Journal of Algal Biomass Utilization
J. Algal Biomass Utln. - Copyright © PHYCOSPECTRUM
ISSN: 2229 – 6905

Optimization of a total acid hydrolysis based protocol for the quantification of carbohydrate in macroalgae

Emily T. Kostas[1], Stuart J. Wilkinson[1], Daniel A. White[2], David J. Cook[1§],

[1]The University of Nottingham, Sutton Bonington Campus, Bioenergy and Brewing Science Building, Loughborough, Leicestershire LE12 5RD, U.K.
[2]Plymouth Marine Laboratory, Prospect Pl, Plymouth, Devon, PL1 3DH

§ Corresponding author: e-mail: david.cook@nottingham.ac.uk. Tel: +44 (0)115 9516245

Abstract:

Accurate quantification of carbohydrate content of biomass is crucial for many bio-refining applications. The standardised NREL two stage complete acid hydrolysis protocol was evaluated for its suitability towards seaweeds, as the protocol was originally developed for lignocellulosic feedstocks. The compositional differences between the major polysaccharides in seaweeds and terrestrial plants, and seaweed's less recalcitrant nature, could suggest the NREL based protocol may be too extreme. Underestimations of carbohydrate content through the degradation of liberated sugars into furan compounds may yield erroneous data.

An optimised analysis method for carbohydrate quantification in the brown seaweed *L. digitata* was thus developed and evaluated. Results from this study revealed stage 1 of the assay was crucial for optimisation however stage 2 proved to be less crucial. The newly optimised protocol for *L. digitata* yielded 210 mg of carbohydrate per g of biomass compared to a yield of only 166 mg/g from the original NREL protocol. Use of the new protocol on two other species of seaweed also gave consistent results; higher carbohydrate and significantly lower sugar degradation products generation than the original protocol. This study demonstrated the importance of specific individual optimisations of the protocol for accurate sugar quantification, particularly for different species of seaweed

1   INTRODUCTION

The goal to produce alternative and cleaner burning sources of biofuels from different biomass feedstocks due to the depletion of fossil fuel reserves and increase in environmental and political pressures, is undoubtedly a crucial step towards achieving a sustainable energy future (Berndes, Hoogwijk et al. 2003).

First generation bioethanol, which has been traditionally produced from sugar-based biomass such as sugarcane and corn, is a potential way of generating an energy-rich source that can be used as a transportation fuel (Karakashev et al. 2007). Although the main commercial production of bioethanol in the USA and Brazil has been successfully achieved by exploiting plants with high sugar contents, the use of such staple food crops as a means to generate fuel has caused great controversy in the food vs fuel debate. Due to this, lignocellulosic biomass has been considered as an alternative feedstock for second generation bioethanol production, which mainly involves the use of agricultural waste products such as wheat straw, corn stover and brewer's spent grains (Lu et al. 2009, Wilkinson et al. 2014). The recalcitrant lignocellulosic complexes however have proved to be somewhat challenging to breakdown and successfully release fermentable sugars which could then be converted to ethanol (Taherzadeh and Karimi 2008). Often, the requirement of a significant input of energy in the

form of a preliminary thermo-chemical pre-treatment is needed in addition to the use of expensive exogenous cellulolytic enzymes.

In contrast, seaweed-derived bioethanol (third/advanced generation bioethanol) has gained an increasing amount of attention due to the advantage that seaweeds have a relatively high growth rate and large biomass yield relative to terrestrial plant based biomass, high carbohydrate content and lignin free compositions (Adams et al. 2009, Meinita et al. 2013). Additionally, they have no direct competition with land crops and no fresh water requirements for their cultivation (Hargreaves et al. 2013) making them an attractive feedstock material for the production of bioethanol. Seaweeds, convenutally known as macroalgae, are classified into three distinct taxonomical groups: phaeophytes (brown algae), rhodophytes (red algae) and cholorophytes (green algae) which results from their different chlorophyll pigmentation. Although seaweeds have high carbohydrate contents, a diverse array of different carbohydrate polymers exists between the three groups. For example, green seaweeds mainly contain the carbohydrate based polymers that are also found in terrestrial plants such as starch and cellulose. However red and brown seaweeds contain polysaccharides which are inherently unique. For example, the most predominant polysaccharide found in red seaweeds is galactan (a combination of carrageenan and agar), which forms a network with cellulose that constitutes the cell wall matrix (Hoek et al, 1998). Carrageenan describes a group of sulphated galactans that are linear in form and water soluble and have a basic structure which consists of repeating units of D-galactose and 3,6-anhydro-D-galactose monomers with α-(1$\rightarrow$3) linkages. Additionally, laminarin (which is a glucose polymer with β1-3 linkages often containing terminating side chains of non-reducing sugar alcohols such as mannitol) and alginate (a polymer of mannuronic and guluronic acids) are polysaccharides that are only present in brown seaweeds. Besides the high carbohydrate contents, the protein and ash contents are relatively high in the dry weight of the seaweed; however the lipid fraction is very low typically less than 5%. In contrast to terrestrial plants, seaweeds contain very little or no lignin in their composition, which makes lignocellulytic biomass based processing methods that have already been developed for terrestrial plant based feedstocks not suitable for seaweeds (Jung et al. 2013).

In order to develop an effective bio-refinery process, for any feedstock not only seaweed based, it is essential to fully characterize the composition of the starting material utilizing both rapid and reliable methods. In the specific case of bioethanol production, particular attention must be paid to the carbohydrate content. For accurate calculations of process yields, quantifying the total carbohydrate content in biomass is essential because it facilitates the working of mass balance and overall process efficiency calculations. Additionally, carbohydrate quantification is directly proportional to ethanol yields in biochemical conversion processes (Montague, et al. 2008) and the determination of achieved percentage theoretical yields of subsequent treatments on pre-treated biomass can only be calculated if the total carbohydrate content has been accounted for accurately. Standardized methods for many compositional components have been developed and are well established. For example the Folch wash is typically used to determine the lipid content (Folch et al. 1957) and either the Kjedahl (Kjedahl, 1883) or Dumas methods (Dumas, 1826) for characterizing protein. These methods have been successfully used for many biomass types however, to date there are no standardized methods for the total quantification of carbohydrates specifically in seaweeds. In order to quantify the carbohydrate content in terrestrial plants, modified versions of the NREL protocol for determining carbohydrates (Sluiter et al. 2008) are typically followed, which usually consists of a two stage complete sulphuric acid hydrolysis followed by the determination of released monosaccharides from the biomaterial. To quantify the release of monosaccharides which are liberated into the liquid fraction, chromatography based (either HPLC or GC-MS) or colourimetric based (Dubois phenol-sulphuric acid colour change) analysis is usually used. Similar modifications to the NREL methods have been used to determine the carbohydrate content of seaweeds, details of which can be seen in Table 1 which depicts the wide range of assays that have previously been used. A number of different methods have additionally been applied to quantify carbohydrates in seaweeds (see Table 1). These range from variations in the type of chemical based deconstructive treatments applied to the seaweed (which are often modifications of the NREL method), to the use of specific enzymes which will cleave off specific polysaccharides and are subsequently measured either spectrophotometrically or chromatographically. Targeting one specific polysaccharide within seaweed for quantification through the use of enzymes could be the most suitable approach if the interest of the work solely relies on the use of that one particular polysaccharide for its conversion to bioethanol or additional high value product/carbon end points. However, this is based on the assumption that a 100% conversion efficiency is achieved. In reality and in practise, achieving 100% conversion (converting 100% of a substrate into its monomeric units) is not theoretically possible due to mass transfer losses, especially if an enzyme is added to a system built up with complex matrices (which is often the case with seaweeds that contain various different polysaccharides), as opposed to a pure substrate. Additionally, the use of specialised exogenous enzymes would likely be relatively expensive and labour intensive in comparison to simpler chemical reagent based protocol, and hence may not be ideal for high throughput rapid screening purposes. Published

literature from various research groups have used a total by difference method, which is a summation of all other non-carbohydrate based compounds (moisture, ash, protein and lipid) which is then subtracted from 100% (Table 1). This method makes the assumption that the remainder is specifically carbohydrate. Although this method of quantification is used to give an estimation of the carbohydrate content, there are considerable limitations to this method as it is not an accurate way of determining carbohydrate content. This could be potentially inaccurate and may lead to the miscalculation of achieved theoretical yields, which could subsequently give inaccurate process efficiency yields.

