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The analysis of macroalgae biomass found around Hawaii for bioethanol production

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Macroalgae commonly found in the ocean around Hawaii were collected from near shore locations and their potential as biomass feedstock for fermentative ethanol was investigated. A green alga, *Ulva reticulata*, was selected for further analysis. This species forms large complex structures that grow quickly and has high dry biomass percentage (20%), soluble carbohydrates (18%), and high total carbohydrates along with low quantities of lignin (13%). During acid saccharification, it was determined that 49% of the total mass was observed as sugars in the hydrolysate; however, fermentation was problematic. Enzymatic saccharification using cellulase from *Trichoderma reesei* was attempted which recovered a measured maximum of 20% glucose based on the initial dry mass. Fermentation successfully converted all the glucose to ethanol. The measured ethanol yield corresponded to approximately 90 L per tonne of dried macroalgae.

Keywords: biofuel; biomass; ethanol; macroalgae; mariculture

1. Introduction

A study was performed to identify and characterize different species of macroalgae biomass found in the waters around Hawaii that have potential use as a lignocellulosic mariculture energy crop, specifically for ethanol fuel production. The investigated species includes both native and invasive macroalgae species.

Cellulosic resources represent the most abundant global pool of biomass. Lignocellulose is the primary waste stream generated by the wood product industry, and also originates from municipal solid waste and agriculture residues including those from dedicated energy crops. [1] Cellulosic ethanol is chemically identical to ethanol from other sources, such as corn starch or sugar, but has the advantage that the lignocellulose raw material is available in greater abundance and, presumably, at a lower price and not derived from a food source.

Cellulosic ethanol production, however, requires a significant amount of processing to make the sugar monomers available for fermentation by microorganisms. Associated costs to convert the starchy or cellulosic materials to fermentable sugars can be prohibitively high and are dependent upon the feedstock.[2] Selection of a suitable substrate is important for reduction in processing cost and total yield.

In areas with limited lignocellulosic waste streams such as Hawaii, cultivation of dedicated biomass crops would be necessary to sustain industrial-scale production of cellulosic ethanol. Recent studies [3] identified the availability of 1.9×10^5 tons of lignocellulosic biomass in the state and concluded that currently Hawaii could not produce enough fuel ethanol to meet current demand levels. Limited fresh water resources, arable agriculture and high land costs limit the options for the development of fuel-producing crops.

In January 2008, the State of Hawaii entered into the Hawaii Clean Energy Initiative, an agreement with the US Department of Energy, to have 70% of Hawaii's energy needs originate from clean and renewable resources by 2030.

Hawaii has a general coastline of over 1200 km, the fourth longest in the U.S., and an offshore exclusive economic zone (EEZ) of about 2.4 million km^2. Utilization of this offshore EEZ for mariculture activity avoids use of Hawaii's limited agricultural land and fresh water resources. Macroalgae do not constitute a major food resource to humans and consequently sidestep the food-or-fuel debate. [4] While the potential use of macroalgae biomass has been recently outlined, [5,6] very little analysis has been done on this resource to date [7] and is non-existent for mariculture suitable species in Hawaii. Macroalgae exhibit high growth rates and can be farmed in mariculture facilities since they form organized structures, unlike microalgae, allowing for controlled spatial containment. [8–11] Environmental impacts and adverse public response can be minimized by careful species selection and management.

Due to cultural and environmental regulations a suitable mariculture crop in Hawaiian waters must be native and not endemic or invasive. Invasive algae species are, however, a serious environmental problem and produce considerable

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waste biomass. Over a period of 5 months, 30 tons of an invasive species of *Gracilaria salicornia* was collected from a limited vicinity of the island of Oahu and the density of this species was as high as 30 kg/m^2 in some areas, with 50% re-growth rates occurring within a month. [12,13] Invasive species have been included in this investigation for waste utility demonstration.

This examination investigates the potential use of Hawaiian macroalgae as a renewable lignocellulosic biomass crop and compares and reports its relevant structural components for the production of fermentative ethanol.

