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Bioethanol Production From Tropical Marine Microalgae Ambon Bay *Navicula* sp. of The Inner Ambon Bay Strain

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Abstract

Microalgae are photosynthetic microorganisms that contain carbohydrates can be converted into glucose through a hydrolysis process so that they can be used as raw materials for bioethanol production. *Navicula* sp. strain TAD is a type of marine microalgae that is spread in the inner Ambon Bay (TAD) sea waters and is used to manufacture bioethanol. This study aims to determine the content of carbohydrates and bioethanol produced from tropical marine microalgae Ambon bay *Navicula* sp. strain TAD. The research was conducted through several stages, namely the cultivation of *Navicula* sp. strain TAD to obtain biomass, determine carbohydrate content in biomass, hydrolyze biomass and manufacture bioethanol through a fermentation process using *Saccharomyces cerevisiae*. The results showed that *Navicula* sp. strain TAD cultivation for seven days had the highest cell density of $72.167 \times 105 \pm 0.946$ cells mL^{-1} with dry biomass of 0.933 ± 0.062 g and productivity of 0.166 ± 0.011 gL^{-1} $hour^{-1}$. The sulfuric acid hydrolysis process was analyzed for a sugar content of 146.5695 ± 0.758 mg. A fermentation process follows the results of the hydrolysis to produce bioethanol. The bioethanol content obtained was 6.357%. These indicated that *Navicula* sp. strain TAD has the potential to produce bioethanol.

Keywords: Acid hydrolysis, Ambon bay, Bioethanol, Fermentation, Navicula sp. strain TAD, tropical marine microalgae

INTRODUCTION

Climate change, increasing demand for fuel, and exploitation of fossil fuel natural resources continuously become a problem for fuel availability. Indonesia's energy needs have increased along with the increasing economic growth and population of Indonesia. The average annual increase in energy demand has been 36 million barrels of oil equivalent (BOE) since 2000. Meanwhile, non-renewable energy reserves, such as oil, natural gas, and coal, are dwindling (Soedomo, 2001).

The development of renewable energy sources is a fundamental aspect of future energy availability sustainability. Alternative fuel sources that have great potential to be developed come from biological resources because they contain various potential components ((Yusuf, Nafie, & Dali, 2016; Damayanti, Azmilia, Ainun, Amin R., & Nurdin, 2021; Hasti et al., 2022; Telussa, Hattu, & Sahalessy, 2021). One type of biological resource developed is biofuels, such as bioethanol. Bioethanol is an energy source with good prospects as a substitute for liquid fuels whose raw materials are renewable, environmentally friendly, and very profitable from an economic point of view (Harun, Singh, Forde, & Danquah, 2010).

So far, the raw materials used to make bioethanol are still constrained by the availability and competition with food ingredients. In addition, the use of raw materials containing lignocellulose is challenging to produce due to the presence of lignin, so it is necessary to do pretreatment, and the resulting conversion is small. Therefore, currently starting to develop the manufacture of bioethanol with raw materials derived from microalgae (Lakatos et al., 2019; Agustini & Febrian, 2019; Guo et al., 2013; Erlangga, Nugroho, & Miskah, 2015; Putnarubun, Suratno, & Adyaningsih, 2018; Handayani & Ariyanti, 2012).

Microalgae have good prospects to be developed as raw materials for bioethanol. Microalgae were chosen because they can overgrow, do not compete

with food, not require large areas, and grow in seawater, freshwater, and brackish water. *Navicula* sp. strain TAD is a microalgae growing in Maluku Province Ambon Bay waters (Telussa et al., 2021). *Navicula* sp. strain TAD contains carbohydrates, so it has the potential to be used as raw material for making bioethanol. Based on this description, the microalgae *Navicula* sp. strain TAD can be used as raw material for the manufacture of bioethanol