Table 1. A comparison of the different methodologies and quantification methods in published literature to determine the total carbohydrate content in various species of seaweed.

Method	Description	Reference
Colourimetric	Total and reducing sugars of *K. alvarezii* measured spectrophotometrically by phenol-sulphuric acid method (Dubois et al. 1956) and Nelson method (Nelson 1944). Galactose was analyzed by HPLC.	(Khambhaty et al. 2012)
	Total carbohydrates species determined by Dubois et al (1956).	(Manivannan, et al. 2008, Trivedi et al. 2013, Mutripah et al. 2014)
	U. rigida was treated with 1 mL of 2N H_2SO_4 and boiled for 1 h. Carbohydrates determined by phenol-sulphuric acid method (Dubois et al. 1956).	(Korzen et al. 2015)
Chemical	*U. lactuca* treated with 72% H_2SO_4 at 30°C for 1 h, followed by dilution of H_2SO_4 to 1M and hydrolysis for 3h at 100°C. Glucose, glucose, xylose, rhamnose and mannitol in the hydrolysate detected by HPLC. Uronic acids detected spectrophotometrically using *m*-hydroxydiphenyl assay using galactouronic acid as a standard (Blumenkrantz and Asboe-Hansen 1973).	(van der Wal et al. 2013)
	Cellulose and hemicellulose content from floating residues (*L. japonica*) determined by modified NREL method (Sluiter, Hames et al.) Sample was treated with 3 mL 72% H_2SO_4 at 40°C for 120 min, followed by addition of 84 mL deionized water and hydrolysis at 121°C for 60 min. Monosaccharide composition determined by gas chromatography.	(Ge et al. 2011)
	Various Thai seaweed species were hydrolysed with 0.5 mL of 5N HCl at 100°C for 15 h. Hydrolysates were washed with water to remove acid and then rotary evaporated. HPLC analysis was used to determined rhamnose, xylose, fucose, arabinose, mannose, glucose and galactose.	(Chirapart et al. 2014)
Enzymatic	Laminarin content of *L. digitata* was calculated by the use of laminarinase to hydrolyse soluble laminarin to glucose. Glucose was measured using the Megazyme GOPOD enzyme assay.	(Adams et al. 2009, Adams et al. 2011, Adams et al. 2014)
Total by difference	Carbohydrate content calculated by subtracting the sum of moisture, ash, crude fat, crude fiber, and crude protein from 100.	(Borines et al. 2013, Wu et al. 2014)

It is evident that there are distinct differences in both the subunits (monomers) and also the specific linkages between the monomers that comprise the polysaccharides found in seaweeds when compared to terrestrial plants. Even still such differences additionally exist between the distinct seaweed taxonomic groups. The majority of the chemical-based methodologies which are used to determine the carbohydrate content (based around the two stage complete acid hydrolysis method set by the NREL as principally designed for lignocellulosic biomass) suggests that they could possibly be too thermo-chemically harsh for seaweeds, since seaweeds do not contain lignin and are suggested to have an overall lower recalcitrance. As such there does not appear to be a

standardised and validated protocol for carbohydrate determination in seaweed. It would appear crucial to develop a suitable, yet effective hydrolytical method which could be applied to seaweeds to achieve this goal in a fast and reliable manner.

The primary objective of this research was to optimize the NREL complete acid hydrolysis protocol for the accurate quantification of the total carbohydrate content found in the brown seaweed *L. digitata*. This new and novel developed protocol was then applied to both a red and a green seaweed species (*C. crispus* and *U. lactuca*), to determine its suitability across all seaweed taxa. The secondary objective was also to validate the efficacy of the rudimentary colourimetric based assay from the original protocol outlined in Dubois (1956), against a modern high-throughput chromatography based analytical system (HPLC) for the quantification step.

2 MATERIALS AND METHODS

2.1 Reagents

All reagents were of AnalaR grade and were obtained from Sigma-Aldrich (UK) and Fischer Scientific (UK). Water used was deionized reverse osmosis of ≥18 mega-Ω purity (purite Select Ondeo IS waster system purite, UK).

2.2 Seaweed and wheat straw collection and preparation

Laminaria digitata, *Chondrus crispus*, *Ulva lactuca* were sampled from the coast around Plymouth, UK, during the spring of 2013. The seaweed samples were rinsed in distilled water to remove salt and debris, before being dried in a fan oven at 80°C for a minimum of 48 h until perceived to be dry. The seaweed samples were then milled using a ball mill (Fritsch, Idar-Oberstein, Germany) to obtain a fine homogeneous powder and stored away from direct sunlight and moisture until further use. Wheat straw (Orbit, harvested from Sutton Bonington) was used as a control substrate for comparison of the release of sugars for the method outlined in section 2.4. The wheat straw was milled to ca 2mm particle size prior to use using a knife mill (Fritsch, Idar-Oberstein, Germany).

2.3 Standardised control protocol for quantification of total carbohydrate content in biomass

A modified version of the NREL protocol for the quantification of carbohydrates was used as the standard bench mark (control protocol) in this investigation. This protocol involves 2 distinct stages and then a subsequent quantification step afterwards (Figure 1). Stage 1 utilises concentrated acid (12 M H_2SO_4) at a relatively low temperature (37˚C) for 1 h and then stage 2 involves diluting the acid strength to 1 M and boiling the reaction vessels for 2 h. The 2 step native of this protocol thus highlights 2 potential stages regarding optimisation for seaweeds. Sugars were then quantified by one of two methodologies. Firstly a colourimetric based assay measuring reducing sugar content and secondly HPLC was used for the specific quantification of individual monosaccharide quantifications.
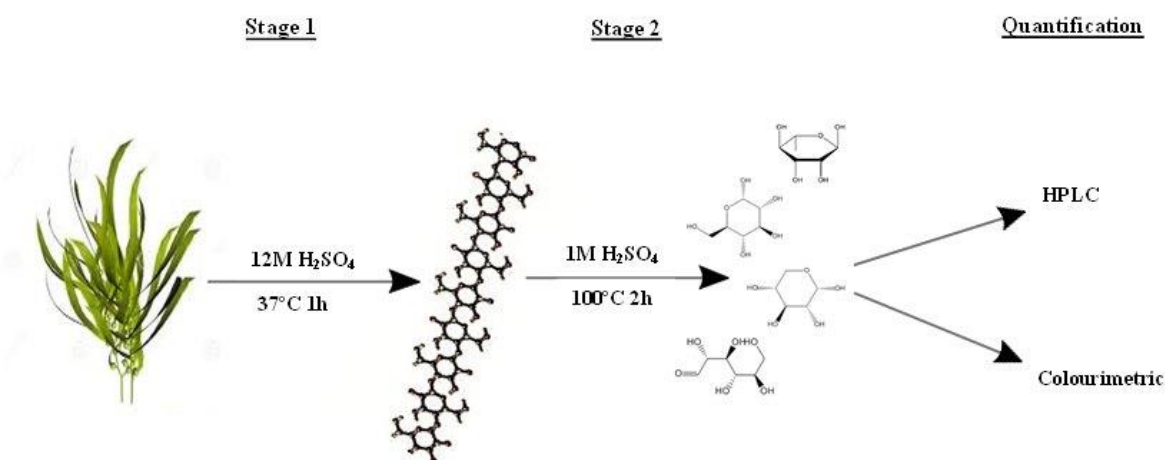

