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Marine integrated culture of carbohydrate rich *Ulva rigida* for enhanced production of bioethanol

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Macro algal seaweeds are a promising feedstock for biofuels production. Yet, their relatively low fermentable carbohydrate content and the inefficient methods used for their conversion hamper their utilization. The optimized production of *Ulva rigida* co-cultured with fed-fish in an offshore mariculture (fish cages) system is reported. Enhanced production of biomass with elevated content of desired carbohydrates is achieved. The farmed biomass was further converted to bioethanol by a one-step sonication assisted SSF process. An ethanol yield of 16 wt% (based on the dry weight of algae) is obtained.

1. Introduction

Marine macro algae are considered as a promising biomass feedstock for the production of bioethanol. Their use as an alternative feedstock compared to the conventional terrestrial biomass offers advantages like high growth rates, low concentration of lignin, no diversion of natural habitats for farming or agricultural land from food to fuel production.[1–4] Moreover, mass culture of macro algae act as an environmental bio-mitigating agent by lowering the eutrophication impact of farmed waters.[5,6] In addition, capture of CO_2 facilitates greenhouse gas mitigation.[7] However, the option of marine biomass was largely ignored, due to difficulties in producing large quantities consistently and also the relatively low, readily available sugar content for fermentation, as compared to the terrestrial counterparts. If marine algal exploitation for bioethanol production were to be scaled up the production of a carbohydrate-rich biomass need to increase substantially.

In addition, advanced conversion techniques should be developed, and sugar yields need to be improved.[8–10]

Integrated Multi-Trophic Aquaculture (IMTA) is a suitable alternative to the commonly practiced algal mono-culture. This strategy could provide a sustainable solution for the feedstock requirement of bioethanol production. IMTA systems are based on the concept of ecological sustainability. In aquaculture this concept refers to the reuse and recycling of internal feedback within a culture system. This minimizes the input and the output wastes of resources, such as nutrients, water and energy in effluent water.[11–13] A common practice among land-based aquaculture operations during the last few decades is the integration of seaweed farming and aquaculture operations where seaweeds are cultured in the effluent water of abalone, prawns, oysters, clams or fish.[11,14] Thus, when integrated with fed aquaculture (finfish), extractive organisms (seaweeds and suspension feeders) may turn waste into productive resources there by intensively reducing the impact of derived waste on the local ecosystem.[11,13,15]

The benefits of integrating seaweed cultivation with fed mariculture in order to recapture waste nutrients are well documented.[11,13,15–17] A study conducted at a Mediterranean offshore mariculture farm, has shown intensified growth rates, and elevated cellular contents of fermentable sugars in the marine algae *Ulva rigida* cultured downstream to fish culture net pens.

Moreover, by the exposure of the cultured biomass to low ambient nutrient levels, a further enhancement of the fermentable sugar fraction was achieved.[18] Nevertheless, most of the studies related to the integrated culture of fish and algae in marine open-water systems have focused mainly on the environmental and economic effects. Little attention has been paid, however, to the use of such systems for the production of marine biomass as a feedstock for bioethanol generation.

In this study, we co-cultured the seaweed *Ulva rigida* with fed-fish culture (*Sparus aurata*) in an offshore fish cage aquaculture complex. The culture system was focused on obtaining high yields of biomass with high content of fermentable sugars. The carbohydrate rich cultured biomass was then processed by the optimized conversion method so as to achieve optimal yields of bioethanol. Compared with the values in Table 1, 0.16 g of ethanol from 1 g of dry macro algae is the highest yield reported so far.

The green macro alga *Ulva* (Chlorophyceae) is a common marine algae abundantly found in eutrophicated coastal waters. This marine alga could be considered a potential energy crop due to its high growth rates and relatively high carbohydrate

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Table 1 State-of-the-art strategies for bioethanol production from marine macro algae

