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Evaluation of the Potential of *Chlorella vulgaris* for Bioethanol Production

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Supporting Information

ABSTRACT: For bioethanol to be a sustainable transportation fuel, appropriate feedstock needs to be established. The focus of the current work is to evaluate if the microalga *Chlorella vulgaris* could be the feedstock of choice. Exclusive formation of glucose was observed upon the acid (HCl) hydrolysis of *C. vulgaris*. Microwave irradiation as well as hydrothermal reaction were employed as heating methods. Under optimal hydrolysis conditions using microwave irradiation (100 °C, 1 M HCl, and 10 min), the glucose yield was 20 ± 3.5 wt % compared to 23 ± 4 wt % under the optimal hydrothermal reaction conditions (120 °C, 1 M HCl, and 60 min). The hydrothermal-based hydrolysis process was further scaled up from a 0.2 g batch to a 2.0 g batch, and the glucose obtained was converted to bioethanol in a fermentation process at 30 °C for 28 h using *Saccharomyces cerevisiae*. An ethanol yield as high as 13.2 ± 0.5 wt % was obtained from *C. vulgaris*.

1. INTRODUCTION

Fast growth of the world population and rapid development of emerging economies have led to a sharp increase in global energy consumption.[1] The world's major energy resources, namely, fossil oils, are being depleted.[2] This situation prompts research for alternate sustainable energy sources.[3] Biomass is one of the promising renewable resources used to generate different types of biofuels (biodiesel and bioethanol).[4,5] Bioethanol is a potential transportation fuel that could substitute fossil-based fuels.[6] Consumption of biofuels (bioethanol and biodiesel) is increasing. By 2050, biofuels will account for 27% of the world's transportation fuel.[7] Use of lignocellulose materials (e.g., rice straw and switch grass)[8] and algae as feedstock are in the exploratory stages, requiring intense research.[9] Green seaweed *Ulva*, which proliferates fast and occurs abundantly worldwide, was used by Trivedi et al. as a feedstock for production of bioethanol by enzymatic hydrolysis.[10] In addition, methods of isolation of marine microbes capable of hydrolyzing cellulose-rich green seaweed *Ulva fasciata* for bioethanol production were also studied.[10] Even though the use of agricultural crops or agricultural waste as feedstock for bioethanol production is advantageous from energy and environmental perspectives, several problems need to be addressed. The cost involved in the conversion of lignocellulosic materials into ethanol is relatively high, owing to the necessity of pretreatment. This is due to the high lignin content in the lignocellulosic biomass, making the saccharification (hydrolysis) process difficult.[11]

Algae are a large group of simple photosynthetic phytoplankton. Algae can be divided into two major categories based on their size. Microalgae are small free-living microorganisms that can be found in a variety of aquatic habitats. Algae, considered as the third-generation biomass, have proven to be superior to any other biomass as a result of their environmental and economic sustainability. Among several biomass feedstocks, marine algae hold promise as an alternate, renewable feedstock for the production of biofuels, especially bioethanol. Use of algae as feedstock has several advantages over terrestrial biomass because of their high productivities (estimates of 5200−7500 gallons acre^{-1} year^{-1}), short life cycle, use of marginal or non-arable land, and avoidance of feedstock and food conflict. In comparison to other advanced cellulosic feedstock for biofuel production, algal genomics and basic research are more advanced and gaining momentum.[12] *Chlorella* have high photon conversion efficiency and can synthesize and accumulate large quantities of carbohydrate biomass for bioethanol production.[13] The high carbohydrate content makes *Chlorella* (20−30 wt % dry) a potential feedstock.[14]

Chlorella vulgaris is capable of accumulating a high content of lipids that could be converted to different forms of "drop-in" fuels, such as biodiesel.[15−18] During the past decade, several researchers were actively involved in the evaluation of the feasibility of algae, for instance, *Chlorella*, for bioethanol production.[19−22]

A comparison of saccharification conditions, such as the temperature, acid concentration, pH, and duration, using three different algae species with ethanoic *Escherichia coli* W3110 strains was made.[19] Zhou et al. suggested *Chlorella* sp. TIB-A01 as a potential feedstock for ethanol, yielding a sugar concentration of 12 wt % and ethanol amount of 0.47 g/g of sugars.[19] Some of the recent work on the conversion of biomass to bioethanol is summarized in Table 1.