Figure 1. Schematic overview of the standard control protocol for the total carbohydrate analysis of lignocellulosic biomass. Protocol: Two stage complete acid hydrolysis followed by the quantification of liberated sugars. Stage 1: 1ml of 12M H_2SO_4 is added to biomass and incubated at 37°C for 1h, liberating the larger polysaccharides from the biomass. Stage 2: acid strength

diluted with DI water to 1M and incubated at 100°C for 2 h which hydrolyses the polysaccharides into their monomeric constituents. Quantification then achieved either using HPLC or colourimetric method.

For the primary quantification method, reducing sugar content was calculated by using the Dubois colorimetric phenol-sulphuric acid method, where 100 μL of sample was transferred into a test tube and mixed with 1mL 2.5% (v/v) phenol and 2.5mL concentrated H_2SO_4. The absorbance's at 490nm were read in a spectrophotometer (Jenway7315 Staffordshire UK) and concentrations were determined by comparison to glucose standards of 0, 200, 400, 600, 800 and 1000 μg/ml.

For the secondary quantification method the liberated monosaccharides (mannitol, fucose, arabinose, galactose, glucose and xylose) and the concentrations of sugar degradation products (in particular furfural and 5-hydroxymethylfurfural) were also quantified via HPLC using the methods outlined in section 2.8.

2.4 Individual evaluation of stage 1 and stage 2 of the control protocol using the two different quantification methodologies

A hydrolysis trial was conducted using brown (*L. digitata*) and red seaweed (*C. crispus*) using only the low temperature concentrated acid step (stage 1) to establish if this alone was sufficient for accurate quantification of total carbohydrates. Wheat straw was run alongside as a control substrate. This single stage dataset was then compared to the same species of seaweed (and again wheat straw) that had been subjected to the fill protocol including the second step (stage 2). The 2 different quantification methods (colourimetric and HPLC based) were then both used to determine the total carbohydrate contents from the 1st step trial (stage 1 only) and the complete (both stage 1 and 2) protocol. This would then indicate whether both hydrolysis steps would be required for seaweed and therefore identify how many optimization possibilities existed.

2.5 Stage 1 optimization

The first stage of the two stage hydrolysis method was investigated to determine whether heating for 1 h with 12M H_2SO_4 concentration at 37°C was too harsh for the *L. digitata* biomass and whether more accurate total sugar yields could be achieved by using less extreme hydrolysis conditions during this first stage.

Preliminary experimentation of stage 1 involved using a range of weaker sulphuric acid concentrations (3-12 M) and a range of different reaction times (15-60 min), whilst maintaining the original 37°C temperature of the benchmark protocol. DesignExpert (Stat-Ease, USA) was used to create a *D*-optimal experimental design that simultaneously varied the 2 experimental variables (acid concentration and reaction time). Whilst reducing the total number of experimental runs from that of a full factorial experimental design yet still providing statistically significant data, the data could then be modelled using liberated sugar concentration as the response factor. The standard control procedure was ensured to be included in the design of this experiment and replicates were inbuilt into the design. The experimental design matrix can be seen in Table 2.

Table 2. Experimental design used to optimize stage 1 (37°C) of the total acid hydrolysis methodology for quantifying carbohydrates in seaweed species *L. digitata*. Optimisation conducted through screening different sulphuric acid concentrations (3-12M) and reaction times (15-60 min) at 37°C, according to a *D-optimal* design space.

Run	Factor 1 A: H$_2$SO$_4$ Conc (M)	Factor 2 B: Time at 37°C (min)
1	12	15
2	3	15
3	3	15
4	7	15
5	7	35
6	3	60
7	5	45
8	12	35
9	7	35
10	5	25
11	12	60

12	10	25
13	3	35
14	3	60
15	12	15
16	7	60
17	12	60
18	7	35
19	7	35
20	10	45
21	5	60
22	10	60
23	10	15
24	12	60
25	12	60
26	12	15
27	12	15
28	8	15
29	8	15
30	8	60
31	12	35
32	8	35
33	10	15
34	10	25
35	10	25
36	10	45
37	10	45
38	10	60
39	9	20
40	9	50
41	11	20
42	11	50

30 mg of *L. digitata* was mixed with 1 ml of H_2SO_4 (3M-12M) and incubated at 37°C at the different temperatures (15-60 min). RO water was added to dilute the strength of the acid to 1M and then the samples were heated at 100°C for 2 h. Reducing sugars were measured following the method outlined by Dubois et al (1956) and individual monosaccharides and sugar degradation products were quantified by HPLC analysis (see section 2.8).

2.6 Stage 2 optimization

After the optimisation of stage 1 of the 2 stage complete hydrolysis assay, these optimal conditions were then used as a basis for the subsequent optimisation of the second step (stage 2).

Optimisation of stage 2 again involved the evaluation of a range of weaker acid concentrations (0.5-2 M) as with the stage 1 optimisation but this time a range of different reaction temperatures (70-100°C) were also evaluated, whilst maintaining the 2 h reaction time as with the benchmark protocol. As with the stage 1 optimisation, DesignExpert was employed to create a *D*-optimal experimental design. The experimental design can be seen in Table 3.

Table 3. Experimental design used to optimize stage 2 of the total acid hydrolysis methodology for quantifying carbohydrates in *L. digitata* by screening different sulphuric acid concentrations (0.5-2 M) and temperatures (20-100°C) for 2 h, according to a *D-optimal* design space. All stage 2 optimisation attempts were conducted using the previously optimised stage 1 parameters prior (11 M H_2SO_4 at 37°C for 1 h).

Run	Factor 1 A: H_2SO_4 Conc (M)	Factor 2 B: Time at 100°C (min)
1	0.5	20
2	1	60
3	0.5	80
4	2	60
5	1.25	80
6	1.25	60
7	2	20
8	1	60
9	0.5	60
10	1.25	20
11	0.5	100
12	2	40
13	0.5	100
14	0.5	100
15	2	100
16	1.25	100
17	2	80
18	2	100
19	1	40
20	1	80
21	1.25	60
22	2	100
23	0.5	80
24	1.25	60
25	0.5	60
26	1.5	40
27	1.5	90
28	0.75	90
29	0.75	30
30	1.8	80
31	1.8	30
32	1.8	90
33	1.8	60

Both colourimetric and HPLC based quantification methods were again used as with the stage 1 optimisation experiments.

2.7   Validation of the fully optimised method (both stage 1 and 2) on other seaweed biomass types

The conditions which were suggested to be the most optimal for *L. digitata* were evaluated on two other seaweed species; *U. lactuca* and *C. crispus* to establish whether each distinct taxonomical seaweed group would require additional specific optimisation. The control protocol was additionally run alongside for both species as a comparison. Liberated monosaccharides and inhibitory compounds were analysed via HPLC (see section 2.8).

