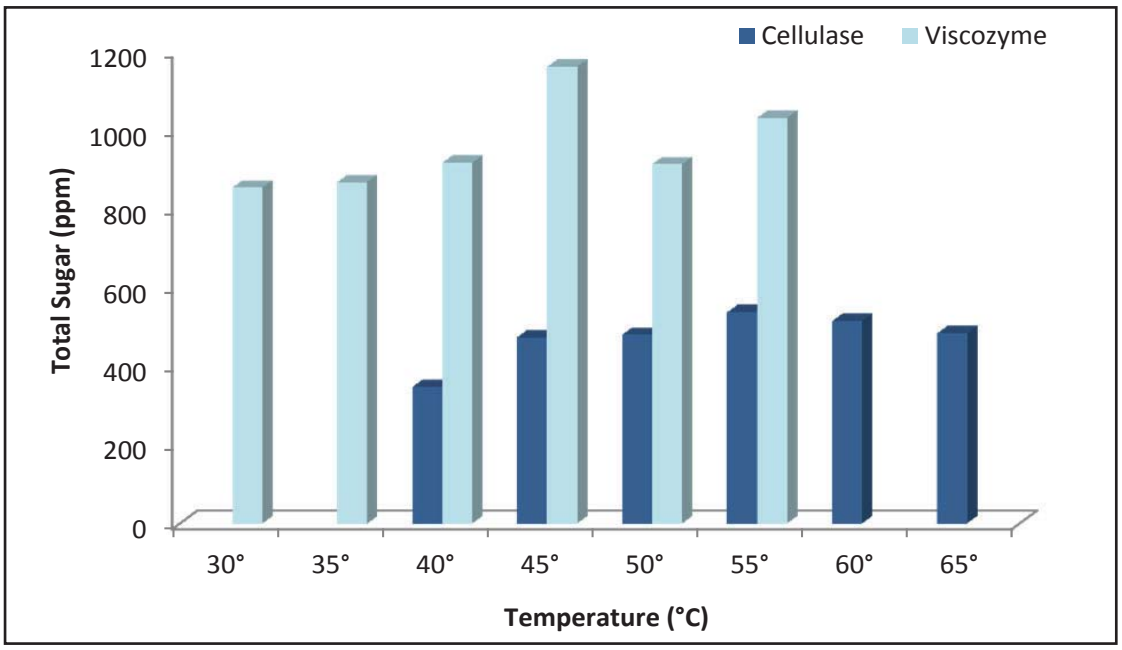


Figure 3
Sugar produced after the hydrolysis process with cellulase enzymes with temperature treatment.