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Table 1. Recent Developments in the Conversion of *Chlorella* sp. to Bioethanol

microalgae	hydrolysis condition	sugar yield	fermentation condition	ethanol yield	reference
C. vulgaris FSP-E	enzymatic hydrolysis (cellulase and amylase enzymes)	0.461 g/g of biomass	simultaneous saccharification and fermentation	11.7 g L^{-1} and an 87.6% theoretical yield	9
Chlorella sp. TIB-A01	hydrothermal process (180 °C for 10 min and 120 °C for 60 min) with HCl–$MgCl_2$	12% (total reducing sugars)	ethanologenic fermentation of *Chlorella* hydrolysates (*S. cerevisiae*)	0.47 g g^{-1} of sugar	19
C. vulgaris	enzymatic treatment (35–55 °C and pH 3.6–5.6 for 60 min)	0.14 g of sugar/g of biomass	5 L fermentor (containing 2 L of LB media with 20 g/L glucose pretreated *C. vulgaris* sugar)), four different ethanolic *E. coli* W3110 strains used	0.4 g g^{-1} of biomass	21
C. vulgaris cake	enzymatic hydrolysis (mixture of cellulases, amylases, and endoglucanases and 60 °C for hydrolysis)	0.55 g g^{-1} of cake	fermentation with *S. cerevisiae* at 33 °C for 24 h	0.17 g g^{-1} of cake	23
Chlorella biomass (*C. vulgaris*)	hydrolysis: 3 h dissolution in [Emim]Cl and then 3 h hydrolysis with 7 wt % HCl at 105 °C, 75% of *Chlorella* biomass	~90% of total sugar released	fermentation with *S. cerevisiae*, Y01 *cerevisiae* at 30 °C	20.53 g L^{-1} ethanol from *E. coli*, 56 g L^{-1} of glucose in hydrolysate after 24 h	24
C. vulgaris	hydrolysis: microwave (5 min at 90 °C) and hydrothermal (1 h at 120 °C), 1 M HCl	24 ± 4 wt % glucose	fermentation with *S. cerevisiae*, at 30 °C	13 wt %	present study

Unlike the work of Zhou et al.,[19,24] our work presented here avoids the use of either $MgCl_2$ or ionic liquids that facilitates the release of fermentable sugars or dissolution of biomass, respectively. Moreover, the use of microwave irradiation and the hydrothermal reactor in the present report facilitate faster release of fermentable sugars from *C. vulgaris*. Microwave irradiation accelerated the release of fermentable sugar from the biomass. Use of microwave irradiation saves time and energy because the target compounds are heated directly without the requirement of heating the entire furnace or oil bath as required in conventional methods of heating. Localized heating during microwave irradiation causes biomass degradation, resulting in the catalytic species to access the reaction site and lead to the release of the monosaccharides.[25] Koberg et al. made a detailed study of the energy consumption for a transesterification process under microwave (batch as well as continuous flow) versus conventional heating methodology. It was found that the energy consumption in a microwave batch process is of the same order of magnitude as that of conventional heating in an air oven but consumes 3 times lower energy in a continuous flow microwave-based process, signifying the benefits of using the microwave-based process.[26]

The aim of the current research is to develop a fast and environmentally benign process for bioethanol production from *C. vulgaris*. Production of biofuels and value-added chemicals from algae is promising, but technological innovation in the conversion of algae to fuels and chemicals is awaited. Herein, we report a rapid method for the hydrolysis of *C. vulgaris* to glucose in the presence of HCl. The glucose thus produced was subsequently converted to ethanol using baker's yeast (*Saccharomyces cerevisiae*).