2. Materials and methods

2.1. Sample collection and determination

Macroalgae samples were collected from the south shore of Oahu between June and July 2009. The samples were manually harvested within the intertidal and tidal regions at depths between 0–2 m. The collected samples were immediately brought back to the laboratory then photo-documented and stored at 4°C. The macroalgae were classified by observed phenotypic characteristics. [8,10,14]

2.2. Dry weight determination

Collected samples were first rinsed with distilled and deionized water then immediately dried. Triplicate samples (5–10 g) of each seaweed were placed in pre-weighed aluminium pans and wet weights determined using a Mettler AB-104S microbalance. Samples were then placed in a bench top incubator set at 50°C and allowed to dry until weights were stable.

2.3. Lignin content determination

Lignin content was gravimetrically determined using a method described by Hill et al. [15] Triplicate 0.5 g of dried samples were weighed with the Mettler microbalance and transferred to a 100 mL beaker. 15 mL of a 6:1 v/v mixture of concentrated sulphuric acid/phosphoric acid was added and stirred until the samples gelatinized. The beaker was then immersed in a 35°C water bath for 2 min. and its contents added to 350 mL of deionized water. The mixture was brought to a boil and left for 20 min. The beaker was allowed to cool for 30 min. The contents were then filtered through a pre-weighed GF/C glass fibre filter, triple rinsed with 10 ml of distilled and deionized water and allowed to dry at 50°C until the weights were stable. Terrestrial biomass samples of *Leucaena leucocephala* [16] and *Pennisetum purpureum* (bana grass) [17] were used for method verification and yield comparisons with commercial analyses.

2.4. Holocellulose and cellulose content determination

Triplicate 10 g wet samples were manually homogenized using a 0°C 70% ethanol solution (pH 7.0) with a pre-chilled mortar and pestle for removal of soluble carbohydrates, amino acids, organic acids, inorganic salts and lipophilic compounds. Five volumes of 0°C ethanol were added to the homogenate and placed in a freezer overnight. [18] Ethanol was removed by centrifugation at 6000 rpm for 10 min using a Sorvall RC-3 floor-model centrifuge. The collected ethanol solutions were retained for carbohydrate analysis. The collected solids were then re-suspended in a phenol–acetic acid–water solution (2:1:1 w/v/v) and allowed to stand for 30 min. [19] Solids were again collected by centrifugation and washed with the phenol solution three times and finally with distilled water, then allowed to dry in pre-weighed aluminium pans in an incubator at 50°C until weights were stable.

The recovered extract is a combination of lignin, hemicellulose, cellulose and other structural carbohydrates. For determination of cellulose content, the dry material was re-suspended in 20 mL of a 17.5% w/v solution of sodium hydroxide at 20°C (Tappi Standard T 203 OS-74) and allowed to incubate for 30 min. The samples were then filtered through pre-weighed GF/C filters and triple rinsed with distilled water. The filters were dried in an incubator at 50°C until weights were stable. Lignin content obtained by the method previously described were used and calculated as a percentage of the holocellulose. Terrestrial biomass samples of *L. leucocephala* and *P. purpureum* were used for method verification and yield comparisons with commercial analyses.

2.5. Carbohydrate content determination

The ethanol solution prepared previously was used for carbohydrate analysis. Total carbohydrates of the homogenates were assayed using a colorimetric phenol–sulphuric acid method [20] and compared against a glucose standard curve.

2.6. Acid hydrolysis

Wet macroalgae samples (150 g) were autoclaved in a 10% v/v sulphuric acid solution at 121°C for >8 h. The acidic solution was allowed to cool and solids removed using a GF/C filter. The filtrate was then adjusted to pH 7.0 using a saturated NaOH solution. Estimates of total sugar concentrations were determined using the colorimetric phenol sulphuric acid method prior to NaOH neutralization. A 5 mL sub-sample was filtered through a 0.2 μm syringe filter, serially diluted, and compared against a glucose standard curve. The sample was not acidified further prior to the addition of phenol. Background determination was performed prior to the addition of phenol and compared against the measured absorbance change.