METHODOLOGY

Materials and Instrumentals

All the chemicals used in this research are pro-analysis grade (Merck, Germany): Ethanol, H_2SO_4, Dinitrosalysilic acid (DNS), $Na_2S_2O_3$, NaOH, $(NH_4)_2SO_4$, K_2HPO_4, KH_2PO_4, $MgSO_4$, $CuSO_4$. $5H_2O$, HNO_3, KI, Na-K Tartrate, Natrium Metabisulfite, *Saccharomyces cerevisiae*, and yeast extract. The equipment used includes glassware, analytical balance, refractometer, falcon tube, Eppendorf micropipette size 10-100 and 100-1000 µL, hemocytometer, Tomy ES-315 autoclave, Thermo Scientific S16 centrifuge, water bath, GC–FID, Nikon YS-100 light microscope, Scanning Electron Microscopy (SEM), Rotary Vaccum Evaporator, Agilent UV-Vis spectrophotometer.

Methods

Cultivation of *Navicula sp.* strain TAD

Cell *Navicula* sp. strain TAD was grown in a modified medium (Telussa, Rusnadi, & Zeily Nurachman, 2019). Cultivation was carried out with an initial cell density of 5×10^5 cells mL^{-1} in a simple photobioreactor at room temperature under a light intensity of 67.5 mmol m^{-2} s^{-1} with photoperiod 12:12 hours (dark: light), salinity 28 ppt, pH 8.2–8.5 and aerated with free air bubbles. The simple photobioreactor used in this study was made of a transparent glass bottle with a height of 25 cm, an external diameter of 9 cm, and a working volume of 900 mL. Cell growth in culture was measured by counting the number of cells (in units of cells mL^{-1}) using a Neubauer Haemocytometer under a light microscope.

Harvesting of Biomass *Navicula* sp. strain TAD

Cell *Navicula* sp. strain TAD cultivated were harvested using sedimentation and filtration techniques using synthetic cloth. Wet biomass *Navicula* sp. strain TAD was weighed using an analytical balance to get the wet biomass weight. Furthermore, the wet biomass was dried using a freeze dryer for 24 hours and weighed to obtain the dry biomass weight. FTIR characterized dry biomass.

Observation of changes in cell morphology

Observations of cell morphology changes were observed using a light microscope and electron microscope (Scanning Electron Microscope). The shape and size of *Navicula* sp strain TAD using a light microscope were observed at 400 times magnification. While observing SEM, cell cultures were centrifuged, and cell pellets were fixed with 2.5% glutaraldehyde for 2.5 hours at 4 ^oC. After that, they were washed with concentrated HNO_3 at 4 ^{o} C and dehydrated with ethanol 20, 60, and 90%. Then dried and observed with SEM.

Bioethanol Production

Enzyme Hydrolysis

The hydrolysis of *Navicula* sp. strain TAD biomass using enzymes (α-amylase, glucoamylase, and cellulase) was carried out on 0.86 g of dry biomass. Hydrolysis using α-amylase enzyme added 40 mL of BPS pH 7, then added 0.08 g of commercial enzyme α-amylase (liquefaction) and dissolved in 10 mL of pH 7 BPS and the combination of α-amylase with amyloglucosidase (saccharification) was 0.002 g using BPS pH 5.4 then hydrolyzed. The hydrolyzed substrate was filtered to obtain the hydrolyzate. Meanwhile, hydrolysis using the cellulase enzyme was carried out by adding 48 mL of acetate buffer pH 4.8 into dry biomass, then adding 2 mL of cellulase, and then heating it at 64 ^0C for 2 hours. The hydrolysis process using the α-amylase enzyme was carried out for 30 minutes at 80 ^oC and amyloglucosidase for 55 minutes at 60 ^oC (Choi, Nguyen, & Sim, 2010). Total reducing sugar was analyzed on the hydrolyzate resulting from the liquefaction and saccharification processes.