2. MATERIALS AND METHODS

Freshwater microalga *C. vulgaris* was grown outdoors in an open pond using Bristol's medium [2.94 mM $NaNO_3$ (Fisher BP360-500), 0.17 mM $CaCl_2{\cdot}2H_2O$ (Sigma C-3881), 0.3 mM $MgSO_4{\cdot}7H_2O$ (Sigma 230391), 0.43 mM K_2HPO_4 (Sigma P 3786), 1.29 mM KH_2PO_4 (Sigma P 0662), and 0.43 mM NaCl (Fisher S271-500) in H_2O].[27] Algal biomass was harvested, dried in a sunbath for 24 h, and ground into a fine powder using mortar and pestle. The microalgae thus produced were subsequently used for bioethanol production. Aqueous HCl (99.9% pure and 36 wt %) was purchased from Sigma-Aldrich, Ltd. Baker's yeast was procured from a supermarket. The materials were used without further purification.

Hydrolysis of *C. vulgaris* was carried out in a commercial microwave oven (MARS, CEM) operated at 1200 W and 100% power. Typically, known amounts of *C. vulgaris* (e.g., 0.2 g), and HCl (e.g., 1–5 M, 5 mL) were taken in a polytetrafluoroethylene (PTFE)-lined reactor of 30 mL volume followed by microwave irradiation. The reaction conditions, such as the microwave irradiation time (5–30 min), temperature of hydrolysis (80, 100, and 120 °C), and concentration of the catalyst (0.25–5 M) were optimized to obtain the highest possible yields of glucose. In addition to microwave irradiation, the hydrolysis process was also carried out by hydrothermal means in a PTFE-lined stainless-steel autoclave at varying temperatures (80, 100, and 120 °C). The hydrolyzate obtained by both techniques was qualitatively analyzed for fermentable sugars using ^{13}C nuclear magnetic resonance spectroscopy (NMR, Bruker Advance DPX 300 instrument, with D_2O as a solvent) and quantified using high-performance liquid chromatography (HPLC) analysis.[28,29] The residual algae that did not react were dried under vacuum at room temperature overnight. The weight percent conversion of the algae was calculated from the difference in the weight of the initial and final biomasses.[29] The fermentation reaction to convert the fermentable sugars to ethanol was carried out at 30 °C for 28 h in an incubator using *S. cerevisiae*. Prior to fermentation, the pH of the hydrolyzate was adjusted to 6 using 1 M

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NaOH. A total of 2 g of yeast (baker's yeast) was added to the neutralized hydrolyzate (35 mL from 2 g of algae), and the fermentation reaction was initiated. Thus, the fermentation medium comprises of 35 mL of the neutral hydrolyzate to which 2.0 g of baker's yeast was added. The flasks were closed with a cotton plug and placed in an incubator.[30] Ethanol production was monitored as a function of time using HPLC and ^{1}H NMR.[31] D_2O was used as the solvent for ^{1}H NMR analysis. HCOONa is used as an internal reference to quantify the amount of ethanol formed as a function of time. HPLC analysis was carried out on a Merch-Hitachi LaChrom system L-7000 equipped with a L-7455 diode array detector and a Schambeck SFD R1 2000 refractive index detector, Bad Honnef, Germany, using a 300 × 7.8 mm Rezex-ROA ion-exclusion chromatography column (Torrance, CA) equipped with a matching guard column.

