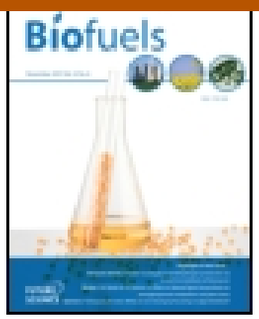

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Bioethanol production from algae *Spirogyra hyalina* using *Zymomonas mobilis*

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ABSTRACT

Algae Spirogyra have the potential to be developed as a raw material for bioethanol production. This study aims to determine the effect of the hydrolysis process of algae Spirogyra on total sugar production. The hydrolysis process of *Spirogyra hyalina* was carried out using a variation of heating duration and type of enzyme. This study also aimed to determine the effect of fermentation duration and the addition of nutritional fermentation on the reducing sugar, pH changes, microbial biomass and ethanol production from algae *Spirogyra hyalina* by using *Zymomonas mobilis*. The fermentation process was carried out in anaerobic conditions using a modified fermentor for 96 hours. Results showed the differences the hydrolysis process of algae *Spirogyra hyalina* affected on sugar levels. The highest sugar level was achieved by a combination of α-amylase and β-amylase enzymes. Fermentation duration and nutritional fermentation affected reducing sugar, pH substrate, microbial biomass and ethanol levels produced from the fermentation of algae Spirogyra by using *Zymomonas mobilis*.

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KEYWORDS
Algae; fermentation; ethanol; Spirogyra; *Zymomonas mobilis*

Introduction

The genus Spirogyra is a member of Zygnemataceae (Zygnematophyceae, Streptophyta) which is usually found in fresh water.[1−3] It comprises unbranched, filamentous green algae that are characterized by spirally coiled chloroplasts and sexual reproduction by means of conjugation.[4,5] Spirogyra algae have the potential to be a raw material for bioethanol production.[6,7] Spirogyra algae can be converted to ethanol through a process of hydrolysis and fermentation.[7,8] Hydrolysis of Spirogyra algae, which generally contains starch, effectively uses a single enzyme alpha amylase.[9] Starch should be hydrolyzed by the combination of the amylase enzyme.[10,11] Therefore, research on optimization of algae Spirogyra hydrolysis is important. Appropriate hydrolysis of algae Spirogyra will maximize the sugar levels which would then be fermented to produce bioethanol with maximum results.

Fermentation of algae Spirogyra using *Zymomonas mobilis* bacteria without the addition of nutritional fermentation can produce ethanol at 9.35% (v/v).[7] Nutritional fermentation is an important factor for the growth of microbial fermentation to produce ethanol.[12−14] Based on these facts, the effect of adding nutritional fermentation on the ethanol production from algae Spirogyra is important. This study aims to determine the effect of the hydrolysis process of algae Spirogyra on total sugar production. This study also aims to determine the effect of fermentation duration and the addition of nutritional fermentation on the reducing sugar, pH changes, microbial biomass and ethanol production from algae *Spirogyra hyalina* by using *Zymomonas mobilis*.

Materials and methods

Spirogyra hyalina strain

Algae Spirogyra used in this study was *Spirogyra hyalina*. *Spirogyra hyalina* was derived from wetlands in the Regency of Malang, East Java, Indonesia (GPS location: −8.012935 and 112.612991). The intake process of algae was done during the dry season, August−October 2015. Algae *Spirogyra hyalina* was identified using a microscope in a glass observation Sedgewick Rafter Cell based on indicators provided by Zarina et al.[15]

Spirogyra hyalina culture

Spirogyra hyalina was cultured in growth medium with 12 hours of light and 12 hours of dark using fluorescent lamp TL 36 W with 3.000 Lux of light intensity.[16] *Spirogyra hyalina* was cultured using sulfahri-01 medium (KN_3 40 mg/L, P_2O_5 30 mg/L, K_2O 30 mg/L, $MgSO_4$ 2 mg/L, $Ca(NO_3)_2.4H_2O$ 2 mg/L $Na_2EDTA.2H_2O$ 0.2 mg/L, $Mn_2Cl_2.2H_2O$ 0.15 mg/L, $FeCl_3.6H_2O$ 0.1 mg/L, Na_2CO_3 0.2 mg/L, $(NH_4)6Mo_7O_{24}.4H_2O$ 0.1 mg/L, H_3BO_3 0.1 mg/L, $CuSO_4.5H_2O$ 0.1 mg/L, $ZnSO_4.7H_2O$ 0.1 mg/L, Aneurine 0.5 ug/L, Lactoflavine 0.5 ug/L, Nicotinic acid