2.7. Hydrogen peroxide pretreatment and enzymatic hydrolysis

Dried macroalgae (0.5 g) were incubated in triplicate using varying concentrations of hydrogen peroxide ranging from 1–10% v/v and incubated for 4 h. Low concentrations of

hydrogen peroxide pretreatment of lignocellulosic biomass for enzymatic hydrolysis has been previously described. [21–23] After incubation, the pretreated material was centrifuged at $10,000 \times g$, rinsed with deionized water and allowed to dry at 50°C until weights were constant. Mass % reductions were then calculated. The dried material was reconstituted in 50 mM acetate buffer and excess cellulase from *Trichoderma reesei* added and allowed to incubate at 50°C for 12 h. The glucose concentration was determined in a 2 mL sub-sample filtered through an Acrodisc syringe filter. A 10 μL sample was injected into an Alltech ELSD 2000 LC system. Separation was performed at a flow rate of 0.45 mL min^{-1}, with a distilled deionized water mobile phase using a Shodex KS-801 column maintained at 75°C. Concentrations were determined using prepared standard solutions of D(+) glucose (w/v). Samples of terrestrial biomass were also used with the same pretreatment and hydrolysis methodology for yield comparisons.

2.8. Fermentation of hydrogen peroxide-pretreated sample and enzymatic hydrolysis

The saccharified hydrolysate was supplemented using a modified Wickerhams YM media with final concentrations of: 0.3% yeast extract, 0.5% peptone, 0.2 g/L chloramphenicol, 6 g/L $(NH_4)_2SO_4$, 7.5 g/L KH_2PO_4, 2.4 g/L K_2HPO_4, 0.6 g/L $MgSO_4$, 0.0001 g/L $CaCl_2$, 0.0001 g/L $CuSO_4$, 0.0001 g/L MnCl, 0.0001 g/L $ZnSO_4$, 0.0001 g/L $CoCl_2$, and 0.0001 g/L Na_2MoO_4. The fermentation broth was calculated to contain a total of 0.1% sugar. A commercially available high specific gravity *Saccharomyces cerevisiae* (WLP 099) purchased from White Labs, Boulder, Colorado, USA, was used for the fermentation. *S. cerevisiae* is considered the best yeast for the fermentation of hexose sugars in lignocellulosic hydrolysates. [24] Batch fermentation was performed in a Hungate tube. After inoculation and a 24 h aerobic incubation at 25°C, cell concentrations were determined using a hemocytometer. An initial concentration of 8.0×10^7 cells/mL was determined and the media sparged with argon gas for 15 min. The fermentation was allowed to proceed for 5 days. At the end of the fermentation period, a final cell count was performed and ethanol content determined using high-performance liquid chromatography (HPLC): a 2 mL sample was filtered through an Acrodisc filter and 10 μL was injected into an Agilent system with refractive index detection. Separation was performed at a flow rate of 1.0 mL min^{-1} with a distilled deionized water mobile phase employing a Shodex KS-801 column maintained at 75°C. Concentrations were determined using prepared standard solutions of ethanol (v/v) and D(+) glucose (w/v).

3. Results and discussion

3.1. Identification of macroalgae

Ten common representative species of native and invasive macroalgae were collected from inter-tidal and near-shore reef locations on the island of Oahu for analysis. Photographs and descriptions of these species are provided in Figure 1.

3.2. Macroalgae compositional determination

A summary of the results of the composition analyses are presented in Table 1. Three of the collected candidates had dry weights exceeding 20% of wet weight (*P. japonica*, *S. rigidula* and *U. reticulata*); the lowest dry weight measured was about 8% (*D. cavernosa and S. polyphyllum*). *P. japonica* had the highest % dry weight, but this appeared to be attributable to visible calcification. *Padina* sp. is the only calcareous genus of the *Phaeaphyceae* and can have calcium carbonate contents reaching 21% on a dry weight basis. [35]

Among the collected species, five were found to have soluble carbohydrates above 15% dry weight (*A. spicifera*, *D. cavernosa*, *H. cervicornis*, *U. reticulata*, and *S. echinocarpum*), with the highest percentage of 23% occurring in *S. echinocarpum*. Three species were determined to have holocellulose contents exceeding 45% of dry weight (*S. polyphyllum*, *S. rigidula* and *U. reticulata*). The amount of lignin was lower than or comparable with terrestrial biomass sources, [36] with the highest percentage found in *D. cavernosa* (33%). Investigations of lignin and lignin-like compounds in algae have been previously reported. [37–39] It should be noted, however, that the chemical composition of macroalgae, including carbohydrate content, can vary widely within species depending on when and where they are collected. [40] The present samples were collected during summer months when solar irradiation is high, and near-shore where allochthonous inputs are variable.