3. RESULTS AND DISCUSSION

3.1. Hydrolysis of *C. vulgaris* under Microwave Irradiation. *3.1.1. Effect of the Reaction Temperature on the Hydrolysis of C. vulgaris.* The microwave irradiation (10 min) was carried out at different temperatures (80, 100, and 120 °C) with 1 M HCl. The hydrolysate obtained in each case was analyzed using ^{13}C NMR for the identification of the fermentable sugars. ^{13}C NMR is a unique qualitative analytical tool for carbohydrate analysis. Fine details of the chemical nature of the hydrolyzate could be deduced from the ^{13}C NMR spectra. Judicious interpretation and distinct identification of individual monosaccharides, such as xylose and glucose, which are usually generated from biomass hydrolysis, could be possible using ^{13}C NMR analysis.[32] In addition, the degradation products of sugars, such as levulinic and formic acids, if any, present in the hydrolyzate could also be identified from their fingerprint signals using this analytical tool.[33]

At the lower reaction temperature (80 °C), no glucose was observed in the hydrolyzate. A glucose yield of 20 ± 4 wt % was obtained with an increase in the reaction temperature to 100 °C. A further increase in the reaction temperature to 120 °C did not result in any increase in the glucose yield. The results indicate that a reaction temperature of 100 °C and an irradiation time of 10 min with 1 M HCl are the optimal hydrolysis conditions, and the corresponding ^{13}C NMR spectrum of the hydrolyzate is depicted in Figure S1 of the Supporting Information.

Each signal in the spectrum is attributed to a specific carbon nuclei of glucose.[31] Peaks typical of glucose [61.2 (C6), 76.1 (C5), 69.5 (C4), 73.1 (C3), 71.8 (C2), 92.5 (C1α), and 96.5 (C1β)] are observed in the hydrolyzate, indicating the effectiveness of microwave irradiation for the fast release of fermentable sugar.

When the algae are irradiated for longer times (30 min), degradation products of glucose, namely, levulinic and formic acids, were observed in the hydrolyzate and no trace of glucose was seen (Figure S2 of the Supporting Information). Irradiation of the algae for less than 10 min yielded lower amounts of glucose, as evident from the poor signal-to-noise ratio of the glucose signals in the ^{13}C NMR spectra (Figure S3 of the Supporting Information).

3.1.2. Effect of the Concentration of HCl. The hydrolysis of *C. vulgaris* was carried out with HCl of varying concentrations (0.25−5 M) at 100 °C with 10 min of microwave irradiation. With the lowest concentration of HCl, the hydrolysis reaction did not proceed and no trace of glucose was observed in the hydrolyzate. Even though glucose could be produced from the algae with 0.5 M HCl, the signal-to-noise ratio in the ^{13}C NMR spectrum of the hydrolyzate is low as a result of the low glucose concentration. A significant amount of glucose (20 wt %) could be produced when 1 M HCl was used, and at higher concentrations, no further improvement in the glucose yield was observed.

3.2. Hydrolysis of *C. vulgaris* under Hydrothermal Conditions. *3.2.1. Effect of the Temperature of the Hydrothermal Hydrolysis Process.* In addition to microwave irradiation, the effect of the hydrothermal mode of heating on the hydrolysis of *C. vulgaris* was evaluated. The hydrothermal reaction was carried out at different reaction temperatures (80, 100, and 120 °C) for a duration of 1 h in the presence of 1 M HCl. At the lowest reaction temperature (80 °C), only traces of glucose could be observed in the hydrolyzate (Figure S4 of the Supporting Information). With an increase in the reaction temperature to 100 and 120 °C, glucose yield values of 18 ± 3 and 23 ± 4 wt % were observed, as deduced from the HPLC analysis. A maximum glucose yield value of 23 ± 4 wt % could be obtained at 120 °C. Reaction temperatures above 120 °C were not tested because the present study is a comparison between microwave and hydrothermal hydrolysis processes and the optimal temperature in the former was 100 °C. Moreover, higher reaction temperatures might cause the degradation of glucose to levulinic acid.[33] In addition, the exclusive presence of glucose was observed in the hydrolyzate obtained at 120 °C, showing the selective nature of the hydrolysis process (Figure 1). A representative HPLC trace for the hydrolyzate obtained