Macro algae	Hydrolysis conditions & sugar yield (g g^{-1} dry algae)	Fermentation conditions & ethanol yield (g g^{-1} sugar)	Reference
Ulva fasciata	Enzymatic (cellulase 22119) hydrolysis; 36 h; 45 °C; **0.207**	*S. cerevisiae*; 28 °C; 24 h; 120 rpm; **0.45**	22
Ulva rigida	Enzymatic (cellulase, amylase) hydrolysis; 37 °C; 3 h; sonication; **0.196**	SSF process; cellulase, amylase, *S. cerevisiae*; 37 °C; 3 h; sonication; **0.33**	25
G. tenuistipitata; *R. riparium*; *G. salicornia*; *U. intestinalis*;	HCl (0.1 to 1 M); 95 °C; 15 h; **0.539, 0.0233, 0.014, 0.0503 respectively**	*S. cerevisiae* TISTR no. 5339; 30 °C; 18 h; 120 rpm; **4.17×10^{-3}; 0.86×10^{-4}; 0.31×10^{-4}; 0.74×10^{-4} respectively**	26
Ulva fasciata	Cellulase produced from *Cladosporium sphaerospermum* was used for hydrolysis; 24 h; 40 °C; **0.112**	*S. cerevisiae* MTCC no. 180, 12 h, 28 °C, 120 rpm; **0.47**	27
Ulva meridionalis	2 mM phosphotungstic acid, HPA, 160 °C, microwave irradiation; synergistic effect between HPA and microwave irradiation; **0.336 neutral sugars**	—	28

content. Most of the studies related to the conversion of marine algae, namely *Ulva* species, to bioethanol used pretreatment processes prior to the enzymatic hydrolysis of the biomass.[18–21] Pretreatment processes are often accompanied with several disadvantages: thermochemical treatments with dilute acids are energy consuming and also generate toxic residues such as hydroxymethyl furfural (HMF) and other furfurals due to the harsh pretreatment conditions.[22–24] Korzen *et al.*, reported recently on a one-step sonication assisted simultaneous saccharification and fermentation (SSF) process devoid of pretreatment for the production of bioethanol from *Ulva rigida.* However, the ethanol yield achieved was still lower (6.2 wt%) than the potentially available fermentable fraction.[25] Recent strategies developed for the conversion of marine macro algae to bioethanol are summarized in Table 1.

2. Materials and methods

2.1. Seaweed culture scale up

Cultivation scale up experiments were carried out for three periods during 2013 in an open sea fish farm (Lev-Yam Aquaculture Ltd.) located off the coast of Michmoret, Israel (Fig. 1(a)). Algal thalli, *Ulva rigida*, (about 500 g) were housed in nylon net cages (length = 3 m, width = 0.5 m, mesh size = 5 mm) (Fig. 1(e)). The cages (n = 6) were attached to buoys, and positioned at 2 sites, one site was located within the fish cage surroundings, 15 m downstream to the cages along the main water current direction, and the other at a control site 150 m upstream to the fish cages (Fig. 1(b)). Algal culture cages were maintained at a depth of 3 m (Fig. 1(c)). Cultivation experiments were carried out from 16th September to 3rd October (27.8 °C, average current speed 8.6 cm s^{-1}, average current direction azimuth 0.7°), 7th November–25th November (24.1 °C average current speed 7.1 cm s^{-1}, average current direction azimuth 8.2°), and from 2nd December–10th December (22.3 °C average current speed 5.1 cm s^{-1}, average current direction azimuth 4.3°). The September and October experiments were further followed by culture manipulation trial, (29.9–3.10, 20.11–25.11), in order to increase the carbohydrate and starch contents of the biomass.

Culture manipulation trials took place consecutively after the two week grow out culture phase. Algal culture cages housed with cultured *U. rigida* (n = 3) were moved from the 15 m downstream culture site and were repositioned at the low nutrient control site for 5 more days. So as to monitor the growth and biochemical content of the seaweeds during the culture manipulation, two days after the repositioning of the culture nets, the nets were taken out of water, seaweeds were gently drained with surplus water and weighed (on board), the seaweeds were promptly brought back into the nets and back into the water. 10 g of sampled seaweed from each net (n = 6, control nets were sampled as well) were rinsed with distilled water and stored frozen (−20 °C) for subsequent tissue analysis. At the end of each culture experiment the seaweeds were processed as specified above.

2.2. Growth measurements

Specific growth rates (SGR) were calculated using the following formula:

$$\mathrm{SGR} = [\ln(W_t/W_0)]/t \times 100$$

in which W_t is the biomass (wet weight) after t days culture and W_0 is the initial biomass. SGR were expressed as percentage of daily increase or decrease in seaweed biomass (% d^{-1}).