Favourable characteristics of candidate macroalgal feedstocks for efficient production of ethanol include high growth rates, % dry mass, carbohydrates and cellulose content. Low amounts of lignin and hemicellulose are also desirable. Soluble carbohydrates and cellulose yield easily fermentable sugars, while saccharification of hemicellulose results in pentose sugars that require non-commercialized fermentations. [41] Low lignin is desirable, as lignin is the major factor in cellulosic recalcitrance, particularly during enzymatic hydrolysis. [42] Although lignin and lignin-like compounds are often low in macroalgae, selecting feedstock with lower lignin content can reduce pretreatment, reducing costs. [43]

In consideration of their properties, the brown algae *S. rigidula* and the green algae *U. reticulata* appeared to have the best potential for fermentative ethanol production. As seen from Table 1, *S. rigidula* had the highest % dry weight and holocellulose content, and the lowest amount of lignin of the collected species. The green algae *U. reticulata* had the second highest % dry weight, holocellulose content and 40% more carbohydrates but contains 65% more lignin than *S. rigidula*. *U. reticulata* was initially determined to have the highest cellulose fraction.

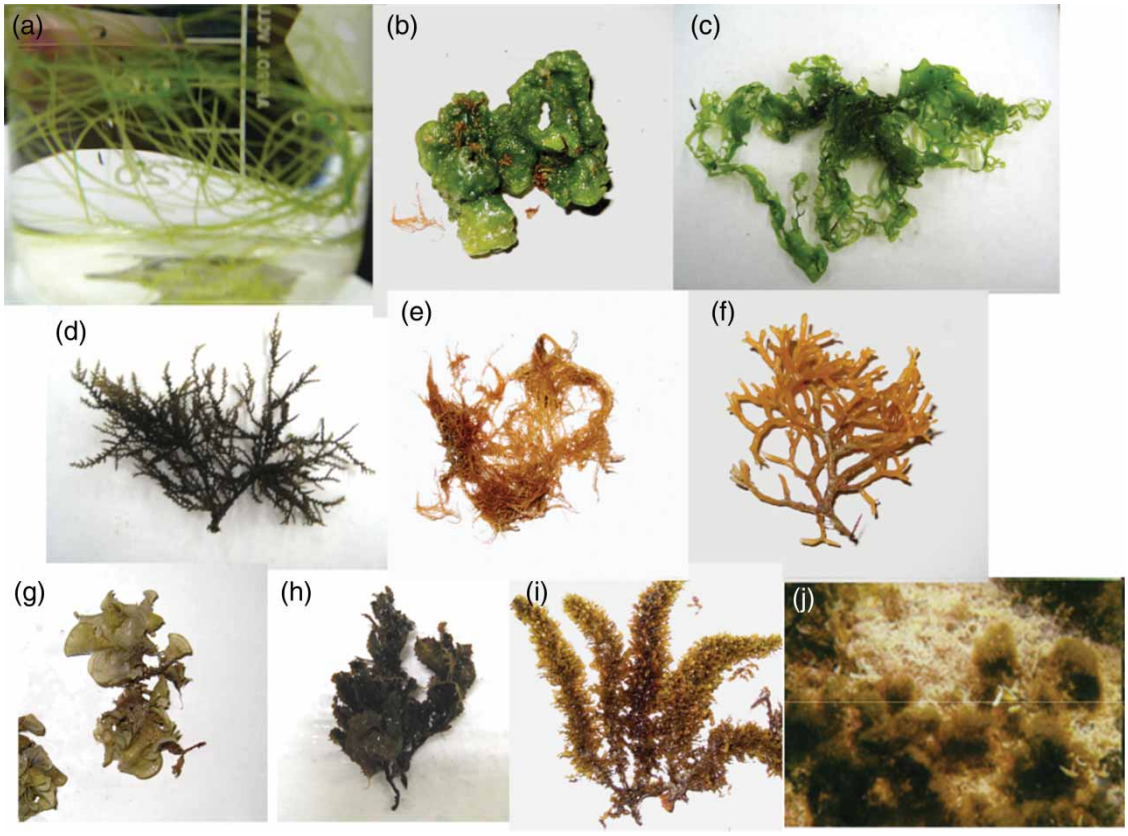

Figure 1. Macroalgae collected along the South Shore of Oahu Hawaii. ***Chlorophyta*** (a) *Cladophoropsis luxurians* (Indigenous) [25] (b) *Dictyosphaeria cavernosa* (Indigenous) [26] (c) *Ulva reticulata* (Indigenous); [27] ***Rhodophyta*** (d) *Acanthophora spicifera* (Alien) [28] (e) *Hypnea cervicornis* (Indigenous) [29] (f) *Gracilaria salicornia* (Alien); [30] ***Phaeophyta*** (g) *Padina japonica* (Alien) [31] (h) *Sargassum echinocarpum* (Endemic) [32] (i) *Sargassum polyphyllum* (Endemic) [33] (j) *Sphacelaria rigidula* (Indigneous) [34] (*S. rigidula* Photo from Magruder and Hunt, 1979 [10]).

Table 1. Analysis of different macroalgae collected along the Oahu, Hawaii coastline for potential ethanol fermentation. Holocellulose, lignin, carbohydrate, and Other content reported on a % dry mass basis.

	Dry Weight % ± stdev.	Holocellulose % ± stdev.	Lignin % ± stdev.	Carbohydrate % ± stdev.	Other % ± stdev.	Division