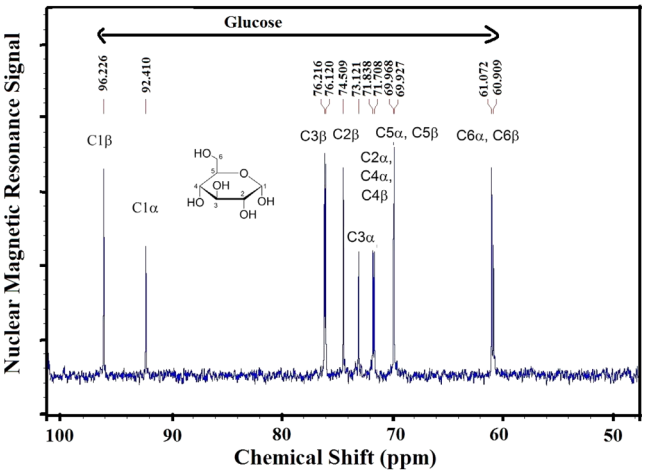

Figure 1. ^{13}C NMR spectrum of the hydrolyzate obtained under hydrothermal conditions (120 °C, 1 M HCl, and 1 h).

under the optimal hydrothermal conditions (120 °C, 1 M HCl, and 1 h) is shown in Figure S5 of the Supporting Information.

3.3. Upscaling Studies on the Hydrolysis of *C. vulgaris* under Hydrothermal Conditions. The purpose of upscaling the hydrothermal reaction to higher batches is to subsequently use the hydrolyzate for producing bioethanol through fermentation. Such an upscaling would not only facilitate the elimination of experimental errors in the quantification of the bioethanol product but also demonstrate the applicability of the hydrolysis process for the conversion of larger amounts of algal biomass. Both the hydrothermal- and microwave-based hydrolysis processes resulted in the selective conversion of *C. vulgaris* to glucose. The microwave-based hydrolysis process is faster, whereas hydrothermal process yielded a slightly higher glucose relative to the microwave-based process. Hydrothermal-

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based processes are well-established at the industrial scale, whereas large-scale production process based on microwave irradiation are at the incipient stages. A minimum of a kilogram quantity of algae would be required to obtain consistent results in the microwave flow process. Owing to the limitation of the small quantity of *C. vulgaris* harvested, the upscaling of the hydrolysis process was studied only using the hydrothermal process. The hydrothermal-based hydrolysis process has been scaled up from 0.2 to 2.0 g. The hydrolysis reaction was carried out at 120 °C for 2 h using 1 M HCl. The ^{13}C NMR spectrum of the hydrolyzate showed the exclusive presence of glucose (Figure 2), with a yield of 24 ± 4 wt %. Signals characteristic of α and β isomers of D-glucose were clearly distinguished from the ^{13}C NMR of the biomasss hydrolysate (Figure 2).

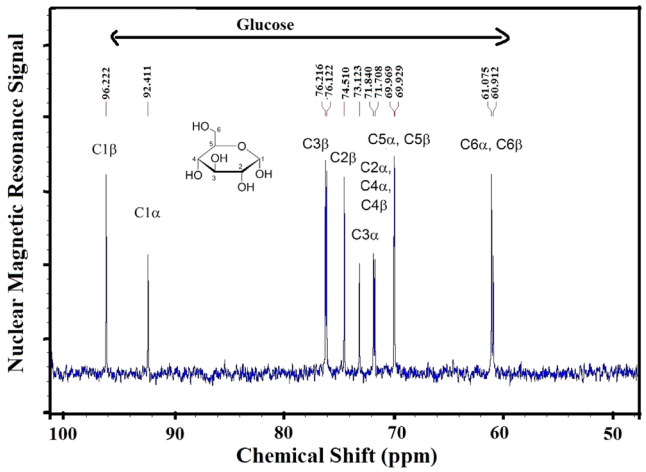

Figure 2. ^{13}C NMR spectrum of the hydrolysate from 2 g of *C. vulgaris* under hydrothermal reaction conditions (120 °C, 1 M HCl, and 2 h).

