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Production of acetone, butanol, and ethanol from biomass of the green seaweed *Ulva lactuca*

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HIGHLIGHTS

- *Ulva lactuca* was characterized as feedstock for the acetone, butanol and ethanol fermentation.
- Hydrolysates were obtained using mild pretreatment conditions and commercial cellulases.
- *Ulva lactuca* hydrolysate was used as substrate for fermentation by two different strains.
- Rhamnose was utilized by *C. beijerinckii* for production of 1,2-propanediol.

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ABSTRACT

Green seaweed *Ulva lactuca* harvested from the North Sea near Zeeland (The Netherlands) was characterized as feedstock for acetone, ethanol and ethanol fermentation. Solubilization of over 90% of sugars was achieved by hot-water treatment followed by hydrolysis using commercial cellulases. A hydrolysate was used for the production of acetone, butanol and ethanol (ABE) by *Clostridium acetobutylicum* and *Clostridium beijerinckii*. Hydrolysate-based media were fermentable without nutrient supplementation. *C. beijerinckii* utilized all sugars in the hydrolysate and produced ABE at high yields (0.35 g ABE/g sugar consumed), while *C. acetobutylicum* produced mostly organic acids (acetic and butyric acids). These results demonstrate the great potential of *U. lactuca* as feedstock for fermentation. Interestingly, in control cultures of *C. beijerinckii* on rhamnose and glucose, 1,2 propanediol was the main fermentation product (9.7 g/L).

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1. Introduction

Different types of plant biomass, including energy crops, cereals and agricultural by-products, are promising starting materials for the production of fuels and chemicals, using the so-called biorefinery approach, in which the whole biomass is being valorized into a range of different products. The most developed biorefinery concepts are currently based on lignocellulosic feedstocks, where sugars are fermented into ethanol or other energy carriers, and other fractions (such as lignin) are converted into high value chemical additives (Cherubini, 2010). In order to meet the expected increasing demand for biofuels and biochemicals, and to diversify the feedstock and product portfolio of biorefineries, there is a need to find additional suitable biomass sources, in particular those that do not rely on using large amounts of agricultural land. This necessity has led to an interest into the use of biomass from aquatic environments, such as seaweeds (macroalgae) (Subhadra and Grinson, 2010; John et al., 2011; Kraan, 2011).

Seaweeds are fast growing marine plants that may reach large sizes, for example, 3–20 m length for certain kelp species (Lüning, 1993). Growth rates and yields of material per surface area that can be obtained in seaweeds forests are significantly higher than those reported for terrestrial plants (Gao and McKinley, 1994; Horn et al., 2000), mostly due to the lower energy required for the production of supporting tissue compared to that for land plants and a higher capacity of nutrient uptake over their entire surface. Seaweeds have a wide range of commercial applications, traditionally as feed or food, and soil fertilizer, and nowadays as source of hydrocolloids for the food industry, personal healthcare and pharmaceutical industry (McHugh, 2003). Seaweeds are classified into three broad groups based on pigmentation: Brown (Phaeophyceae), red (Rhodophyceae) and green (Chlorophyceae). Green seaweeds have

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traditionally been a part of local diets due to their high protein content and nutritional value (Bobin Dubigeon et al., 1997), however, hydrolysates from this algae can also serve as feedstock for the production of butanol (Potts et al., 2012). Pre-treatment and hydrolysis methods needed for the solubilization of seaweed sugars are less severe than those needed for pre-treatment of lignocellulosic materials, most probably due to the absence of lignin in their cell walls and the relatively lower crystalline of sugar polymers.

Some species of *Clostridium* are able to produce acetone, butanol and ethanol (ABE) by anaerobic fermentation from a wide variety of sugars, both hexoses and pentoses, a process known as the ABE fermentation. Solvent-producing clostridial species are able to utilize sugars in a variety of substrates, including (lignocellulosic) hydrolysates derived from plant biomass (Qureshi et al., 2010). Since seaweeds are composed of a mix of different sugars, these organisms are expected to efficiently convert most sugars into an ABE mixture (Huesemann et al., 2012). The ABE process is being commercially re-introduced for the production of biologically derived butanol (biobutanol) to be used as biofuel or to replace petrochemically produced butanol in the bulk chemical market (López-Contreras et al., 2010; Green, 2011). Some species of green seaweeds, including *Ulva lactuca*, are rich in the deoxysugar rhamnose (Lahaye, 1991), that can be fermented anaerobically into commercially interesting 1,2 propanediol by a number of microbial species, including some clostridial strains (Forsberg et al., 1987; Saxena et al., 2010).

In the present study, *U. lactuca* L., harvested along the Dutch coast of the North Sea was biochemically characterized as fermentation feedstock. Solubilization of sugars in the biomass was carried out using different pre-treatments followed by enzymatic hydrolysis. Hydrolysates of *U. lactuca* were used as medium for the production of acetone, butanol and ethanol by two different well-studied clostridial strains.

2. Methods

2.1. Source and storage of Ulva samples

U. lactuca was collected along the coast of Zeeland (The Netherlands) in the month of December 2008 and stored at −80 °C for several weeks before being freeze-dried lyophilized and stored at room temperature.