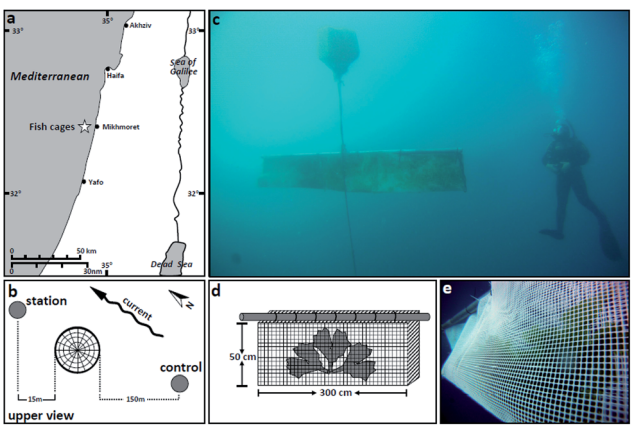

Fig. 1 Schematic (a) map of the study area showing the fish farm, (b) scheme of the fish cage and the algal culture cages, (c) algal culture cage suspended at 3 m depth, (d) scheme of an algal culture cage and (e) *Ulva rigida* in culture cage.

2.3. Determination of starch content

Seaweed samples were dried to a constant weight at 60 °C in an oven, grounded to fine powder and stored in a desiccator for further use. Starch content of the algae was determined by the method developed by Smith and Zeeman.[29] In short, known amount (20 mg) of ground algae were washed twice in 80% (v/v) ethanol, followed by resuspending in sodium acetate (200 mM, pH = 4.8), boiled for 10 min and incubated for 3 h with amylo glucosidase (6 U, Sigma) and α-amylase (1 U, Sigma). Control experiments were also carried out under identical conditions without the addition of enzymes. The release of glucose was determined at 450 nm using a glucose oxidase assay with Bio-Rad Laboratories micro plate reader using a calibration plot from glucose standard.[30]

2.4. NMR (^{1}H & ^{13}C) analysis of SSF broth

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker Avance DPX 300. The amount of ethanol produced from the algae in the SSF process was quantified using ^{1}H NMR.[25] HCOONa was used as internal standard and D_2O is used as solvent. The reaction products formed in the SSF process were further confirmed using ^{13}C NMR.

2.5. Optimization of the SSF process for achieving higher ethanol yields

2.5.1. Effect of algal consistency in the broth. To prove the practical utility of sonication based SSF process, the process has been scaled up from 2 wt%. The reaction conditions employed in the current study were similar to those employed in the simultaneous saccharification and fermentation of algae. Algal consistency in the broth was set to 10%, 15%, and 20%, based on DM (dry mass) while the proportions of enzymes and yeasts were similar at all treatments.

10% constitution of the broth comprised of: 2 g algae in 20 mL solution – (10 mL H_2O and 10 mL buffer), 100 μL glucoamylase from *A. niger* (≥300 U mL^{-1}), 40 μL α-amylase (≥250 units per mL); 0.1 g cellulase (≥0.3 units per mg solid) and 0.5 g yeast (commercial Baker's yeast, *Saccharomyces cerevisiae*); 15% constitution of the broth comprised of: 3 g algae in 20 mL solution – (10 mL H_2O and 10 mL buffer), 150 μL glucoamylase, 60 μL α-amylase; 0.15 g cellulase and 0.5 g yeast; 20% constitution of the broth comprised of: 4 g algae in 20 mL solution – (10 mL H_2O and 10 mL buffer), 200 μL glucoamylase, 80 μL α-amylase; 0.2 g cellulase and 0.5 g yeast.

The SSF process was carried out under mild sonication at 37 °C for 2–4 h in a bath sonicator (MRC Clean-01 Ultrasonic cleaner, 40 kHz ultrasound frequency and 120 W ultrasonic power).[25]

2.5.2. Effect of enzyme loading on the SSF of *Ulva rigida*. After finding the optimum solid consistency to be 15 wt%, the effect of enzyme loading on the conversion of *Ulva rigida* to ethanol in an SSF process is evaluated. Three different enzyme loadings (low, middle and high) were tested. Typical compositions of the three broths are as follows:

Low loading, enz/2: 3 g algae in 20 mL solution – (10 mL H_2O and 10 mL buffer), 75 μL glucoamylase, 30 μL α-amylase, 0.075 g cellulase and 0.5 g yeast.