Even though advanced technologies, such as high rate ponds (HRPs) and photobioreactors (PBRs), were now available for the large-scale cultivation of microalgae (*C. vulgaris*, *Spirulina*, etc.), large quantities of freshwater consumption and poor photosynthetic conversion efficiencies are a major challenge for the sustainable use of microalgae as a feedstock for bioethanol production. However, it should be noted that the photosynthetic conversion efficiencies of microalgae (up to 3.4%) are comparable to that of the macroalgae (3.0%) and an order of magnitude higher than that of the terrestrial crops, such as sugar cane (0.4%).[34] Even though algal systems are more expensive at the moment to establish, operate, and harvest compared to terrestrial biomass, the problem could be surmounted by developing strategies for selectively isolating valuable compounds, such as ω-3, which are contained in algal biomass. Cost-effective strategies for the isolation and purification of valuable compounds, such as ω-3, need to be developed. In addition, after the use of the carbohydrate content of *Chlorella* for bioethanol production, the leftover residue comprising mostly of lipids could be used for biodiesel production, making the biofuel production process more efficient and cost-effective.

3.4. Bioethanol Production from *C. vulgaris*. The hydrolyzate produced with 2.0 g of algae under hydrothermal conditions was subjected to fermentation to convert the fermentable sugars to ethanol. As the hydrolysis reaction was carried out under acid conditions (1 M HCl), the pH of the hydrolyzate was adjusted to 6 using 1 M NaOH prior to fermentation because the yeast performance would be the best in the pH range of 4−6. Aliquots of samples from the fermentation broth were collected at regular intervals, and the ethanol was quantified using HPLC.

The amount of ethanol produced from the fermentable sugars was evaluated as a function of time. In 3 h, an ethanol yield of 4.5 wt % could be obtained, and it reached a maximum value of 13.2 ± 0.5 wt % in 24 h (Figure 3). The analyte at the

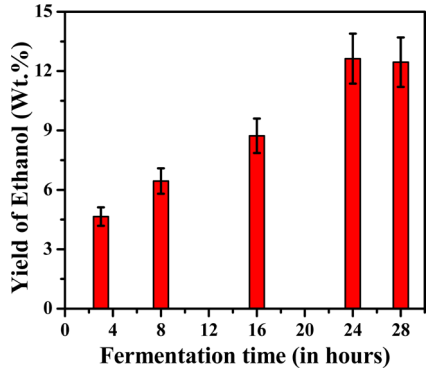

Figure 3. HPLC evaluation of the time course of fermentation of the hydrolysate from *C. vulgaris* [fermentation conditions: 35 mL of neutral hydrolysate, 2 g of baker's yeast, incubation at 30 °C, and 28 h; replicates of n = 3; error bars indicate standard deviation (SD)].

optimal fermentation reaction time (24 h) was further analyzed using ^{1}H and ^{13}C NMR. The ^{1}H NMR spectrum of the aliquot of the sample collected from the fermentation broth toward the end of the fermentation (24 h) is shown in Figure 4.

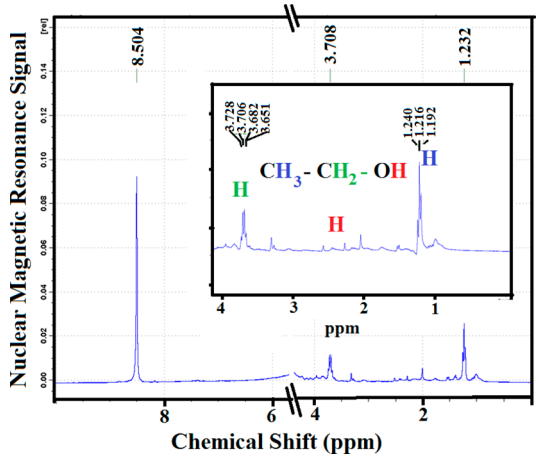

Figure 4. ^{1}H NMR spectrum of the aliquot of sample collected from the fermentation broth at 24 h (hydrolysis conditions, 2 g of algae, hydrothermal treatment at 120 °C, 1 M HCl, and 2 h; fermentation conditions, 35 mL of neutral hydrolysate, 2 g of baker's yeast, incubation at 30 °C, and 28 h).