2.8   HPLC analysis - quantification of liberated sugars and sugar degradation products

The monosaccharide concentrations were quantified via HPLC using Dionex ICS-3000 Reagent-Free™ Ion Chromatography, using electrochemical detection. The CarboPacTM PA 20 column (3 x 150 mm) was used and

the mobile phase was 10 mM NaOH with a flow rate of 0.5 mL/min. The injection volume was 10 µl and the column temperature was 30°C. Authentic standards of monosaccharides (mannitol, fucose, arabinose, galactose, glucose and xylose) with concentrations within range of 1 g/L^{-1} to 0.0625 g/L^{-1} were used for quantification. Other monosaccharides which are inherent specifically to brown seaweeds such as mannuronic and guluronic acids are not currently detectable using the analytical method described above. Although their non-quantification may give a slight underestimation of the 'total' carbohydrate content in *L. digitata*, the summation of the six detectable monosaccharides which are currently quantified can still provide adequate figures for the optimisation trial.

The analysis of sugar degradation products (HMF, furoic acid, furfural, vanillic acid, vanillin, ferulic acid and *p*-coumaric acid) were quantified by HPLC using the method outlined in Wilkinson et al, 2014. This system used UV detection at 270 nm (2695 HPLC system and 996 Photodiode Array Detector, Waters, USA) and a Techsphere ODS C18 column (5 µm, 4.6 mm × 250 mm; HPLC Technologies, UK) was used at room temperature. The sample volume was 10 µl and the mobile phase was a gradient of methanol in 1% acetic acid at an overall flow rate of 0.5 ml/minute. The methanol concentration was increased from 20% to 50% over 30 minutes with a 100% methanol column cleaning phase and a 9 min re-equilibration period. Data were recorded using Millennium Chromatography software (Waters, USA).

2.9   Experimental design and statistical analysis

Experimental design, response surface modeling and ANOVA were all performed using Design Expert v 7.0 (Stat-Ease Inc., Minneapolis, USA). The experimental designs and response surface modelling (RSM) used a *D-optimal* format from which the *D-optimal* algorithm chose the treatment combinations (the exact parameter settings; e.g. acid concentrations, reaction times and temperatures) to include in the design from a specific range for that parameter set by the user. This reduced the total number of experiments needed to be conducted from that of a full factorial experimental design yet still provided statistically valid data. Additional experimental runs were also conducted to improve the statistical validity of the derived models. Additional statistical analyses were conducted using a student's *t-test.*
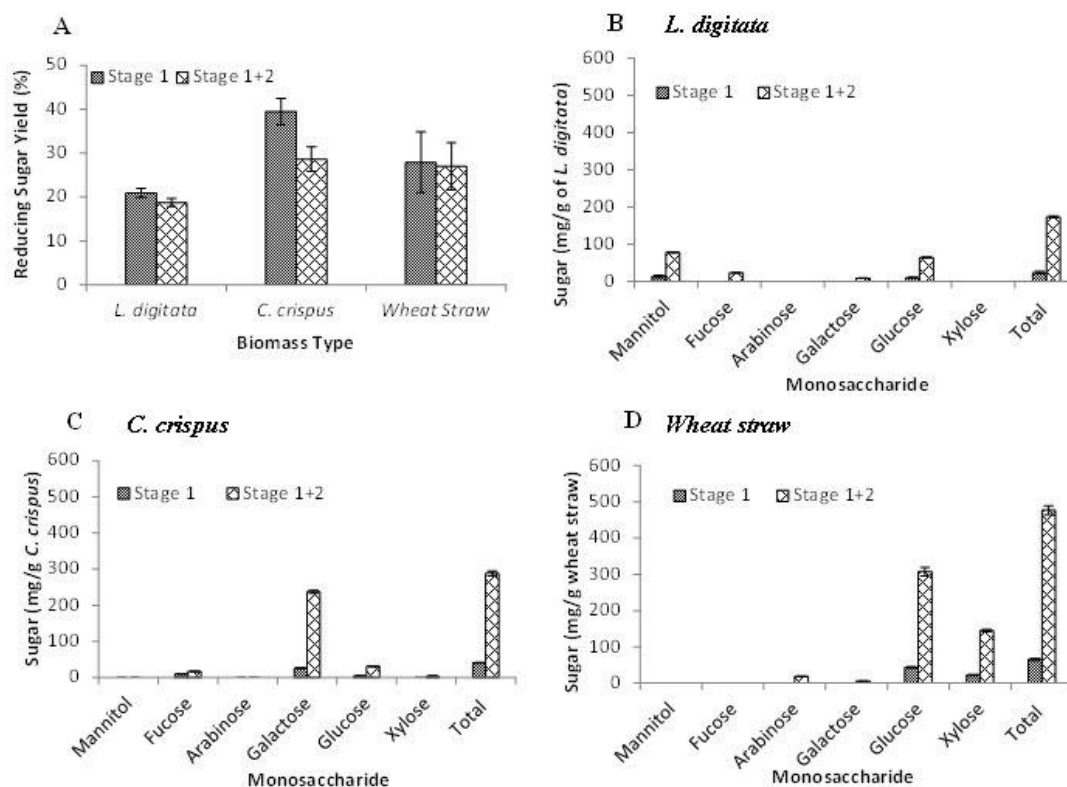
3   RESULTS

3.1   Comparison of stage 1 and stage 2 of the complete acid hydrolysis based carbohydrate assay on L. digitata, C. crispus and wheat straw (using both colourimetric and HPLC quantification)

All experimental data within this results section is presented in terms of achieved total sugars from two different methods of quantification in order to compare the reliability of each current method. Quantification method 1 was the colourimetric based assay outlined in the protocol followed by Dubois et al (1956) which calculates the percentage of reducing sugars liberated from the hydrolysis of biomass compared to glucose standards. Quantification method 2 is the sum of individual monosaccharides (mannitol, fucose, arabinose, galactose, glucose and xylose) liberated from the hydrolysis including sugar degradation products which were analysed chromatographimetrically via HPLC, and yields are expressed as mg of total sugar per g of biomass.

The percentage differences in the release of reducing sugars (quantified using the Dubois based colourimetric assay) and individual monosaccharides (HPLC) between the two stages of the standardised benchmark control protocol, from the three types of biomass, can be seen in Figures 2A-D.

There appears to be little if any significant difference in the yields of reducing sugars when looking at both *L. digitata* and the wheat straw control substrate between the two stages of this control protocol.

However, there was a small (10%) difference in sugar yields observed between stage 1 and stage 2 for the red seaweed *C. crispus* (Figure 2A). Nevertheless, in contrast to the colourimetric derived data, HPLC data revealed a clear and significant difference in the release of monomeric sugars between both stages of the protocol for all three biomasses (Figures 2B-C). Across the three biomass types, higher yields of the specific monosaccharides (mg/g of biomass), particularly glucose, galactose and xylose, were released during the second stage of the protocol, which subsequently increased the total yields of monomeric sugars.