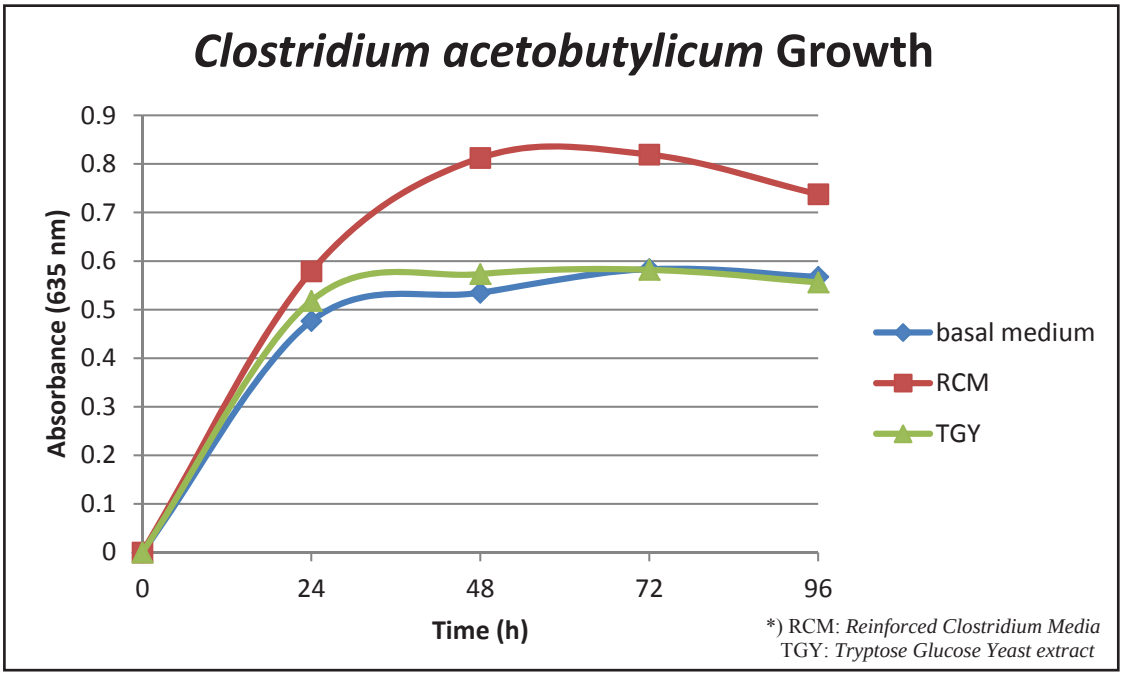


Figure 4
The growth of *c. acetobutylicum.*

absorbance value means higher bacteria growth. The bacterial growth can also be indicated by the turbidity of growth media. RCM is the optimal medium for the growth of *C. acetobutylicum*. It was used as the initial media for bacterial activation because of its exceptional ability to provide the best support for the growth of *C. acetobutylicum*. In Figure 4, it can be seen that the exponential phase of *C. acetobutylicum* occurred from the start and lasts for 48 hours.

Basal medium is the standard medium for *C. acetobutylicum*. This media is easily adapted to the carbon source that will be given; in this case, the carbon source comes from microalgae. Observation of the *C. acetobutylicum* growth cultivated in the basal medium showed a lower growth rate compared to RCM media. The optimal population had been reached within 24 hours then it entered the stationary phase (Figure 4). The *C. acetobutylicum* growth in TGY shared a similar result to the basal medium, where the bacterial growth reached an exponential phase within 24 hours and then entered the stationary phase until 96 hours.

*C. acetobutylicum* has the ability to produce acetone-butanol-ethanol (ABE) from the metabolism. The ability to produce ABE through the fermentation process is the main concern for further development. The *C. acetobutylicum* fermentation result exhibited the formation of butanol from all treatments including control. The only exception was from the use of *Nannochloropsis* sp. biomass without acid treatment. Based on Table 1**,** the highest ABE yields from *C. acetobutylicum* fermentation were obtained in fermented broth containing hydrolysates and biomass which were hydrolyzed with viscozyme. It is in line with the previous results which showed that the results of hydrolysis with viscozyme provided the highest simple sugars quantity.

Ezeji, *et al.* (2007), states that biomass metabolism from solventogenic clostridia starts from the initial processing of lignocellulose, starch, and cellulose hydrolysis to the final stage metabolite, namely butyl Co-A. In the presence of butyraldehyde dehydrogenase and butanol dehydrogenase, butyl Co-A will be converted into biobutanol. However, if the fermentation is incomplete, the butyrate Co-A in the presence of phosphate butyl transferase and butyrate kinase will be transformed into butyric acid.

The chromatogram obtained from the GC analysis showed the levels of each component, even though the quantity was very small (Figure 4). Butanol biosynthesis through the ABE fermentation process by *C acetobutylicum* yielded in a small amount of product; it was due to the toxic nature of the ABE to bacteria. In addition to the production of ABE, there were also some compounds that have not been identified. These compounds were suspected to be alcohols, isomers of ABE compounds, fractions of acids (butyrate/acetate), intermediates, and/or impurities from the hydrolysis process (Kussuryani & Rani, 2015). Even so, it did not appear on the chromatogram because of the very low quantity.

Table 1
The yield of ABE fermentation (% v/v)

| Fermentation Substrates | Ethanol | Acetone | Butanol |
|---|---|---|---|
| Hydrolysates from cellulase hydrolyses | 0,00021 | 0,00054 | 164,848 |
| Hydrolysates from viscozyme hydrolyses | 0,00015 | 0,00012 | 179,439 |
| Hydrolysates and biomasses from cellulase hydrolyses | 0,00002 | 0,00074 | 252,909 |
| Hydrolysates and biomasses from viscozyme hydrolyses | 0,00107 | 0,00092 | 261,058 |
| SSF cellulase | ND | 0,00007 | 255,050 |
| SSF Viscozyme | ND | 0,00011 | 122,812 |
| Biomasses with acid treatment | 0,00005 | ND | 250,145 |
| Biomasses without acid treatment | ND | ND | ND |
| Basal medium | 0,00102 | 0,00127 | 213,258 |