Middle loading, enz*1: 3 g in 20 mL solution – (10 mL H_2O and 10 mL buffer), 150 μL glucoamylase, 60 μL α-amylase; 0.15 g cellulase and 0.5 g yeast.

High loading, enz*2: 3 g in 20 mL solution – (10 mL H_2O and 10 mL buffer) 300 μL glucoamylase; 120 μL α-amylase; 0.3 g cellulase and 0.5 g yeast;

2.5.3. Effect of culture conditions and carbohydrate content of *Ulva rigida* on bioethanol yield. After optimizing the algae consistency and enzyme loading, another important parameter to tune the ethanol yield namely, the carbohydrate content of the *Ulva rigida* was varied by culturing the algae under specific growth conditions to have higher carbohydrate content. The original as received *U. rigida* and the high carbohydrate *U. rigida,* which was co-cultured with fed-fish in an offshore mariculture system and was subjected to a two day culture manipulation were examined for ethanol yield under optimized sonication based SSF process.

3. Results and discussion

3.1. Evaluation of specific growth rate (SGR) of *Ulva rigida*

Specific growth rate, and starch content expressed as % of dry mass for *Ulva rigida* cultured during 14 days (initial culture period or grow out) + 5 days in low nutrient site, during Sep/Oct 2013 and Nov. 2013 were summarized in Fig. 2. Data are expressed as means ± SD.s.

The specific growth rate of *Ulva rigida* grown downstream from the fish cages showed significantly higher (27 times for the September trial (Fig. 2(a)) and 41 times for the November trial (Fig. 2(b))) specific growth rates (SGR) than those grown at the control station upstream from the cages. The availability of inorganic nutrients has been identified as the most important factor controlling the growth and productivity of seaweeds.[25,31–33]

The achieved daily growth rates are similar to the maximal specific growth rates reported by studies with *Ulva* species integrated in land-based multi-trophic aquaculture using tank cultivation,[34,35] and are comparable to the values found for mass cultivation of *Ulva*.[6,36] The decline in the growth of *U. rigida* between the September and the November experiments might be the result of intrinsic seasonality effect on the growth of this species. The mean seawater temperature measured during the September experiment was 29.2 °C and dropped to 24.4 °C during the November experiment, other parameters such as irradiance, although not measured during the study, had likely also changed during the different culture seasons. The effect of seasonality on growth and biochemical composition has been studied and observed in different species of algae.[37–39] However, in order to fully understand the effects of seasonality on the annual production yields of seaweed biomass further long-term experiments should be carried out.

During both culture trials (September, Fig. 2(c) and November, Fig. 2(d)), the starch content was significantly higher (31.5% of DM, September trial) at the control station than downstream to the cages (24% of DM, September trial, $p < 0.01$) (Fig. 2(c)). This is in an inverse proportion to the ambient seawater nutrient concentrations In addition, after two days of culture manipulation at the low nutrient site, the starch contents