Acanthophora spicifera	9.8 ± 0.4	33.2 ± 0.7	25.7 ± 2.1	20.7 ± 1.8	20	*Rhodophyta*
Cladophoropsis luxuirians	11.8 ± 1.6	44.5 ± 3.9	14.8 ± 3.3	7.1 ± 1.7	34	*Chlorophyta*
Dictyosphaeria cavernosa	9.4 ± 2.1	26.1 ± 3.9	33.2 ± 5.2	15.3 ± 2.2	25	*Chlorophyta*
Gracillaria salicornia	14.4 ± 1.2	44.5 ± 2.8	9.3 ± 0.67	12.3 ± 2.5	34	*Rhodophyta*
Hypnea cervicornus	18.3 ± 1.8	42.2 ± 6.2	11.6 ± 1.6	19.5 ± 1.9	27	*Rhodophyta*
Padina Japonica[a]	29.6 ± 3.6	27.7 ± 0.4	11.3 ± 1.5	1.8 ± 2.3	59	*Phaeophyta*
Sargassum echinocarpum	18.9 ± 1.5	42.2 ± 4.7	31.0 ± 1.0	23.2 ± 1.7	60[a]	*Phaeophyta*
Sargassum polyphyllum	8.8 ± 1.5	47.8 ± 0.8	32.3 ± 1.1	4.8 ± 1.4	15	*Phaeophyta*
Sphacelaria rigidula	23.3 ± 2.2	66.5 ± 6.6	9.3 ± 1.3	10.6 ± 2.1	14	*Phaeophyta*
Ulva reticulata	20.2 ± 0.9	65.3 ± 2.1	13.9 ± 1.4	17.8 ± 2.3	3	*Chlorophyta*

[a]High dry weight and low lignin percentages due to the presence of carbonate.

Cellulose contents of various green algae are reported to be major cell wall constituents,[44] having contents as high as 20–30% [45–47] with claims up to 45% wt. [48] However, when compared with commercially obtained data and enzymatic hydrolysis results it was determined that measured cellulose compositions are likely artefacts resulting from the selected methodology. *Ulva* was further analysed by Microbac, a commercial vendor using various NREL methodologies [49–53] (Approximate values: 12% cellulose based on glucan content, 2% hemicellulose based only on xyland content, 13.5% lignin and 10% soluble extractives) and the University of Hawaii Agriculture diagnostic centre (UH-ADSC) (Table 2). Current analytical techniques for the assessment of lignocellulosic

Table 2. Comparison of results from experimental and commercial methodologies.

	Holocellulose % ± stdev.	Lignin % ± stdev.	Cellulose % ± stdev.
Ulva reticulata	65 ± 2	14 ± 1	34 ± 1
Ulva reticulata[a]	44 ± 2	11 ± 1	6 ± 1
Pennisetum purpureum	60 ± 1	26 ± 1	58 ± 2
Pennisetum purpureum[a]	62 ± 1	18 ± 1	56 ± 1
Leucaena leaucocephala	61 ± 5	26 ± 4	59 ± 1
Leucaena leaucocephala[a]	58 ± 1	26 ± 1	42 ± 1

[a]Commercial data are averages of results from the University of Hawaii Agriculture Diagnostic facility.