*) SSF: Simultaneous Saccharification and Fermentation
ND: Not Detected

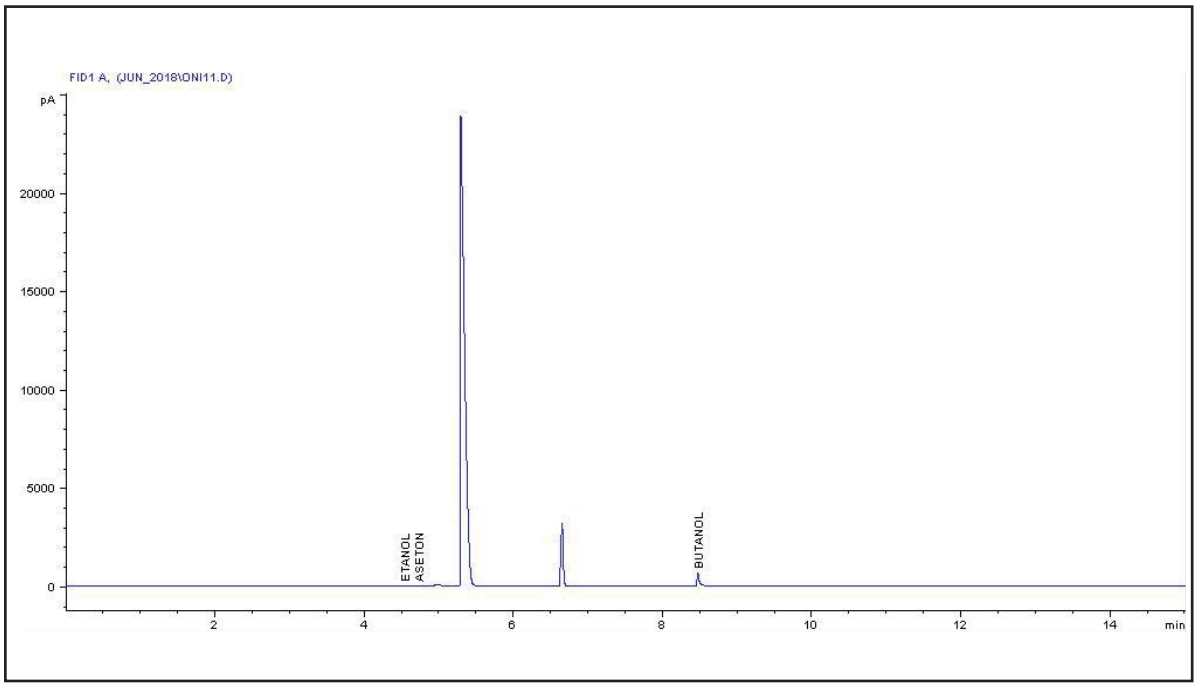


Figure 5
Chromatogram of fermentation results ABE formation in hydrolysates microalgae with cellulase.

The theoretical determination of the microbial products is quite difficult because of the reaction pathway complexity. The maximum theoretical total yield for ABE fermentation is between 38.6% and 39.9%, under biosynthetic conditions where there are no intermediate acids remain in the system (Gottumukkala, *et al.*, 2017). Similarly, the ABE ratio varies according to fermentation conditions and microorganisms.

## CONCLUSIONS

The process of hydrolysis with cellulose and viscozyme can produce simple sugars, with the highest yield of 1738.38 ppm simple sugar was obtained from the hydrolysis using viscozyme. Biobutanol production using *C. acetobutylicum* was able to produce butanol with a percentage of 2.61% v/v. These results were the highest yield percentage obtained from hydrolyzate fermentation and *Nannochloropsis* sp. biomass which resulting from the hydrolysis using viscozyme.

## ACKNOWLEDGEMENT

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## GLOSSARY OF TERMS

| Symbol | Definition | Unit |
|---|---|---|
| RCM | Reinforced Clostridial Medium | |
| DCM | Dichloromethane | |
| GC | Gas Chromatography | |
| TGY | Tryptose Glucose Yeast extract | |
| ABE | acetone-butanol-ethanol | |
| SSF | Simultaneous Saccharification and Fermentation | |

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