Acid hydrolysis

Hydrolysis occurs using a series of condenser devices and a three-neck flask using a magnetic stirrer. In hydrolysis, dry biomass is used and dissolved in sulfuric acid with a concentration of 3.5%. The mixture is then put into a three-necked boiling flask with a condenser installed. To get the best conditions in the hydrolysis process, the process was carried out for 1 hour at a temperature of 110 ^oC and 30 minutes at a temperature of 90 ^oC. The hydrosylate was then filtered, and a neutralization process was carried out with 4 M NaOH to a pH of 4-5; the analysis of reducing sugars was continued (Miranda, Amri, & Utami, 2014).

Fermentation

The sugar from the hydrolysis is then processed to the fermentation stage using *S. cerevisiae*. The process of fermenting sugar into ethanol is carried out under different conditions. Differences in fermentation based on storage time, for 3 and 5 days. Then the fermentation results are filtered and continued with the distillation process. The distillate was analyzed using GC to determine the ethanol content produced.

Bioethanol Qualitative Analysis

Qualitative analysis of ethanol was carried out by color test with $K_2Cr_2O_7$. A total of 1 ml of $K_2Cr_2O_7$ was put into a test tube, and 6 drops of concentrated H_2SO_4 were added. The solution was stirred, and then the fermented distillate was added. The test is declared positive for containing ethanol if the solution changes color from orange to green or blue. After that, further tests were carried out using FTIR.

Bioethanol Quantitative Analysis

The ethanol content of the sample was measured using the GC instrument. To be able to see how much ethanol content is in the sample.

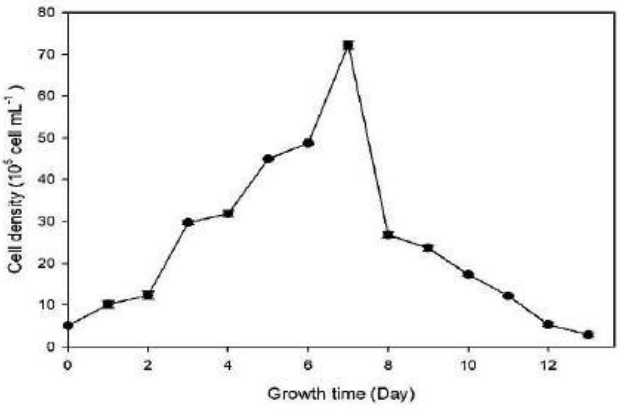
RESULTS AND DISCUSSION

Cultivation *of Navicula* sp. strain TAD

Cell *Navicula* sp. strain TAD was grown in a modified medium to obtain high cell growth and biomass. A modified medium is a simple medium that contains a mixture of nitrate, silicate, iron, and phosphate with the main nutrient composition ratio of nitrogen (N), phosphate (P), and silicon (Si) 140:1:59. By using a 5×10^5 cell mL^{-1} starting cell at a light intensity of 5000 lux, *Navicula* sp. strain TAD was grown in a modified medium. Cells were grown for 13 days and showed different changes in culture color and cell density during growth (Figure 1). The color change of the culture on day 7 confirmed the change in cell density on the growth of *Navicula* sp. strain TAD, where the darker color of the culture indicates a higher number of cells and higher biomass productivity. The highest cell density was obtained on day 7 of $72.167 \times 10^5 \pm 0.946$ cells mL^{-1}.

Meanwhile, the decrease in cells occurred very significantly on day 8. This was because the nutrients in the culture were very low, resulting in a higher level of competition for nutrient absorption between microalgae. According to Telussa et al. (2019), the less nutrient content in the culture, the growth rate of microalgae will decrease.

(a)



(b)

Figure 1. Growth of *Navicula* sp strain TAD. (a) Culture color (days 0, 2,4,6,8,10) (from left to right)); (b). growth curve

Biomass Harvesting *Navicula sp.* strain TAD

Culture *of Navicula sp.* strain TAD were harvested on day 7, where the cells reached a maximum point with excellent cell shape in the exponential phase. The culture harvesting process was carried out using sedimentation and filtration techniques (Figure 2).