(Stage 1: 12M H_2SO_4 at 37°C for 1 h; Stage 1+2: 1M H_2SO_4 at 100°C for 2 h)

Figure 2. Comparison of reducing sugar yields (%) and monosaccharide yields (mg per g biomass) released from various different biomass types during either just stage 1 or stage 1+2 of the control protocol. **A** Reducing sugar yields (%) released from *L. digitata*, *C. crispus* and wheat straw (control substrate) during the 2 separate stages of control method, with quantification via the phenol-sulphuric acid colourimetric assay. Data are the mean ± SD of three replicate measurements. Total sugar yields refer to the sum of the six different monosaccharides. **B-D** HPLC data showing yields of individual monosaccharides released during stages 1 and 2 of the original control method. Data are the mean ± SD of three replicate measurements. **B**: *L. digitata*. **C**: *C. crispus*. **D**: Wheat straw

3.2   *Optimisation of stage 1 of the control benchmark protocol using L. digitata*

Figure 3 shows the influence of variation of time (min) and sulphuric acid concentration (M) at 37 °C on the percentage yield of reducing sugars using quantification method 1 and also the release of monosaccharides and sugar degradation products using method 2 during the first stage of the protocol. The derived response surface model (RSM) (Figure 3A) suggested that time was not a significant factor which had any influence on the final yield of reducing sugars liberated from *L. digitata* (p=0.1684). However, the initial concentration of sulphuric acid used appeared to be highly significant (p=<0.0001). It was evident that there was an increase in the final yield of reducing sugars as the concentration of acid increased however overall yields then started to decrease when the acid concentration exceeded 10M sulphuric acid. Quantification of the individual monosaccharides and the total yield (the sum of the six individual monosaccharides) analysed using HPLC supplied the trend observed in the 3D RSM (Figure 3B). It appeared that using an 11M sulphuric acid concentration released 209 mg of total sugars per gram of *L. digitata* compared to the 12M acid concentration used in the control protocol which released only 166.4 mg/g. In addition, the use of higher acid concentrations (11M-12M H_2SO_4) had a huge effect on the concentration of glucose specifically from the seaweed (*L. digitata*). The higher acid concentrations liberated ca. 65-75 mg/g (glucose per g seaweed) compared to only ca. 2-5 mg/g for the lower concentrations (3-9 M H_2SO_4). This large difference thus confirmed seaweed biomass did still require strong acid concentrations similar to that of lignocellulosic biomass, although with an optimum possibly slightly lower than 12M.
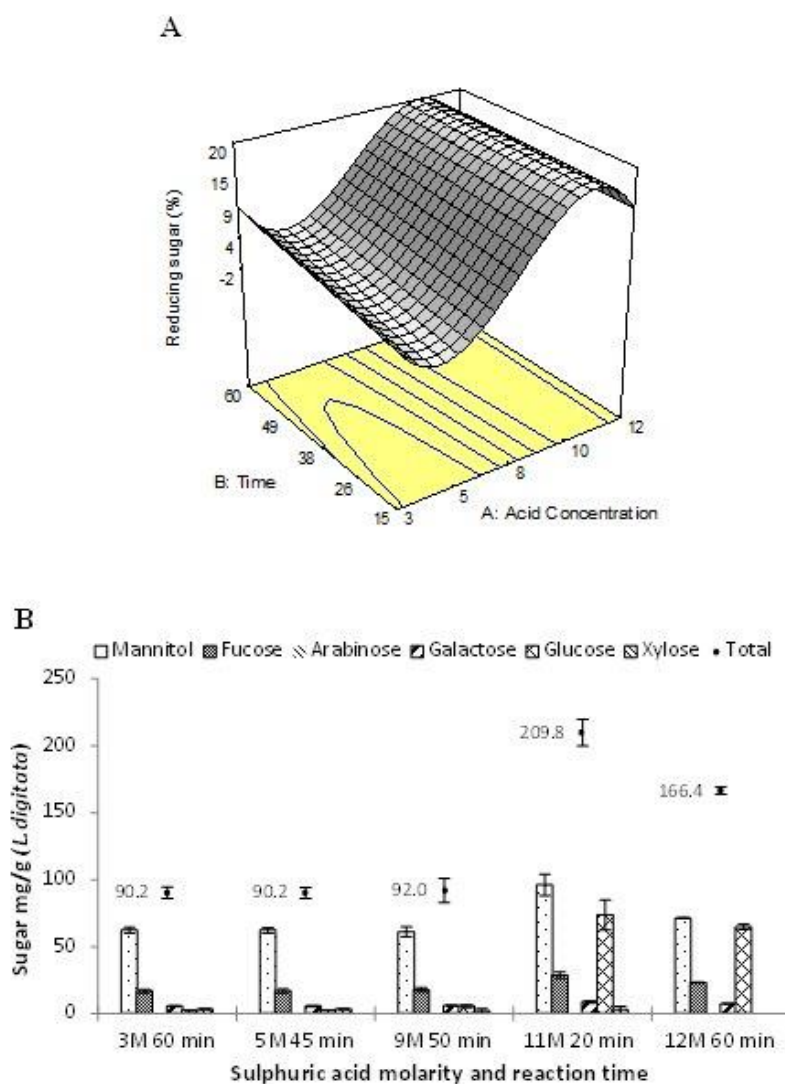
Figure 3. Preliminary screening of the simultaneous impact of variation of the sulphuric acid concentration and reaction time on the release of sugars from *L. digitata* during stage 1 (at 37°C) of the protocol. **A** 3D response surface model showing the impact of different sulphuric acid concentrations (M) and reaction times (min) on the release of reducing sugars (%). Quantification using the phenol-sulphuric acid colourimetric assay. Model R^2: 0.56. **B** HPLC based quantification of the individual monosaccharides yields (mg/g biomass) liberated from seaweed using a range of H_2SO_4 concentrations (3-12 M) and a range of reaction times (15-60 min). Data are the mean ± SD of three replicate measurements.

Furfural was the only identified sugar degradation product that was detected in the preliminary screen. Reaction time was suggested to be somewhat significant regarding furfural concentrations (p=0.0113) (Figure 4A). However, sulphuric acid concentrations played a much more significant role with regards to furfural generation (p<0.0001). There was an increase in the production of furfural with higher concentrations of sulphuric acid and a slight increase with furfural concentrations with increasing reaction time (R^2 = 0.50). Interestingly, 2 other unknown compounds with retention times of 4.4 and 6.6 minutes were detected in various experimental runs (Figures 5A and B). It was not possible to identify or accurately quantify these unknown compounds without a reference standard to compare against, however peak area sizes were evaluated in response to variation parameters. Figure 4B illustrates how the unknown compound with a 4.4 min RT decreases in size (reduction in peak area) as acid concentration and reaction time increased. However the model suggested that reaction time was not likely to be a significant factor (p=0.56). The second unknown compound (with a RT of 6.6 min) was only detected in experimental runs which were treated with highest sulphuric acid concentration of 12M (Figure 4C).
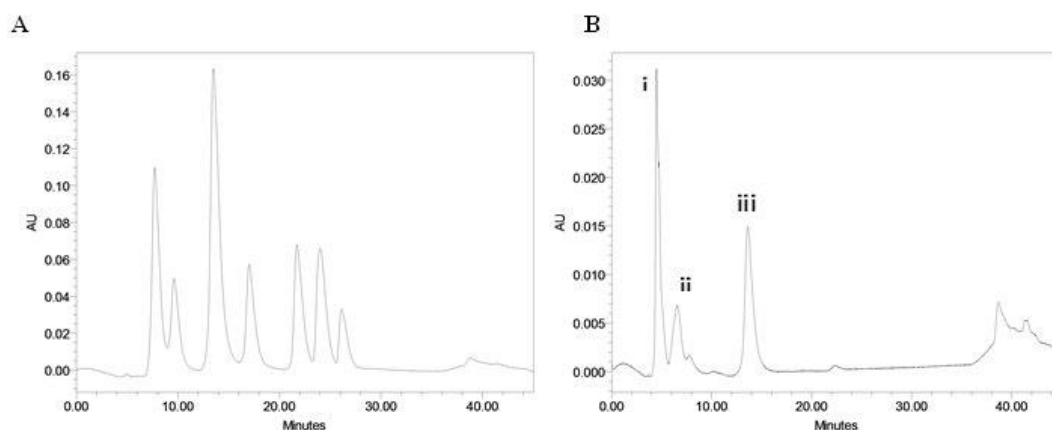
Figure 4. Example chromatograms of sugar degradation products generated during stage 1 optimization of the total acid hydrolysis protocol. **A** HMF, furoic acid, furfural, vanillic acid, vanillin, ferulic acid and *p*-coumaric acid (Left to right). **B** Degradation products released from *L. digitata* after exposure to 12M H_2SO_4 for 60 min at 37°C. **i**. Unknown peak at 4.5 min **ii**. Unknown peak at 6.5 min **iii**. Furfural.