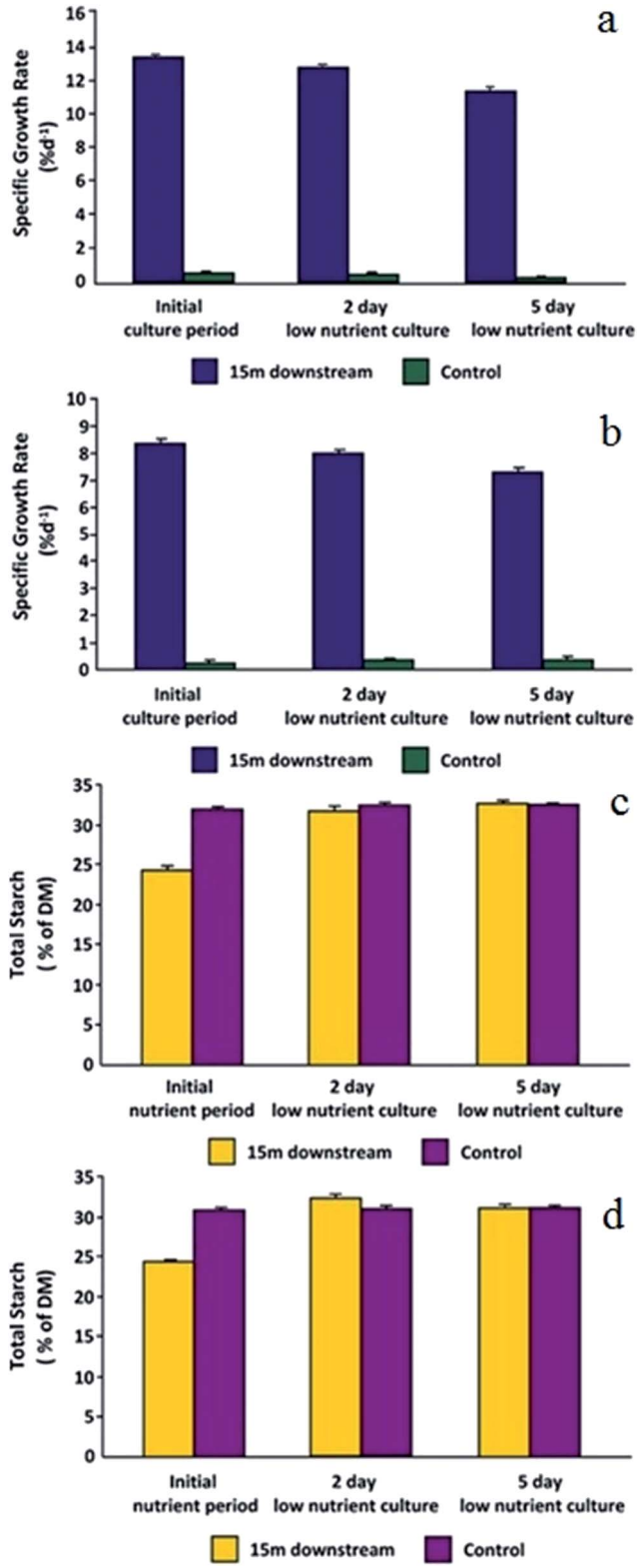

Fig. 2 *Ulva rigida* culture and starch content.

bounced up and levelled with the values of the control site. These results are in line with previous studies on reserve carbohydrates in seaweeds. High nutrient concentrations were found to alter the proximate composition in seaweeds and caused a shift to lower levels of carbohydrates such as starch.[39,40] Moreover, it has been shown that nitrogen-deficient green algae accumulate carbon mainly as starch reserves, which could be further used by respiration during growth and reproduction.[41,42]

Since both high growth rates and high concentrations of desired carbohydrates are crucial parameters for an economically viable biomass for bioethanol production, then two steps must be combined: first a nutrient-rich step for high biomass production and, second, a nutrient-limited phase for the carbohydrate/starch accumulating phase.

3.2. Effect of algae consistency in the broth

So as to prove the practical utility of sonication based SSF process, the process has been scaled up from 2 wt% of algae to 20 wt%. A proportionate increase in the ethanol yield was achieved from ~6 wt% to 12 wt% with an increase in the initial solid consistency (algae amount). 15 wt% of dry weight of the algae was found to be the optimum amount of the algae for high yields as well as ease of operation and separation of ethanol formed by subsequent centrifugation. Above 15 wt% of solid consistency, the SSF was similar to solid state fermentation posing operational difficulties like non-homogenous mixing of the enzymes, yeast and algae and also separation of the formed ethanol. This could be observed from the higher error values in the ethanol yield obtained with a solid consistency of 20 wt% as depicted in Fig. 3.

3.3. Effect of enzyme loading on the SSF of *Ulva rigida*

After finding the optimum solid consistency to be 15 wt%, the effect of enzyme loading on the conversion of *Ulva rigida* to ethanol in an SSF process is evaluated. The loading of enzymes (amylase and cellulase) is found to be another important parameter that could be used to tune the ethanol yield from 8 to 15 wt% (on dry weight basis). Three different enzyme loadings (low, middle and high) were tested. Typical composition of the three broths are as follows:

Low loading, enz/2: (3 g algae in 20 mL solution – 10 mL H_2O and 10 mL buffer, 75 μL glucoamylase, 30 α-amylase, 0.075 g cellulase and 0.5 g yeast).