biomass do not adequately consider macroalgal-specific cell wall polysaccharides, and a standardized methodology for comparison is unavailable. Typically terrestrial biomass is regarded as a complex mixture of structural components that principally contain cellulose, hemicellulose and lignin. Our experimental protocol for structural carbohydrate analysis using terrestrial biomass has been validated with commercially obtained data comparing two sources of common lignocellulosic biomass (Table 2). Included within our experimental determinations are algae-specific structural carbohydrates [54–59] which are not easily distinguished from the cellulosic and hemicellulosic fractions using the described separation methods. These variable macroalgae compositions can comprise a greater majority of the total structural carbohydrates [60,61] and can impact analytical conditions that include pH and acidic/alkaline separations and hydrolysis. Separation of the individual compounds using differential solubilities through pH alteration does not consistently provide adequate separation of the structural polysaccharides. As an example, alginates are anionic polymeric complexes that are typically extracted using alkaline solutions similar to those used for the differentiation of cellulose from hemicelluloses. When treated with alkaline solutions alginates form viscous slurries that do not easily filter or separate from insoluble components, introducing bias to cellulose determinations. Ulvans are sulphated polysaccharides built from repeating disaccharides composed of rhamnose, glucuronic acid, iduronic acid or xylose. [54,62] These polysaccharides have tight associations with hemicelluloses and alkaline extractions promote aggregation. [63]

Published NREL methodology for biomass structural carbohydrates and lignin [51] analysis using total biomass acid hydrolysis and HPLC cannot easily differentiate the origin of macroalgal structural carbohydrates and is likely unsuitable for use with macroalgal biomass. Implementation of a structural carbohydrate separation methodology is necessary for determination of origin using liquid chromatographic analysis. Furthermore, because of the large volumes of acid necessary for hydrolysis due to strong absorption by algal biomass, contents of salts after neutralization can be prohibitively high for direct use in HPLC without prior separation. The subsequent separation of salts would result in significant sugar losses.

In spite of these shortcomings, the current analysis does provide a methodological comparison of different macroalgae resources based reliably upon soluble carbohydrates, lignin, and total structural carbohydrates. While further protocol development is necessary variability in analysis methodology and data reporting are one of the downfalls for providing comparable macroalgae compositional analysis. [64]

U. reticulata was selected, on the basis of commercial production potential, to conduct a first round of exploratory studies of hydrolysis and fermentation. This species of algae forms large complex structures that reach approximately 2 m lengths, and can more easily be contained and harvested during mariculture cultivation, [10,65] furthermore is easily propagated by vegetative growth, with doubling rates occurring in days. [66] Hawaii's cultural and environmental situation will require that a mariculture species of algae be non-endemic and native and non-invasive to shoreline ecosystems. *U. reticulata* is relatively uncommon but can sometimes be abundant in low intertidal to 2 m-deep waters, often as an epiphyte. *Ulva* sp. are opportunistic and exhibit high growth rates, so cultivation activities would require careful monitoring. [67] *U. reticulata* is a native and non-endemic species found in Hawaii; its distribution is not extensive, and currently it is not a problematic species.

3.3. Hydrolysis and fermentation of U. reticulata

Collected *U. reticulata* was hydrolysed using a combination of time, steam, temperature, and moderate acid concentrations. Cellulose is not easily saccharified and varying combinations of treatments using these parameters exist. The disruption of the intra and inter-molecular hydrogen bonding of cellulose typically require the use of high acid concentrations at lower temperatures or low acid concentrations at higher temperatures and pressures, with time varying dependent upon the conditions used. [68] A variety of these parameters, including different acids, are often employed with different combinations and applications. [69–71] The lowest acid content over a long time duration with maximum sugar recovery was utilized with the intent for direct hydrolysate fermentation. Using a modified method described by Dubois et al., [20] 4.9 g of total sugar

are estimated in the hydrolysate from a 10 g (dry weight) sample, or about 49% of the total mass, corresponding to a theoretical ethanol yield of about 60 L per tonne of wet macroalgae.

Direct utilization of the acid hydrolysate was investigated for fermentation. After 5 days, trace production of ethanol and incomplete utilization of glucose was observed. Incomplete sugar utilization appeared to be due to fermentation inhibitors produced during the saccharification and subsequent acid neutralization steps [72–74]; addition of glucose to the hydrolysate did not detectably increase ethanol production. In our experimental protocol, high concentrations of Na_2SO_4 are generated during sulphuric acid neutralization, which is likely the major cause of fermentation inhibition. Solubility separation of the salts and subsequent concentration result in unfavourable sugar recovery, and without separation, sugars could not be directly measuring using HPLC.