Overall, using 11M sulphuric acid appeared to be the optimum acid concentration to use for the successful hydrolysis of *L. digitata* as (regardless of which of the 2 different quantification methods was used) it liberated higher monosaccharide concentrations, more reducing sugars and fewer sugar degradation products than the control protocol. In addition, reaction time was not suggested to be a significant factor for any of the responses measured. An additional experiment was conducted to determine whether reaction time was still not suggested to have any significant contribution with regards to the release of sugars from *L. digitata*. This used 1 ml of acid with the optimum concentration of 11M at the set temperature of 37°C. Figure 6 shows that time still did not play a major role in the release of reducing sugars concentration from *L. digitata*. There was only a ca. 13% difference in the reducing sugar yields between the reaction time that released the lowest yield of reducing sugars (15 min); ca 16% and the highest yield (60 min); 29%. As time was not suggested to greatly influence the release of sugars during the first step (stage 1) at 37°C, and the highest yield was still achieved using the original incubation time of 60 min (as with the control protocol) this was selected as the most appropriate incubation time to use for further experimental optimisations. Therefore, the optimised conditions chosen for stage 1 of the control protocol for *L. digitata* involved using 11M sulphuric acid at 37°C for 60 min. These conditions were then subsequently used for the first stage of the new optimised protocol of which the second step (stage 2) would then also be optimised.

3.3   Optimisation of stage 2 of the control protocol for L. digitata

After having optimized the first stage of the assay, the second stage of the control protocol was investigated in order to attempt to optimise it and to examine whether more sugar could be liberated from *L. digitata* using the new optimised stage 1 conditions. This series of experiments were designed to determine whether varying the concentration of acid to a lower molarity (than the 1 M used in the control protocol) and also whether incubating the seaweed at 100°C for 2 h was actually required or whether lower temperatures were sufficient. Different concentrations of diluted acid (0.5-2 M) were evaluated as well as different incubation times (20-100 min) at 100°C (see Table 2 for experimental design details). The effect of variation of these experimental parameters was then determined through quantification of reducing sugars, which were determined by the phenol-sulphuric acid colourimetric assay (Dubois based). Consequently, the derived model was not deemed to be significant and gave a flat response with a poor R^2 value of 0.17 (Figure 7). Therefore, the original protocol for stage 2 was not changed.

In conclusion it suggested that optimisation of stage 1 of the process was more crucial than stage 2 in the specific case of *L. digitata* species. As such, stage 2 of the process remained unchanged as part of the new optimised process used subsequently for testing other species of seaweed.

3.4   Evaluation of the new optimized protocol for quantification of the total carbohydrate content in alternative species of seaweed

The newly optimized method for determining the carbohydrate content of *L. digitata* (using the optimised first stage reaction conditions of 11M concentrated sulphuric acid at 37°C for 60 min) and also the original, unmodified control protocol were both applied to two other species of seaweed belonging to different taxonomical groups: *C. crispus* (a rhodophyte) and *U. lactuca* (a chlorophyte) to determine whether the newly optimised protocol (developed for *L. digitata*) was also optimal for these other species. Figure 8 shows the difference in the liberation of total sugars (the sum of mannitol, fucose, arabinose, galactose, glucose and xylose concentrations) and sugar degradation product furfural for each *C. crispus* and *U. lactuca*. The newly optimized quantification protocol did liberate a significantly (p = 0.02 - 0.04) higher total sugar yield for both the *C. crispus* and *U. lactuca* species when compared to the total sugar yields liberated from the original control protocol. Total yields of 215 and 254 mg/g were achieved using the new protocol (for *C. crispus* and *U. lactuca*, respectively) compared to only 202 and 244 mg/g respectively from the original control protocol. Additionally, there was a significant difference in the production of furfural (which was the only sugar degradation product detected in the samples), between the newly optimised and original control protocols applied to both *C. crispus* and *U. lactuca* (p < 0.005). Figure 8B shows that higher concentrations of furfural were generated from both species of seaweed when the original control protocol was applied (2.5 and 3 mg/g for *C. crispus* and *U. lactuca*, respectively) when compared to the new optimised protocol (0.7 and 1.3 mg/g, respectively).

4   DISCUSSION

The aims of this study were to determine whether the lignocellulosic biomass derived protocols that are currently applied to seaweeds are suitable for carbohydrate analysis and to develop a more accurate protocol specifically to seaweed. For these purposes, *L. digitata* was chosen as a benchmark species for the initial method development and the formulation of the newly optimized protocol. Two additional species of seaweed (*C. crispus* and *U. lactuca*) were then used to test the effectiveness of the new protocol.

Both stage 1 and stage 2 of the 2 step complete acid hydrolysis method for total carbohydrate quantification of lignocellulosic biomass (which is also used by some research groups for seaweed also) were evaluated and optimised. Additionally, two different quantification methods were compared against each other for the analysis of sugars. The colourimetric method outlined by Dubois et al (1956) is a well-established quantification method that has been extensively used in many fields of research, with more than 32,500 peer-reviewed papers having cited the method for carbohydrate quantification at the time of writing this present paper. The assay is based upon a phenol-sulphuric acid reaction that depends on the dehydration of hydrolysed sugars to furfural derivatives during their reaction with concentrated sulphuric acid (Dubois et al. 1956, Bicker et al. 2003, Lima et al. 2010). When phenol is added, the furfural derivatives react further and a coloured complex is formed which can then be read using a spectrophotometer at a maximum absorbance wave-length of 490 nm (Dubois, Gilles et al. 1956). Although this method is relatively easy to follow and gives rapid results, there are a significant number of limitations mainly related to the actual quantification conducted by comparison to the absorbances of the purely glucose standards. The method of quantification using only glucose as a reference standard compound may lead to inaccuracies in the calculation of the carbohydrate content of biomass types that are composed of carbohydrate polymers which are not entirely glucose based, as it is the case with many species of seaweed (Albalasmeh et al. 2013). Additionally, differences which exist in the chemical reactivity of various carbohydrates with derivatisation reagents (sulphuric acid in this case) resulting from the carbohydrate being either neutral or anionic, could influence their molar absorption co-efficiencies (Mecozzi 2005). The observation that a similar total percentage of reducing sugar concentration was quantified between the two stages of the original control protocol suggested that the Dubois colourimetric assay is not sensitive enough to detect the changes between stage 1 and stage 2 (Figure 2A). This was confirmed when HPLC analysis was conducted on the same samples, which revealed the monosaccharide profiles for *L. digitata, C. crispus* and the wheat straw control substrate were different following the 2 different stages of the original control protocol (Figures 2B-D). This suggests the second step (stage 2) was crucial for accurate quantification of the total carbohydrate content in seaweed and could not be avoided. It indicated the first stage alone was not significant for complete recovery of the total sugar content. It additionally suggests that the Dubois based colourimetric method for the final quantification step was not sensitive enough to detect these differences in sugar yields. The increased galactose sugar yields that were detected using the HPLC based quantification method (liberated from *C. crispus*) using both stage 1 and stage 2 of the protocol (compared to when only stage 1 was used) are as a result of carrageenan hydrolysis. This confirmed the requirement of the second stage of the protocol in order to completely hydrolyse carrageenan. In a