(a) (b) (c) (d)

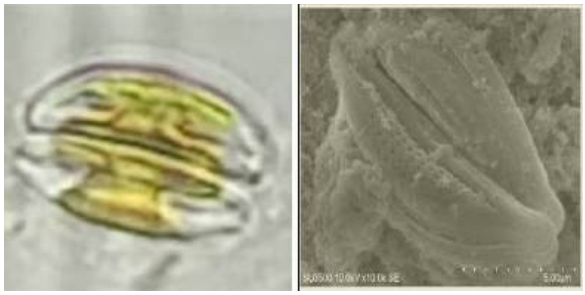
Figure 2. The process of harvesting biomass of *Navicula sp.* strain TAD. (a) Culture on the 7th day, (b) Filtration process, (c) Base biomass, and (d) Dry biomass.

The sedimentation technique was carried out for 30 minutes. The time involved in the sedimentation process is not too long because the cells of *Navicula* sp. strain TAD have a large cell shape and size, so it does not take much time in the sedimentation process (Santoso, 2017). The filtration technique uses a synthetic cloth to ensure the biomass can be separated from the culture. The harvest yield of

800 ml of culture was 0.933 ± 0.062 g of dry biomass with a yield of 0.166 ± 0.011 gL^{-1} h^{-1}.

Morphology of *Navicula* sp. strain TAD

Navicula sp. strain TAD is a microalgae derived from the diatom class, which is used as a raw material in bioethanol production. Cell Growth of *Navicula* sp. strain TAD during cultivation was observed through changes in culture color, cell density, biomass product, and cell morphology. Cell morphology observations were carried out using light microscopy and SEM. The light microscopy image showed the cell morphology of *Navicula* sp. strain TAD is oval (like the letter D) and yellow (Figure 3a). Observation of *Navicula* sp. strain TAD was also observed by SEM, which showed the specific characteristics of *Navicula* sp. Strain TAD with oval-shaped frustule with nanopores that lay on the surface for the exchange of nutrients or other materials (Figure 3b).

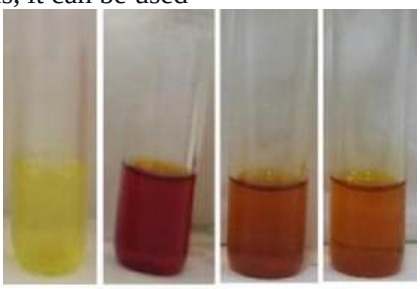


(a) (b)

Figure 3. Cells of *Navicula* sp. strain TAD. (a) Observation under a light microscope, (b) SEM Observations

Hydrolysis by an enzymatic and acid method

The hydrolysis was carried out by comparing the hydrolysis method between the enzymatic method (α-amylase enzyme, glucoamylase enzyme, cellulase enzyme) and acid on the dry biomass of *Navicula* sp. strain TAD to obtain a more efficient hydrolysis method for converting carbohydrates into simpler sugars. The results of the hydrolysis were tested using DNS (Figure 4) with a linear regression equation (y = 0.0038x-0.1023) from the glucose standard curve (Figure 5) so that the glucose concentration was obtained on acid hydrolysis, cellulase enzyme hydrolysis, α-amylase enzyme, and glucoamylase enzyme was 42.2842; 21.3806; 20,016 mg, respectively. These results indicate that hydrolysis using acid can break down cellulose in microalgae more efficiently (Qaishum, Amri, & Utami, 2015). The enzymatic hydrolysis process is more specific than the acid hydrolysis process. However, enzymatic hydrolysis requires pretreatment to increase enzyme breakdown. Treatment with acid is one of the good ways of hydrolysis because the hydrolysis process with acid can dissolve hemicellulose, reduce the crystallinity of cellulose and increase the material's porosity. Cellulose in microalgae can be converted into glucose by hydrolysis. From the results of the hydrolysis, it can be used