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amide 0.5 ug/L). *Spirogyra hyalina* was incubated in the growth medium for four weeks. Incubation was performed at room temperature, approximately 30°C. Algae *Spirogyra hyalina* was harvested by filtrating the water culture using a plankton net. *Spirogyra hyalina* was transferred onto filter papers and was allowed to stand for five minutes to absorb the excess water. The fresh biomass of *Spirogyra hyalina* was then used as a feedstock for bioethanol production.

Pretreatment process

Fresh biomass of *Spirogyra hyalina* was dried in an oven at a temperature of 80°C for 24 hours. *Spirogyra hyalina* which was dried than cut using blender machine until crushed and sieved to 40 mesh size sieve. A total of 130 grams of *Spirogyra hyalina* which passes 40 mesh sieve were added 1 liter of distilled water, then stirred. The blend was hydrolyzed in accordance with the study design.

Hydrolysis process

Spirogyra hyalina was put into an Erlenmeyer flask and heated on a hot plate. The heating process was carried out in accordance with the study design (0 minutes, 30 minutes, 60 minutes, 90 minutes, 120 minutes) with a heating temperature of 100°C and then cooled to 45°C. Some enzymes were added in accordance with the study design (α-amylase, β-amylase and combinations of α-amylase and β-amylase). The enzymes used were obtained from Liquozyme Supra, Novozymes, Denmark, as much as 8.1 KNU (Kilo Novo Unit). The incubation process was carried out for 80 minutes. Once hydrolyzed, the hydrolyzate was filtered using a filter paper to take the supernatant. The supernatant was then centrifuged at 9000 rpm for 15 minutes. After centrifugation the supernatant was sterilized, ready for use as the substrate of fermentation.

Starter preparation of Zymomonas mobilis

Zymomonas mobilis was inoculated into a 50 ml Erlenmeyer flask containing 5 ml of sterile *Spirogyra hyalina* hydrolyzate pH 5 by adding Buffer Na-citrate, then incubated in a rotary shaker with agitation speed of 15 rpm at 30°C for 24 hours (Activation I). A total of 1 ml of Activation I inoculate was placed in a 50 ml Erlenmeyer flask containing 9 ml of *Spirogyra hyalina* hydrolyzate, incubated in a rotary shaker with agitation speed of 15 rpm at a temperature of 30°C for 24 hours (Activation II). A total of 5 ml of Activation II was inoculated into a 100 ml Erlenmeyer flask containing 50 ml of *Spirogyra hyalina* hydrolyzate, incubated in rotary shaker with agitation speed of 15 rpm at a temperature of 30°C and incubated for 8 hours (log phase of *Zymomonas mobilis* in *Spirogyra hyalina* medium/fermentation substrate).

Fermentation process

Starter inoculum that was activated is was added as much as 5 ml (concentration of 10% with OD_{600}nm = 0.5) into a 100 ml fermenter bottle containing 50 ml of substrate *Spirogyra hyalina*. The substrate was enriched with Gandasil-D® fertilizer (N 20%, P_2O_5 15%, K_2O 15%, $MgSO_4$ 1%) and supplied with micro elements and vitamins such as Mn, B, Cu, Co and Zn, Aneurine, Lactoflavine, and Nicotinic acid amide with different concentrations (0.00 g/L, 0.05 g/L, 0.10 g/L, 0.15 g/L, 0.20 g/L, 0.25 g/L). The fermentation was conducted for various times (0 hours, 24 hours, 48 hours, 72 hours and 96 hours) at room temperature (±30°C). The fermentation process was carried out in anaerobic conditions without agitation by using a modified fermentor.

Measurement of biomass, reducing sugar and ethanol levels

Zymomonas mobilis cell biomass measurements were performed using the method of dry cell weight (DCW). Reducing sugar measurements were performed using the Luff Schoorl glucose refractometer method. Measurement of ethanol concentration was by specific gravity method and chromatography method.