similar fashion, the increases in glucose yields from wheat straw were as a result from the hydrolysis of cellulose. This calls into question how sugars can be correctly quantified and which are the most accurate and most suitable analytical methods to use for such assays when accuracy (of both the identification and quantification) is highly important. For rapid screening purposes (for almost immediate data acquisition), a standard colourimetric assay such as that outline by Dubois could still be appropriate in certain circumstances as it can still provide some degree of information on reducing sugar yields, and has thus subsequently been used purely for rapid screening purposes in the remainder of this study. However, it is evidently more appropriate to use higher throughput analytical systems such as HPLC when the quantification of specific individual monosaccharides is required (which have been liberated post hydrolytical treatments). The only significant limitation with the use of an analytical system such as HPLC is the detection of only known compounds, of which authentic standards can be prepared and used to assist in identification and quantification. Most HPLC columns can only specifically separate certain related families of compounds due to the nature of their chemical interactions with the sample matrix. This means that only compounds which one has specifically screened for can be quantified, and the unique monomeric sugars which are inherent to seaweeds might be somewhat more of a challenge to quantify such as mannuronic and guluronic acids found in brown seaweeds. This has led researchers to develop novel and highly specific quantification methods for seaweed monomeric sugars and carbohydrates (Manns et al. 2014, Graiff et al. 2015, Kim et al. 2015).

Results from this study suggested that the original control protocol for the quantification of carbohydrates is too extreme for seaweeds. This was based around a total sugar yield (sum of six individual monosaccharides) of ca. 210 mg (per g of biomass) achieved using the new optimised protocol (using 11 M H_2SO_4) compared to the only ca. 166 mg (per g biomass) total sugar yield achieved using the original protocol (12 M H_2SO_4). Additionally, the lower levels of sugar degradation products generated from the optimised (11M H_2SO_4) protocol also suggests that the original protocol was excessively harsh (Figure 4A). The 3D RSM depicted in Figure 3A clearly shows that 11M H_2SO_4 is likely to be the optimum for maximum release of sugars from *L. digitata*, as the sugar yields begin to drop when the concentration of acid is increased further. It may certainly be possible that treatment with 12M H_2SO_4 could have actually liberated more sugars from the seaweed however the stronger acid concentration would have converted a greater proportion of liberated sugars into degradation products. This is confirmed as furfural concentrations continued to increase as acid concentration increased (Figure 4A). Although the specific identification of the unknown compound that eluted at 4.4 min (Figure 5B) was not achieved, it is suggested to be a sugar that gets hydrolysed as a result from the increase in thermal-chemical treatment it gets exposed to. Alternatively, the unknown compound could already be an inhibitory product that has been produced from milder conditions that were screened in this trial which could be thermo-chemically unstable and is further broken down or converted to another compound as the conditions become more extreme. The second unknown compound (with a retention time of 6.6 min) which was only found in samples treated with the original control protocol (12 M H_2SO_4 and 12 M H_2SO_4 concentrations for 35 min) may similarly be a sugar degradation product and could also help explain the lower sugars yields liberated from using the control protocol.

In contrast to the significant degree of optimisation that was achieved from the modification of stage 1 of the hydrolysis protocol, there appeared to be little variation in the total sugar yields regardless of any of the modification made to stage 2. This implied there was no requirement for any optimization of the second stage of the assay, if similar yields of reducing sugars were being liberated from the biomass regardless of any variation in reaction severity. It highlighted that stage 1 was the most crucial: initially treating the seaweed with a sufficient concentration of acid to release the maximum amount of larger polysaccharides which can then be further broken down to into their corresponding simple sugars, but also minimising the formation of degradation products.

The newly optimized protocol for *L. digitata*, (11M H_2SO_4 at 37°C for 1 h followed by diluting the acid strength to 1M and incubating for 2 h at 100°C) was additionally shown to be somewhat more appropriate to species of seaweed which belong to the other taxonomical groups than the original control protocol (Figures 8A+B). A rhodophyte (*C. crispus*) and a chlorophyte (*U. lactuca*) were selected because the carbohydrate structures and composition differ significantly between the groups (Percival and McDowell 1981, Adams et al. 2011). Any significant difference in carbohydrate composition between species would imply that an altered chemical structure and different bond linkages will additionally exist. As such different severities of treatment may be required to effectively hydrolyse the carbohydrates for accurate quantification. Results from these experiments indicated that the 12M H_2SO_4 control protocol is still too extreme for both *U. lactuca* and *C. crispus* species. A direct comparison of the original control protocol and the newly optimized method developed for *L. digitata* but conducted on *U. lactuca* and *C. crispus* revealed that significantly lower yields of total sugars were released from the application of the original control protocol. In addition higher furfural levels were again detected in the two seaweed species when applying the original control protocol compared to the newly optimised protocol. This yet

again suggests that the original control protocol is still too extreme even for other species of seaweed and its use would provide an under estimation of the true total carbohydrate content unless each and every potential degradation product could be accurately identified and quantified. Furthermore, findings from this study confirm the importance to optimize protocols for each different species of seaweed in order to accurately determine the content of carbohydrates.

A comprehensive optimisation of the total acid hydrolysis based protocol for the quantification of the carbohydrate content of seaweeds has been presented in this study. We conclude the most commonly followed protocol, which is typically a modification of the NREL based assay specifically designed for carbohydrate analysis from lignocellulosic biomass, is excessively harsh for seaweed biomass. This can lead to giving erroneously low total sugar quantification as its severity can degrade liberated sugars to decomposition products. Specifically for *L. digitata*, the optimised protocol which released maximum yields of sugar utilised the two stage acid hydrolysis protocol using 11M sulphuric acid at 37°C for 1 hr followed by a dilution of the acid to 1M and heating at 100°C for 2 h. The new optimised protocol liberated ca. 210 mg total sugars (per g biomass) compared to only ca. 166 mg/g for the original control protocol. In addition the first stage of the hydrolysis protocol was shown to be the most crucial for maximising sugar yields. We also conclude that different seaweed species require specific individual optimisations of the protocol for accurate total sugar quantification, and this was concluded by applying the newly optimized method on samples of *C. crispus* and *U. lactuca*. Additionally, HPLC seemed to be a more appropriate method for accurate quantification of sugars, as opposed to the rudimental colourimetric assay outlined by Dubois et al (1956). In conclusion it would appear crucial to optimise the total carbohydrate quantification methods, not only just seaweed but specifically for each different biomass type used as accurate composition analysis is crucial for the successful development of bio-refinery processes.

5   ACKNOWLEDGEMENTS

REFERENCES

Adams, J., et al. (2011). "Seasonal variation in the chemical composition of the bioenergy feedstock Laminaria digitata for thermochemical conversion." Bioresource technology **102**(1): 226-234.

Adams, J. M., et al. (2009). "Fermentation study on Saccharina latissima for bioethanol production considering variable pre-treatments." Journal of Applied Phycology **21**(5): 569-574.