(a) (b) (c) (d)

Figure 4. DNS test samples. (a) aguades, (b) acid hydrolysis samples, (c) cellulase enzyme hydrolysis samples, and (d) α-amylase and glucoamylase enzymes hydrolysis samples

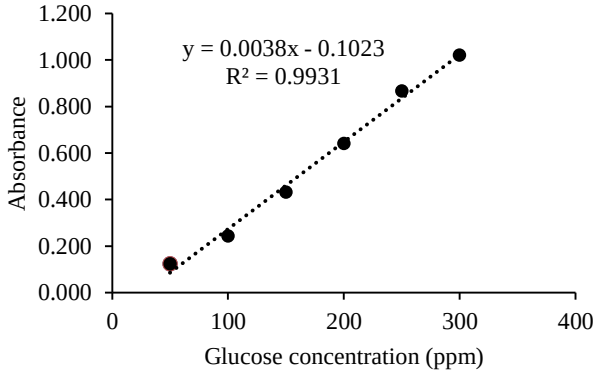


Figure 5. Glucose solution standard curve

In the hydrolysis process using the acid method, the hydrolysis conditions, like time and temperature, are very important factors that must be considered in analyzing the content of reducing sugars in a material/sample. Based on research conducted by Qaishum (2015), the content of reducing sugar produced with the time used for 50 minutes experienced a significant decrease. It is because the H^+ ion in the acid has reached its optimum point in unlinking the glycosidic chain in cellulose. Harun produced the same thing (2011), where at 45 minutes, the glucose content decreased. Therefore, the hydrolysis process was tested under conditions with a hydrolysis time of 30 minutes at 90 ^oC. In this condition, the reducing sugar content was three times higher (146.5695 mg) compared to the hydrolysis carried out for 1 hour at 110 ^oC (42.2842 mg). Thus, the next hydrolysis process will be carried out with a hydrolysis time of 30 minutes at a temperature of 90 ^oC using acid.

Fermentation Process

The fermentation process is a conversion step of glucose, fructose, and sucrose into ATP, ethanol, and CO_2 molecules. In this study, fermentation was carried out anaerobically, and used *Saccharomyces cerevisiae* to produce bioethanol. Biomass is fermented at pH 5 with a temperature of around 25-30 ^oC to obtain maximum fermentation results. In this study, the fermentation process was carried out for 3 and 5 days to increase bioethanol production. The fermentation results showed a decrease in the content of reducing sugars from the hydrolysis of *Navicula* sp. strain TAD after the fermentation process. It can be seen from the decreasing sugar content before and after fermentation on the 3rd and 5th days (Figure 6).

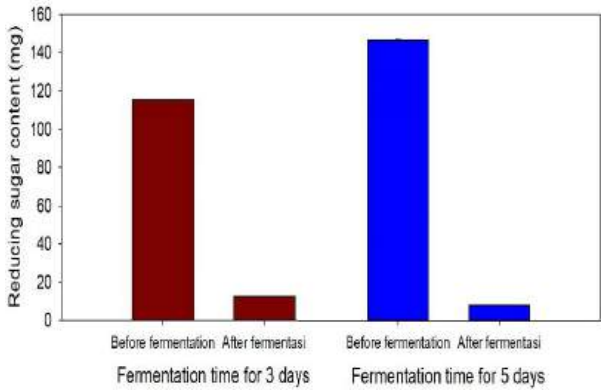


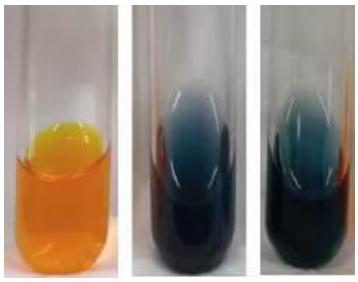
Figure 6. Histogram of reducing sugar content before (blue bar) and after (red bar) Fermentation.