Data analysis

The research design used was CRD (Completely Randomized Design). This study was conducted three times. The parameters measured were sugar levels, pH changes, microbial biomass, reducing sugars and ethanol levels. Data were analyzed statistically using analysis of variance (ANOVA) with level of confidence 95% ($\alpha = 0.05$). Analysis was carried out to determine the effect of the hydrolysis process of *Spirogyra hyalina* on total sugar production and the effect of fermentation duration as well as the addition of nutritional fermentation on the reducing sugar, pH changes, microbial biomass and ethanol production of *Spirogyra hyalina*. Further analysis after interpreting the interaction effects based on ANOVA was conducted with Tukey test with level of confidence 95% ($\alpha = 0.05$) to find the same pair of group data at each treatment.

Results and discussion

Effect of hydrolysis process

The hydrolysis process of *Spirogyra hyalina* was carried out by varying the heating duration and type of enzyme. The results show that the highest sugar level was achieved by the combination of α-amylase and β-amylase enzymes with an average sugar level of 12.60%. Next is α-amylase enzyme with an average sugar level of 9.00%. The sugar levels by treatment with β-amylase enzyme are lower than α-amylase

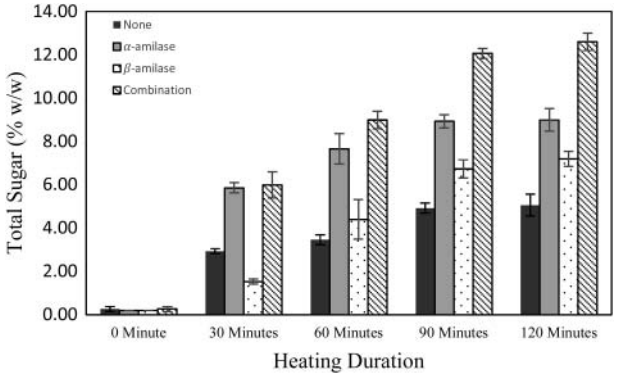

Figure 1. Effect of hydrolysis process on total sugar.

enzyme. Sugar level by treatment with β-amylase enzyme was 7.20%. The lowest sugar level occurred without enzyme treatment and was 5.07% (Figure 1). ANOVA analysis results at 95% confidence interval indicated that the type of enzyme and heating duration affected on sugar levels. Tukey analysis results at 95% confidence interval indicated that the sugar levels are significantly different for all types of enzymes. Thus, different types of enzymes provide a response to different sugar levels. Tukey analysis results at 95% confidence interval indicate that the sugar levels in the heating duration of 90 minutes and 120 minutes are not significantly different. The sugar levels on heating duration of 0 minutes, 30 minutes and 60 minutes were significantly different.

The average sugar levels obtained with the most effective and efficient treatment (combination of α-amylase and β-amylase enzyme with 90 minutes of heating duration) was is 12.07%. These results were higher than other studies which hydrolyzed *Spirogyra hyalina* using a single α-amylase enzyme for 120 minutes of heating duration, with a sugar content of 9.01%.[7] Starch was hydrolyzed by a combination of α-amylase and β-amylase enzymes.[10] The sugar levels that were obtained in this study were within the range that can be used for ethanol fermentation using *Zymomonas mobilis*. Sugar levels obtained in this study are in accordance with *Zymomonas mobilis* sugar ranges of 6.0−20.7%.[17-18]

Zymomonas mobilis biomass

Nutritional fermentation was added to the fermentation medium to optimize the growth of *Zymomonas mobilis* bacteria with different concentrations. Nutritional fermentation used was Gandasil-D® fertilizer as a source of NPK (chemical symbol for Nitrogen [N], Phosphorous [P], Potassium [K]) and micronutrients that was not presented in the fermentation medium, or presented in low concentrations in the fermentation medium. The results show that the highest biomass of *Zymomonas mobilis* bacteria reached is on nutritional fermentation of 0.20 g/L and 0.25 g/L which has an average biomass almost the same: 1.7973 (0.20 g/L) and 1.7893 (0.25 g/L) on 96 hours of fermentation duration (Figure 2). Next is nutritional fermentation of 0.15 g/L with an average biomass of 1.5793 g/L on 96 hours of fermentation duration. The average of *Zymomonas mobilis* biomass on 0.10 g/L of nutritional fermentation was 1.4320 g/L on 96 hours of fermentation duration. *Zymomonas mobilis* biomass on 0.05 g/L of nutritional fermentation is 1.2893 g/L on 96 hours of fermentation duration. The lowest biomass occurred without the addition of nutritional fermentation, with biomass only 1.660 g/L on 96 hours of fermentation duration.