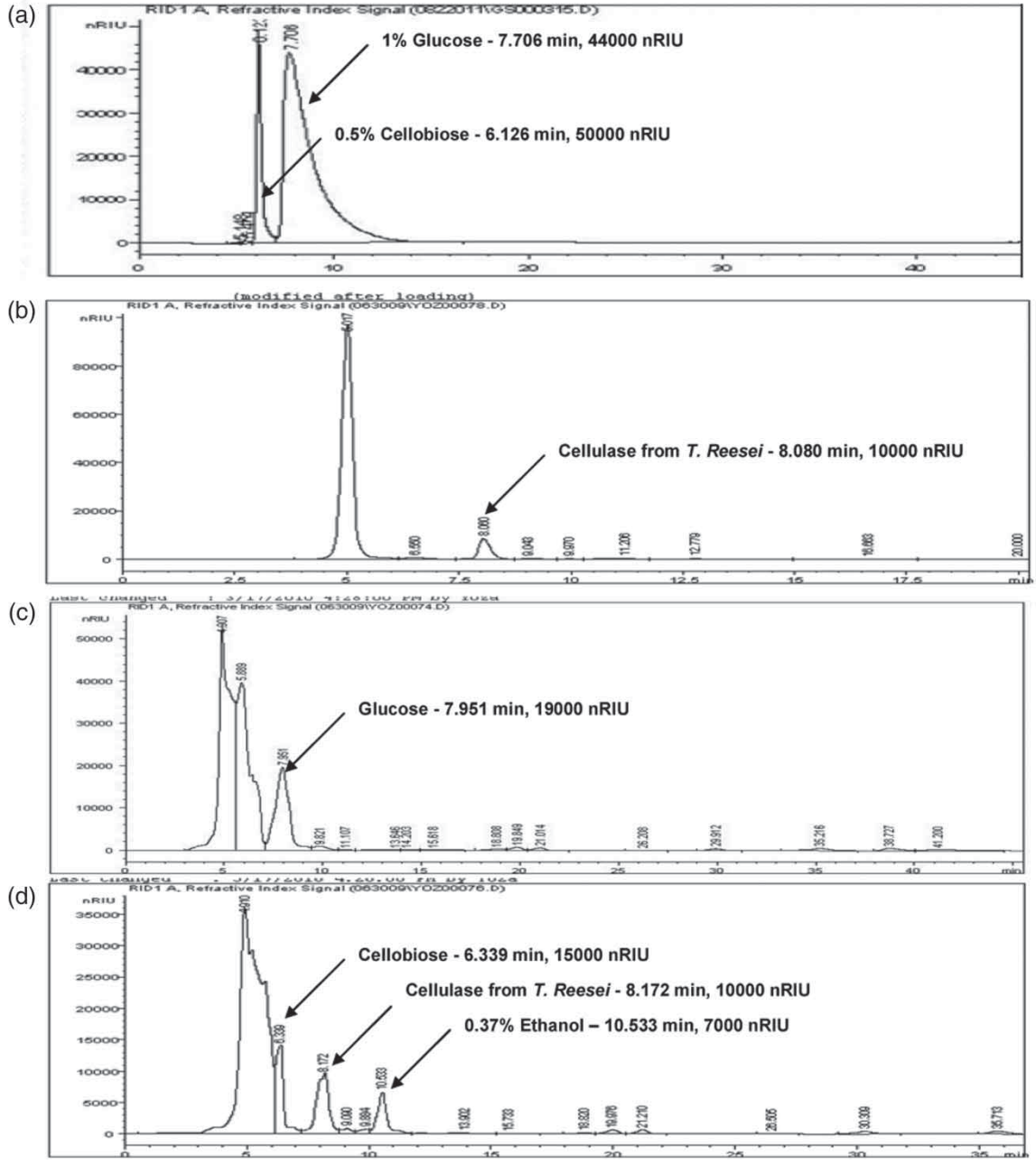

Figure 2. HPLC chromatogram of H_2O_2 pretreated *Ulva reticulata* after enzymatic hydrolysis using *T. reesei* cellulase. Determined using a Shodex KS-801column and Agilent system with refractive index detection. (a) 1% cellobiose w/v and 1% w/v glucose in distilled deionized water. (b) Modified YM media with cellulase (50 units) from *Trichoderma reesei*. (c) Post saccharification and pre fermentation of *U. Reticulata*, supplemented with modified YM media. (d) post fermentation showing cellulase, ethanol and residual cellobiose.