The presence of 3H (t, 1.2 ppm) and 2H (q, 3.7 ppm) signals indicate the formation of ethanol. The signal at 8.5 ppm was characteristic of HCOONa used as an internal standard. The amount of ethanol in the broth at 24 h was found to 13 wt % from the relative integral values of ethanol (3H t, 1.2 ppm) and HCOONa (H s, 8.5 ppm) (Figure 4). A detailed method of estimation of bioethanol from the hydrolysate of *C. vulgaris* using ^{1}H NMR spectroscopy was provided in the Supporting Information.[28]

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The presence of ethanol and the complete disappearance of fermentable sugars in the fermentation broth in the 24 h aliquot is depicted in the ^{13}C NMR spectrum (Figure 5). The presence

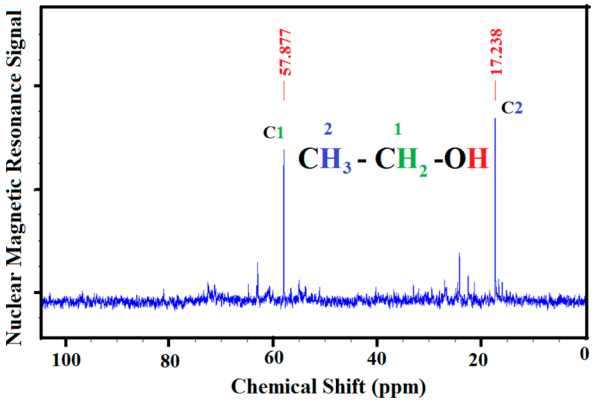

Figure 5. ^{13}C NMR of the aliquot of the sample from the fermentation broth at 24 h (hydrolysis conditions, 2 g of algae, hydrothermal treatment at 120 °C, 1 M HCl, and 2 h; fermentation conditions, 35 mL of neutral hydrolysate, 2 g of baker's yeast, incubation at 30 °C, and 28 h).

of ethanol is confirmed from the peaks located at 17.2 and 57.8 ppm, which are characteristic of ethanol. The peaks at 62 and 72 ppm were typical of glycerol, which is a secondary metabolite formed during the fermentation of glucose.

Interestingly, the signals typical of glucose in the region of 60−100 ppm were absent, indicating the complete consumption of the fermentable sugars in 24 h, which is in accordance with the HPLC analysis (Figure 5). Thus, the maximum yield of ethanol that could be produced from the microalgae *C. vulgaris* is 13 wt %, corresponding to 0.46 g of bioethanol/g of glucose, and this value is close to the theoretical yield (0.51 g of bioethanol/g of glucose).[30]

4. CONCLUSION

Selective production of glucose from *C. vulgaris* is achieved in acid-catalyzed hydrolysis processes. Use of microwave irradiation for the hydrolysis process facilitated faster production of glucose (20 ± 3.5 wt %) from *Chlorella*. In comparison to the microwave-based hydrolysis process, hydrothermal-based acid hydrolysis yielded a slightly higher glucose (23 ± 4 wt %). Microalgae could be a promising feedstock for bioethanol production. Exclusive production of glucose is possible with algae because they are devoid of lignin and hemicellulose components that are usually present in terrestrial biomass.[28] Moreover, the absence of lignin is advantageous because no pretreatment is required. In addition, the absence of hemicellulose means the absence of acetic acid in the hydrolyzate. Hemicellulose was found to be the source of acetic acid during biomass conversion.[33] Acetic acid is a known fermentation inhibitor, and for algae biomass, the inhibition of fermentation is not encountered. In summary, under optimal hydrolysis and fermentation reaction conditions, a maximum of 13 wt % yield of ethanol, on a dry weight basis, could be obtained from *C. vulgaris*.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.energyfuels.6b00253.

Details of ^{13}C NMR spectra of certain compounds (PDF)

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Notes

The authors declare no competing financial interest.

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