The highest biomass of *Zymomonas mobilis* bacteria was reached on nutritional fermentation of 0.20 g/L (1.7973 g/L biomass) on 96 hours of fermentation duration. It is higher than other studies which show the highest biomass of *Zymomonas mobilis* in the medium sucrose (200 g/L) enriched with glucose 100 g/L, yeast extract 10 g/L, $(NH_4)\ 2SO_4$ 2 g/L, KH_2HPO_4 3 g/L, $MgSO_4.H_2O$ 0.3 g/L, peptone 0.5 g/L and $FeSO_4$ 0.2 g/L only 1.6500 g/L.[19] Nutritional fermentation with Gandasil-D® concentration of 0.20 g/L is a nutrient that can be used to enrich the fermentation medium from *Spirogyra hyalina*.

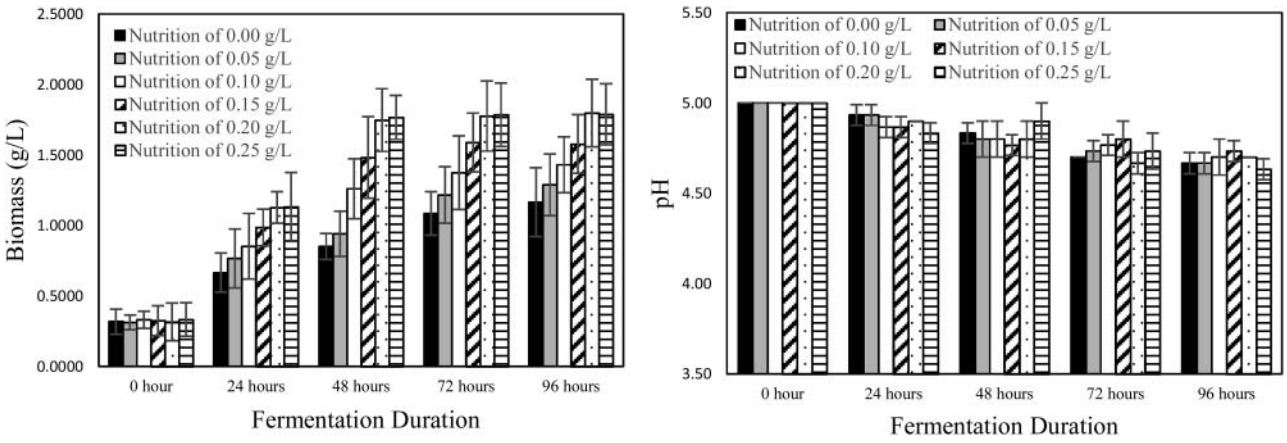

Figure 2. *Zymomonas mobilis* biomass (left) and pH change during fermentation (right).

pH change during the fermentation process

Changes in pH during the fermentation process were measured. The fermentation medium was adjusted to pH 5 by addition of Na-citrate buffer. *Zymomonas mobilis* bacteria used was also adapted to the fermentation medium with a pH of 5 for 3 times. The results show that pH fluctuates during the fermentation process. pH fluctuation generally shows a decreasing trend with increasing fermentation duration. Decreasing pH levels occurred during the fermentation process until the end of the fermentation (96 hours). The lowest decreasing pH level occurred on addition of nutrients of 0.25 g/L (pH 5 to pH 4.63). The highest decreasing pH level occurred on treatment without the addition of nutrients (pH 5 to pH 4.67) (Figure 2).