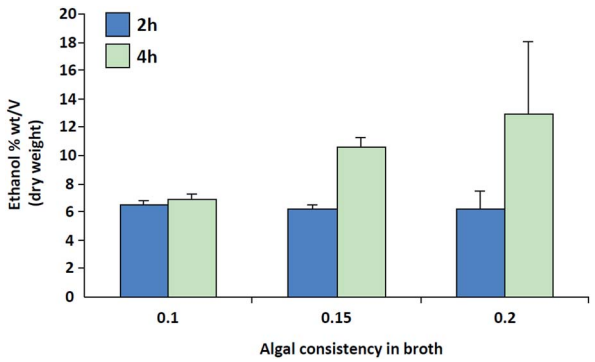

Fig. 3 Efficiency of ultrasonication process for the SSF process with high solid (algae) content (10–20 wt%) (replicate no. $n = 3$; error bars indicate standard deviation, SD).

Middle loading, enz*1: (3 g in 20 mL solution – 10 mL H_2O and 10 mL buffer, 150 μL glucoamylase, 60 α-amylase; 0.15 g cellulase and 0.5 g yeast).

High loading, enz*2: (3 g in 20 mL solution – 10 mL H_2O and 10 mL buffer) 300 μL glucoamylase; 120 α-amylase; 0.3 g cellulase and 0.5 g yeast;

Near 2-fold increase in ethanol yield is observed by increasing the enzyme loading by 4 times as depicted in Fig. 4. In view of the cost of the enzymes, the optimum loading of enzymes is the middle loading, enz*1, which could yield 11 wt% ethanol upon sonication for 4 h.

3.4. Effect of starch content of *Ulva rigida* on bioethanol yield

After optimizing the algae consistency and enzyme loading, another important parameter to tune the ethanol yield namely, the carbohydrate content of the *Ulva rigida* was varied by culturing the algae under specific growth conditions to have higher carbohydrate content. The original as received *Ulva rigida* and the cultured *Ulva rigida* were examined for ethanol yield under optimized sonication based SSF process. A 2-fold enhancement in the ethanol yield is observed in the cultured algae (16 wt% on dry weight basis) compared to the as received *Ulva rigida* (8 wt% on dry weight basis) as shown pictorially in Fig. 5.

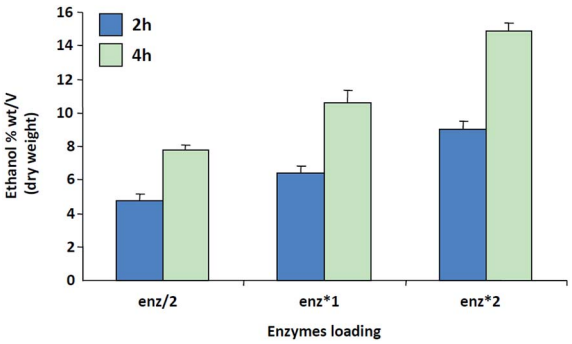

Fig. 4 Effect of enzyme loading on the ethanol yield in the SSF process with a solid consistency of 15 wt% (replicate no. $n = 3$; error bars indicate standard deviation, SD).

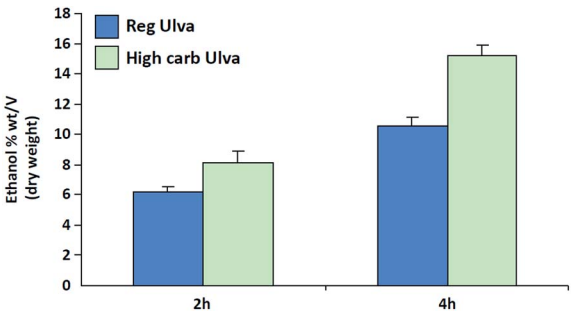

Fig. 5 Effect of tailoring the carbohydrate content of *Ulva rigida* on the ethanol yield in the sonication based SSF process with 15 wt% solid consistency and 1 wt% enzyme loading (replicate no. $n = 3$; error bars indicate standard deviation, SD).

The ^{1}H NMR spectrum of the aliquot of sample from the fermentation (SSF) broth under optimal reaction conditions (15 wt% solid consistency, high carbohydrate *Ulva rigida*, 1 wt% enzyme loading, 4 h sonication) is depicted in Fig. 6.