Although acid hydrolysis has a number of favourable characteristics, including simplicity, speed, and relatively low costs, enzymatic methods offer an alternative which could avoid the generation of fermentation inhibition. The efficient use of enzymatic digestion often requires pretreatment of the material to allow for more efficient enzyme access to cellulose cleavage sites for the release of monomeric glucose. [75] Use of hydrogen peroxide has many benefits over acidic pretreatments. [23] Oxidation of the peroxide leaves no toxic residues. Pretreatment with hydrogen peroxide due to the already thin algae does not require extensive milling. This removes the need for energetic preprocessing costs and the need for reclamation of environmentally toxic acid wastes. After H_2O_2 pretreatment and centrifugation, 75% of the original material can be recovered using dilute 2% peroxide and maximum glucose recovery after enzymatic saccharification. Soluble carbohydrates were previously extracted using the method previously described. Subsequent enzymatic saccharification after H_2O_2 pretreatment of whole *Ulva* using *T. reesei* cellulase resulted in 20% recovery of glucose (based on the total mass). Without exact cellulose measurements it is difficult to conclude where the glucose contributions originate from. The total recovered glucose is likely the result of contributions from the residual soluble carbohydrates but mostly from structural cellulose. Analysis of glucose yield after soluble carbohydrate extraction and enzymatic digestion are nearly identical.

Figure 2 presents chromatograms for H_2O_2 pretreated *U. reticulata* before (2(c)) and after (2(d)) fermentation of the hydrolysate generated using cellulase. Also included are chromatograms of a reference solution of 0.5% w/v cellobiose and 1% w/v glucose (2(a)) that was employed for calibration, and of the modified Wickerhams YM media with 50 units of cellulase from *Trichoderma reesei* added (2(b)). Comparison of Figure 2 (a) and (b) indicates that the elution of cellulase partially overlaps with glucose. This complicates quantification of glucose levels in the hydrolysate and fermentation products.

As seen in Figure 2(d), fermentation produced 0.37% ethanol (v/v). This exceeds the alcohol yield corresponding to complete conversion of all the glucose in the hydrolysate measured in Figure 2(c). We believe that this is due to residual enzymatic saccharification occurring during the fermentation. The cellobiose elution peak at around 6.4 min (2(a)) appears to be present in Figure 2(c) but is not well resolved. Residual cellobiose is expected resulting from the incomplete hydrolysis of cellulose using *T. reesei*. Enzymatic hydrolysis of the cellobiose would have increased overall glucose yields. [76] Chromatographic separation is observed after fermentation in Figure 2(d); slight shifts in retention are due to changes in the sample.

The results of this preliminary trial suggest that hydrogen peroxide pretreatment and enzymatic saccharification can be used as an alternative to acid hydrolysis to produce a hydrolysate from a local macroalgae that can be successfully fermented applying enzymes and techniques being developed for terrestrial lignocellulosic biomass. The 0.37% v/v ethanol concentration in the 25 mL sample volume containing 1 g of dried *Ulva* corresponds to an approximate ethanol yield of 90 L per dry tonne of macroalgae (about 19 L per wet tonne).

4. Conclusion

Marine macroalgae have previously been exploited as sources of fermentable sugars [77] for ethanol production. However, a suitable species that can be grown in consideration of Hawaii's restrictions needs development. The current investigation compares different macroalgae found around the Hawaiian islands using specifically lignocellulosic methods and enzyme technologies. With these methods we have demonstrated the feasibility of ethanol production using a macroalga as a cellulosic resource. While the calculated yields are low compared with terrestrial fuel crops such as corn that can produce 370 L of ethanol per tonne, [78] when compared with weight yields of lignocellulosic biomass feedstocks such as waste bagasse, productivites are comparable (90 L/tonne). [79] Detailed characterization of the total carbohydrates, growth potential and regulation is, however, necessary and will be further investigated.

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