During the fermentation process, *Zymomonas mobilis* will produce various acids, such as pyruvic acid and acetaldehyde,[20] that will affect the pH of the fermentation medium.[21,22] The more acid formed causes a decrease in pH, also higher. The decreasing pH did not significantly occur during the fermentation process until the end of the fermentation (96 hours). The highest decreasing pH occurred in a condition of 0.25 g/L. There is no significant decreasing pH due to the Na-citrate buffer 0.1 M. pH range of cultures in all the fermentation medium in this study is in optimal conditions of *Zymomonas mobilis* bacteria, which is in the range of pH 4 - pH 5.[7,9,17]

Reducing sugar

Reducing sugar during the fermentation process was monitored to view the reducing sugar consumption by *Zymomonas mobilis* bacteria. Reducing sugar levels continued to decrease with increasing fermentation duration. The average of reducing sugar levels at the beginning of the fermentation duration was 12.71%, and continued to decrease concurrently with the fermentation duration (Figure 3). Reducing sugar levels at the end of the fermentation duration had reached 1.01±0.09% (Table 1).

From Table 1, the highest decrease in reducing sugar levels occurred on the fermentation medium to which was added fermentation nutrition of 0.25 g/L with the use of 91.93%, and fermentation medium to which was added nutrition of 0.20 g/L with the use of 91.54%. The lowest consumption of reducing sugar occurred in the fermentation medium that had no added nutritional fermentation, which the reducing sugar consumption was 70.65%. Reducing sugar is an important factor for the *Zymomonas mobilis* cells as a source of energy for metabolism, affecting the levels of ethanol produced.

Reducing sugar continuously decreased with increasing fermentation duration. The average reducing sugar in the initial fermentation was 12.71%, and continued to decrease as time increased. The more reducing sugars that were used by the microbial cells, the higher the levels of ethanol produced.[23,24] The use of the higher reducing sugar will produce higher ethanol levels.[25,26] The same results were reported by other studies, that the higher ethanol levels lead to the decrease in reducing sugar contained in the fermentation substrate.[9] *Zymomonas mobilis* bacteria converted the reducing sugar into ethanol, biomass and CO_2 gas.[27]

The consumption of reducing sugar in this study (91.93%) was lower compared to other studies which also used *Spirogyra hyalina*, with the use of reducing sugar of 96.61% in 72 hours of fermentation duration with the addition of nitrogen gas in the space fermenter.[7] Therefore, the addition of nitrogen gas in the space fermenter is more effective in triggering the use of reducing sugar than enriching of nutritional fermentation as shown in this study. This was supported by other studies.[28]

The consumption of reducing sugar was lower, but the levels of ethanol in this study were higher, than other studies.[7,9] The initial reducing sugar in this study was higher by 35.65% than other studies.[9] Reducing sugar levels in this study were higher due to

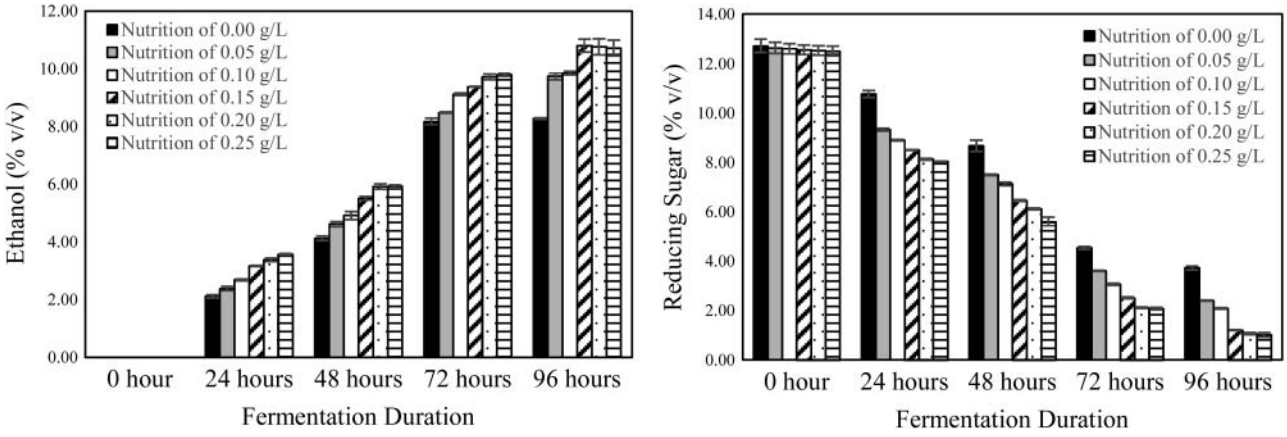

Figure 3. Ethanol levels (left) and reducing sugar levels (right).