The presence of 3H (t, 1.18 ppm) and 2H (q, 3.64 ppm) indicate the formation of ethanol. The signal at 1.9 ppm is due to the sodium acetate buffer present in the broth. The signal at 8.5 ppm (1H, s) is characteristic of the internal standard, HCOONa. The amount of ethanol estimated based on the relative integral values of the internal standard and ethanol peaks is 16 wt%. From 31.5 wt% starch in the high carbohydrate algae, the expected glucose amount upon complete hydrolysis is 35 wt% (ref. 43) which corresponds to a theoretical ethanol yield value of 17.8 wt%.[10]

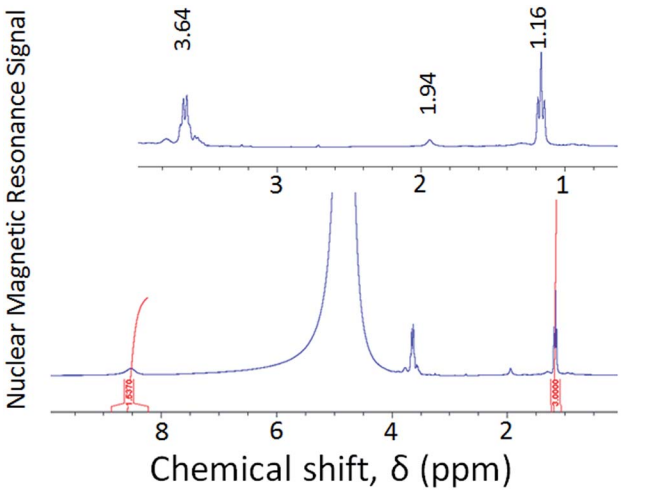

Fig. 6 ^{1}H NMR spectrum of aliquot of sample collected from the fermentation (SSF) broth under optimal reaction conditions.

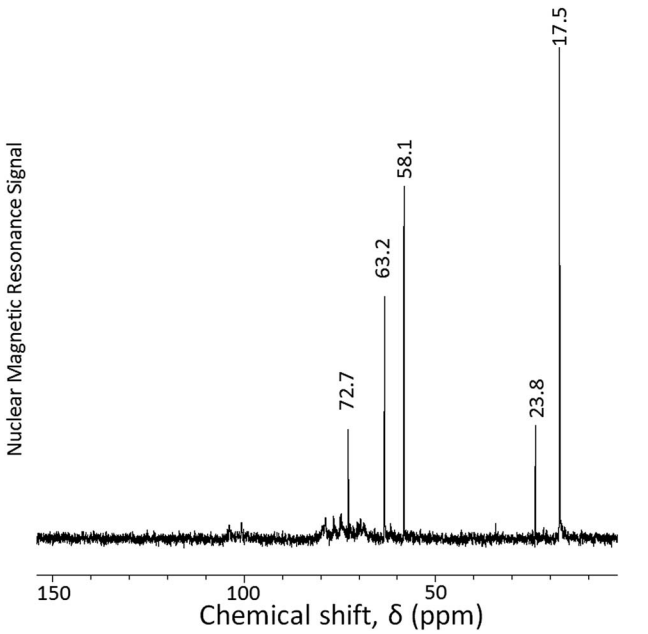

Fig. 7 ^{13}C NMR spectrum of aliquot of sample collected from the fermentation (SSF) broth under optimal reaction conditions.

Under the optimized process conditions, 16 wt% ethanol could be obtained from the high carbohydrate algae which corresponds to a process efficiency of 89%. This value is relatively higher than the process efficiency of 65% reported previously.[25]

The formation of ethanol from *Ulva rigida* in the SSF process is further confirmed using ^{13}C NMR as represented in Fig. 7. The signals at 17.5 and 58.1 ppm are typical of ethanol. The presence of secondary metabolite, glycerol is also evident from the signals at 63.2 and 72.7 ppm. The signal at 23.8 ppm is due to CH_3COONa buffer used in the SSF process. No fermentable sugars are detected in the region of 60–100 ppm typical of glucose indicating the effectiveness of the SSF process.

4. Conclusion

Specific growth as well as saccharification and fermentation conditions that facilitate optimal production of ethanol from the marine macro algae *Ulva rigida* have been elucidated. Ethanol yield as high as 16 wt% (based on dry mass of algae) could be produced from *Ulva rigida* by carrying out the SSF process with 15 wt% solid consistency using high carbohydrate *Ulva rigida*, 1 wt% enzyme (cellulase and amylase) loading and for a short duration of 4 h sonication.

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