In Figure 6, the reduced sugar content decreased from 146.569 ± 0.415 mg to 8.168 ± 0.134 mg. Based on the results of this study, it can be seen that the content of reducing sugar converted into bioethanol in 3-day fermentation is lower than that of 5-day fermentation. Research by Ho et al., (2013) in ethanol production from microalgae biomass shows that the glucose content will decrease as the ethanol content increases during the fermentation process. It shows that time is very influential on the content of bioethanol that will be produced, where a longer fermentation time will cause *Saccharomyces cerevisiae* to develop more so that it can convert sugar into higher ethanol.

Bioethanol Analysis

Bioethanol Qualitative Analysis

Qualitative analysis of the distillation of bioethanol from Navicula sp. strain TAD was conducted using $K_2Cr_2O_7$ to ensure the presence or absence of ethanol in the fermented distillate. $K_2Cr_2O_7$ will oxidize ethanol under acidic conditions to aldehyde compounds if the solution contains ethanol. According to Clark., (2019), the reaction that occurs when ethanol is oxidized, $K_2Cr_2O_7$, will undergo reduction, which is marked by the color of the solution, which was originally orange in color and will change to green/blue. The orange dichromate (VI) ion will be reduced to a green/blue chromium (III) ion. The test results on bioethanol samples from *Navicula* sp. strain TAD (Figure 7) using pure ethanol and aguades as comparison are marked by the final color of the reaction is green/blue, which indicates the formation of bioethanol during the fermentation process.



(a) (b) (c)

Figure 7. Results of bioethanol qualitative test. (a) Aquades, (b) Ethanol standard, (c) Bioethanol from *Navicula* sp. strain TAD

Bioethanol Characterization by FTIR

The results were obtained from the distillation process, then continued with qualitative analysis using FTIR (Fourier Transform Infra-Red). The spectrum of bioethanol test results from *Navicula* sp. strain TAD can be seen in Figure 8 and Table 1.

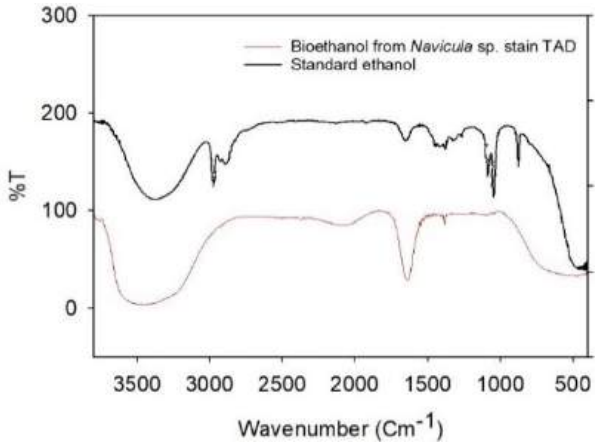


Figure 8. FTIR spectrum of bioethanol from *Navicula* sp. strain TAD

Based on the results, the fermentation product of *Navicula* sp. strain TAD strain contains ethanol, characterized by the absorption of –OH, CH_3, CH_2, and CO groups. The OH bond in alcohol absorbs at a

higher wave number than an acid, which is between 3230 - 3550 cm $^{-1}$. This absorption is even greater wavenumber if the alcohol does not contain hydrogen bonds as in the gaseous state. The absorption of CH bonds is slightly below 3000 cm $^{-1}$, and the absorption in the region at 1000 and 1100 cm $^{-1}$ is one from CO bonds.

Table 1. FTIR Data of Bioethanol from *Navicula* sp. strain TAD

Bond type	Wavenumber (cm^{-1})	
	Bioethanol of *Navicula* sp. strain TAD	Standard Ethanol
OH	3443	3445
C-H	2999	2998
C-O	1061	1050

Quantitative Analysis of Bioethanol Using GC

The bioethanol content of *Navicula* sp. strain TAD was determined using GC. The detector is used to analyze the flame ionization detector (Flame Ionization Detector); this detector is used to analyze the components that have CH alkyl groups in the sample. Determination of bioethanol content based on the linear equation Y = 384.52x obtained from the standard curve of ethanol between area and content (Figure 9).