Table 1. Average of reducing sugar at the beginning and at the end of fermentation.

Nutrition (g/L)	Initial reducing sugar (0 hour)	Final reducing sugar (96 hours)	Reducing sugar consumption
0.00	12.71±0.27	3.73±0.08	70.65%
0.05	12.63±0.23	2.40±0.02	81.00%
0.10	12.60±0.20	2.08±0.03	83.49%
0.15	12.55±0.19	1.20±0.02	90.44%
0.20	12.53±0.20	1.06±0.04	91.54%
0.25	12.51±0.20	1.01±0.09	91.93%

the modification of the hydrolysis process. Modifications were done by using a combination of α-amylase and β-amylase enzymes that leads to increased reducing sugar levels. The higher initial reducing sugar causes *Zymomonas mobilis* bacteria to produce more ethanol.

Ethanol fermentation

Increased ethanol levels occurred with increased fermentation duration. Ethanol levels in the medium were 0% for all fermentors at the beginning of fermentation (0 hours). Ethanol levels ranged from 2.12−3.18% in 24 hours of fermentation duration. Ethanol levels ranged from 4.13−5.93% in the 48 hours of fermentation duration. Ethanol levels ranged from 8.18−9.60% in the 72 hours of fermentation duration. Ethanol levels ranged from 8.28−10.77% at the end of the fermentation (96 hours) (Figure 3). ANOVA analysis results at level of confidence 95% suggests that nutritional fermentation and fermentation duration are the effect of the ethanol levels produced. Tukey analysis results at level of confidence 95% shows that the ethanol levels in the fermentation culture that were added nutrients of 0.20 g/L and 0.25 g/L is not significantly different. This shows that the addition of nutritional fermentation as much as 0.20 g/L can be considered as the best treatment, because the levels were not significantly different from ethanol with nutritional 0.25 g/L.

Zymomonas mobilis biomass and ethanol levels increased along with increasing fermentation duration. This is supported by other studies, that the ethanol fermentation is fermentation type of growth associated, namely the higher cell growth will cause the higher product of the cell.[29−31] The results showed that nutritional fermentation was affect to the levels of ethanol. Highest ethanol concentration was achieved with the addition of nutritional fermentation of 0.20 g/L. This shows that the hydrolysis of *Spirogyra hyalina* requires nutrient enrichment to optimize the formation of ethanol levels. Without the addition of nutritional fermentation, the highest the ethanol levels reached was only 8.28%. There is a positive correlation between the addition of nutrients such as nitrogen to the production of ethanol from *Zymomonas mobilis* bacteria. [19,28,32] The highest ethanol level achieved in the 96 hours of fermentation duration is 10.77%. These results are higher than the research of Sulfahri et al., which also used *Spirogyra hyalina*, with the highest ethanol yield of 9.70% in the 96 hours of fermentation duration.[7,9] Higher yield can be due to the difference in the substrate fermentation and the difference in fermentation conditions.

Ethanol fermentation by *Zymomonas mobilis* bacteria with the fixation of nitrogen gas in the space fermentor can increase ethanol production up to 97% and accelerate ethanol production up to 50%.[28] Fixation of nitrogen in the space fermentor will accelerate the fermentation duration.[9] The results of this study show that the additional nutrition in substrate fermentation increased the ethanol levels up to 30.07%. Therefore, the treatment combination between nitrogen fixation in space fermentor and the addition of nutritional fermentation are important for future research.

Conclusions

The differences of the hydrolysis process of *Spirogyra hyalina* affects the glucose levels that can be utilized by *Zymomonas mobilis* bacteria. The best result of hydrolysis process is the combination of α-amylase and β-amylase enzymes and 90 minutes of heating duration with average sugar levels of 12.60%. The differences in fermentation duration and nutritional fermentation was the effect on reducing sugar level, pH substrate, biomass and ethanol levels that were produced from the fermentation of *Spirogyra hyalina* using *Zymomonas mobilis*. The results show that the highest levels of ethanol were achieved on addition of 0.20 g/L of nutritional fermentation within the 96 hours of fermentation duration, with the ethanol levels reaching 10.77%.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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