Adams, J., A. Schmidt and J. Gallagher (2014). "The impact of sample preparation of the macroalgae Laminaria digitata on the production of the biofuels bioethanol and biomethane." Journal of Applied Phycology: 1-7.

Albalasmeh, A. A., et al. (2013). "A new method for rapid determination of carbohydrate and total carbon concentrations using UV spectrophotometry." Carbohydrate polymers **97**(2): 253-261.

Berndes, G., et al. (2003). "The contribution of biomass in the future global energy supply: a review of 17 studies." Biomass and bioenergy **25**(1): 1-28.

Bicker, M., et al. (2003). "Dehydration of fructose to 5-hydroxymethylfurfural in sub-and supercritical acetone." Green Chemistry **5**(2): 280-284.

Blumenkrantz, N. and G. Asboe-Hansen (1973). "New method for quantitative determination of uronic acids." Analytical biochemistry(54): 484-489.

Borines, M. G., R. L. de Leon and J. L. Cuello (2013). "Bioethanol production from the macroalgae Sargassum spp." Bioresource technology **138**: 22-29.

Chirapart, A., J. Praiboon, P. Puangsombat, C. Pattanapon and N. Nunraksa (2014). "Chemical composition and ethanol production potential of Thai seaweed species." Journal of applied phycology **26**(2): 979-986.

Dubois, M., et al. (1956). "Colorimetric method for determination of sugars and related substances." Analytical chemistry **28**(3): 350-356.

Folch, J., et al. (1957). "A simple method for the isolation and purification of total lipids from animal tissues." J biol chem **226**(1): 497-509.

Ge, L., P. Wang and H. Mou (2011). "Study on saccharification techniques of seaweed wastes for the transformation of ethanol." Renewable energy **36**(1): 84-89.

Graiff, A., et al. (2015). "Chemical characterization and quantification of the brown algal storage compound laminarin—A new methodological approach." Journal of Applied Phycology: 1-11.

Jung, K. A., et al. (2013). "Potentials of macroalgae as feedstocks for biorefinery." Bioresource technology **135**: 182-190.

Hargreaves, P. I., et al. (2013). "Production of ethanol 3G from Kappaphycus alvarezii: Evaluation of different process strategies." Bioresource technology **134**: 257-263.

Karakashev, D., et al. (2007). "Anaerobic biotechnological approaches for production of liquid energy carriers from biomass." Biotechnology letters **29**(7): 1005-1012.

Khambhaty, Y., K. Mody, M. R. Gandhi, S. Thampy, P. Maiti, H. Brahmbhatt, K. Eswaran and P. K. Ghosh (2012). "Kappaphycus alvarezii as a source of bioethanol." Bioresource technology **103**(1): 180-185.

Kim, T. H., et al. (2015). "A novel alginate quantification method using high-performance liquid chromatography (HPLC) for pretreatment of Saccharina japonica." Journal of Applied Phycology **27**(1): 511-518.

Kjeldahl, J. (1883) "Neue Methode zur Bestimmung des Stickstoffs in organischen Körpern" (New method for the determination of nitrogen in organic substances), Zeitschrift für analytische Chemie, 22 (1) : 366-383.

Korzen, L., I. N. Pulidindi, A. Israel, A. Abelson and A. Gedanken (2015). "Single step production of bioethanol from the seaweed Ulva rigida using sonication." RSC Advances **5**(21): 16223-16229.

Lima, S., et al. (2010). "Acid-catalysed conversion of saccharides into furanic aldehydes in the presence of three-dimensional mesoporous Al-TUD-1." Molecules **15**(6): 3863-3877.

Lu, X., et al. (2009). "Optimization of H 2 SO 4-catalyzed hydrothermal pretreatment of rapeseed straw for bioconversion to ethanol: focusing on pretreatment at high solids content." Bioresource technology **100**(12): 3048-3053.

Manns, D., et al. (2014). "Methodology for quantitative determination of the carbohydrate composition of brown seaweeds (Laminariaceae)." RSC Advances **4**(49): 25736-25746.

Manivannan, K., G. Thirumaran, G. K. Devi, A. Hemalatha and P. Anantharaman (2008). "Biochemical composition of seaweeds from Mandapam coastal regions along Southeast Coast of India." American-Eurasian Journal of Botany **1**(2): 32-37.

Mecozzi, M. (2005). "Estimation of total carbohydrate amount in environmental samples by the phenol–sulphuric acid method assisted by multivariate calibration." Chemometrics and Intelligent Laboratory Systems **79**(1): 84-90.

Meinita, M. D. N., et al. (2013). "Comparison of agarophytes (Gelidium, Gracilaria, and Gracilariopsis) as potential resources for bioethanol production." <u>Journal of Applied Phycology</u> **25**(6): 1957-1961.

Montague, L., et al. (2002). "Lignocellulosic Biomass to Ethanol Process Design and Economics Utilizing Co-Current Dilute Acid Prehydrolysis and Enzymatic Hydrolysis For Corn Stover: technical report. Colorado: National Renewable Energy Laboratory; 2002, Report No. NREL/TP-510-32428.

Mutripah, S., M. D. N. Meinita, J.-Y. Kang, G.-T. Jeong, A. Susanto, R. E. Prabowo and Y.-K. Hong (2014). "Bioethanol production from the hydrolysate of Palmaria palmata using sulfuric acid and fermentation with brewer's yeast." <u>Journal of Applied Phycology</u> **26**(1): 687-693.

Nelson, N. (1944). "A photometric adaptation of the Somogyi method for the determination of glucose." <u>J. biol. Chem</u> **153**(2): 375-380.

Percival, E. and R. McDowell (1981). "Algal walls—composition and biosynthesis." <u>Plant Carbohydrates II</u>, Springer**:** 277-316.

Sluiter, A., B. Hames, R. Ruiz, C. Scarlata, J. Sluiter and D. Templeton Determination of Structural Carbohydrates and Lignin in Biomass: technical report. Colorado: National Renewable Energy Laboratory; 2008, Report No. NREL/TP-510-42618.[Links].

Sluiter, A., et al. Determination of Structural Carbohydrates and Lignin in Biomass: technical report. Colorado: National Renewable Energy Laboratory; 2008, Report No. NREL/TP-510-42618.

Taherzadeh, M. J. and K. Karimi (2008). "Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review." <u>International journal of molecular sciences</u> **9**(9): 1621-1651.

Trivedi, N., V. Gupta, C. Reddy and B. Jha (2013). "Enzymatic hydrolysis and production of bioethanol from common macrophytic green alga Ulva fasciata Delile." <u>Bioresource technology</u> **150**: 106-112.

van der Wal, H., B. L. Sperber, B. Houweling-Tan, R. R. Bakker, W. Brandenburg and A. M. López-Contreras (2013). "Production of acetone, butanol, and ethanol from biomass of the green seaweed Ulva lactuca." <u>Bioresource technology</u> **128**: 431-437.

Van den Hoek, C., et al (1995). "Algae : An introduction to phycology. " <u>Cambrudge. Univ Press</u>

Wilkinson, S., et al. (2014). "Optimisation of alkaline reagent based chemical pre-treatment of Brewers spent grains for bioethanol production." <u>Industrial Crops and Products</u> **62**: 219-227.

Wu, F.-C., J.-Y. Wu, Y.-J. Liao, M.-Y. Wang and L. Shih (2014). "Sequential acid and enzymatic hydrolysis in situ and bioethanol production from Gracilaria biomass." <u>Bioresource technology</u> **156**: 123-131.