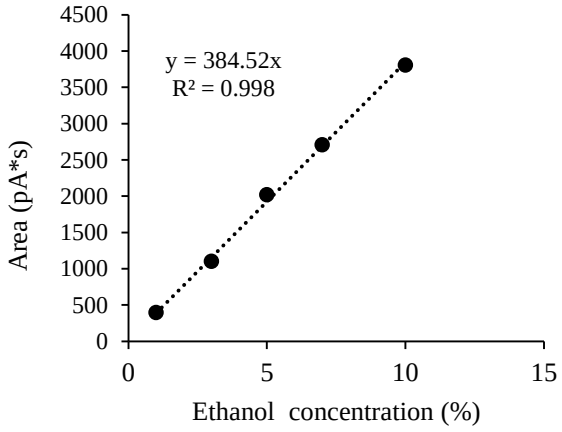


Figure 9. Graph of Relationship between Area and Ethanol Content

The results of the GC Chromatogram (Figure 10) show that the distillate sample has three peaks. The peak with a retention time of 3.151 is the peak of ethanol. Figure 10 shows that the distillation results of the bioethanol are not completely pure ethanol. The determination of ethanol content from the results showed that the determination of ethanol content using the GC-FID method and ethanol standards could provide good separation. In Table 2, the highest ethanol content is in the Navicula sp. strain TAD sample with an ethanol content of 6,357%, which was fermented for five days. This follows research by Baharuddin, Sappewali, Karisma, & Fitriyani (2016) on producing ethanol from rice straw and Dao tree bark with a fermentation time of 3 days to produce ethanol of 0.071 and 0.094 %, respectively.

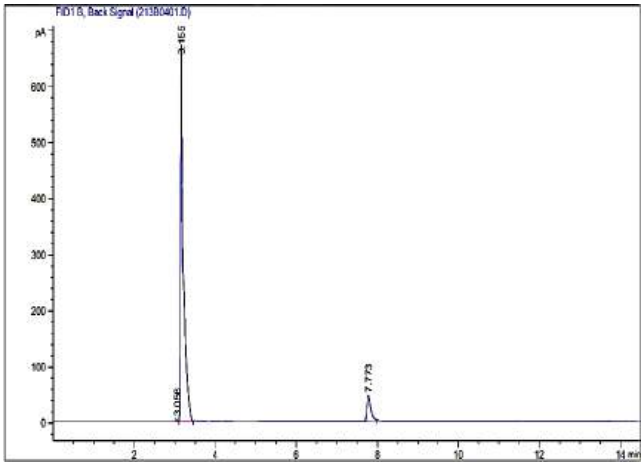


Figure 10. Chromatogram of GC bioethanol from *Navicula* sp. strain TAD

Table 2. Bioethanol content data from *Navicula* sp. strain TAD using GC

Fermentation time (Day)	Ethanol content (%)
3	0.0706
5	6.357

In comparison, the treatment with a fermentation time of five days produced ethanol of 0.122 and 0.292 %. This shows that for five days, glucose is more maximally converted into ethanol through Saccharomyces cerevisiae metabolism than fermentation for three days.

CONCLUSION

Navicula sp. strain TAD cultivation for seven days had the highest cell density of 72.167 × 105 ± 0.946 cells mL^{-1} with dry biomass of 0.933 ± 0.062 g and productivity of 0.166 ± 0.011 gL^{-1} hour $^{-1}$. The sulfuric acid hydrolysis process was analyzed for a sugar content of 146.5695 ± 0.758 mg. A fermentation process follows the hydrolysis results to produce bioethanol. The bioethanol content obtained was 6.357%.

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