2.2. Analytical protocols

2.2.1. Chemical composition of U. lactuca

Freeze-dried *Ulva* was milled over a 2 mm screen. Ash content was determined by incineration at 575 °C for 4 h. Solvent and water extractives were determined by successive extraction with toluene/ethanol (2:1 v/v), 95% ethanol and boiling water. Sugar content was determined on the residue after solvent extraction and the whole biomass after hydrolysis with 72% w/w H_2SO_4 at 30 °C for 1 h, followed by dilution to a H_2SO_4 concentration of 1 M and hydrolysis for 3 h at 100 °C. The hydrolysate was neutralized with bariumcarbonate and analyzed for neutral sugars on a HPAEC (High Performance Anion Exchange Chroamtography) equipped with a CarboPac PA1 (250 × 4 mm) column with a CarboPac PA1 (50 × 4 mm) guard-column (Dionex), and pulsed amperometric detection, using fucose as internal standard (no fucose was found in *Ulva* samples), as described previously (van den Oever et al., 2003). Uronic acids in this hydrolysate were determined spectrophotometrically by the *m*-hydroxydiphenyl assay using galacturonic acid as standard (Blumenkrantz and Asboe Hansen 1973).

2.2.2. Elemental analysis

Elemental analysis (CHNS) was carried out on a Thermo Quest EA 1110 elemental analyzer. For determining total phosphorus *Ulva* was subjected to sulfuric and nitric acid treatment according to the Dutch Standard Method NEN-6662. Phosphorus was detected spectrophotometrically using molybdate blue at 885 nm according to Dutch Standard Method NEN-6479.

2.2.3. Aminoacids analysis

Amino acid analysis (free or protein-bound amino acids) was performed by Ansynth BV (Roosendaal, The Netherlands) using an Alpha Plus II, Biochrom 20 or Biochrom 30 amino acid analyzer. Standard methods were used for the determination of Tryptophan (AOAC, 1993) and other amino acids (AOAC, 1990).

2.3. Pre-treatment and enzymatic hydrolysis of Ulva

2.3.1. Pre-treatment

Freeze-dried *U. lactuca* was milled over a 2 mm screen and resuspended at 10% (w/w) in water. Mild alkaline pre-treatment was carried out by addition of NaOH at 6% (wt NaOH/wt dry matter) and incubation of the slurry at 85 °C for 4 h. Acid pre-treatment was carried out at pH 2 by addition of H_2SO_4 (7.5% wt H_2SO_4/wt dry matter) and incubation of the slurry at 150 °C for 10 min. Pre-treatment at 85 °C was performed under atmospheric conditions in a stirred stainless steel vessels. For pre-treatment at 150 °C, closed 316L stainless steel cylindrical reactors fitted with thermocouples, were submerged in a silicon oil Haake B bath equipped with a Haake N3 temperature controller (Thermo Fisher Scientific, Waltham, MA, USA) The sample core temperature was recorded (Picotech data collector and software, Picotech, UK), the core temperature was held at 150 °C for 10 min and the cylinders were immersed in an ice bath. Samples were centrifuged at 10,000*g* for 15 min at room temperature. The sugar composition of pellet and supernatant was determined as described in Section 2.2.1. The supernatant was analyzed before and after hydrolysis with 1 M H_2SO_4 at 100 °C for 3 h to distinguish between soluble oligomeric and monomeric sugars and enable the determination of total sugars.

2.3.2. Enzymatic hydrolysis of the pre-treated material

The insoluble fractions of the pre-treated material (pellets) were subjected to enzymatic hydrolysis by a commercial cellulase cocktail (GC220, Genencor). Pellets were dispersed in 50 mM sodium acetate buffer, pH 5.0, containing the antibiotic Pen Strep (Sigma–Aldrich) at 0.02 mL/g of *Ulva*. GC220 was added at 0.3 mL/g of pre-treated *Ulva* (35 FPU/g (Kabel et al., 2006)). Enzymatic hydrolysis was performed at 50 °C for 96 h and the supernatant was analyzed for sugar composition.

2.3.3. Preparation of hydrolysate for fermentation

A hydrolysate was prepared by re-suspending milled *Ulva* in milliQ water at 15% DM (w/w) and incubating the suspension at 150 °C for 10 min in a pressurized 2-L vessel**.** This vessel mixes by head over tail rotation and was equipped with a thermocouple to record the core temperature (Picotech data collector and software, Picotech, UK). Heating of the vessel was achieved by circulation silicon oil with a Haake B bath equipped with a Haake N3 temperature controller (Thermo Fisher Scientific, Waltham, MA, USA).

Enzymatic hydrolysis of the *Ulva* slurry obtained after pretreatment was carried out as described in Section 2.3.2 at 50 °C for 24 h, but without the addition of antibiotics. The pH of the hydrolysate was 5.8. The hydrolysate was centrifuged at 10,000*g* for 15 min, at 4 °C, after which the supernatant was stored at −20 °C.

2.4. Fermentation of U. lactuca hydrolysate

Clostridium acetobutylicum ATCC 824 and *Clostridium beijerinckii* NCIMB 8052 were stored as spore suspensions and cultivated as previously described (López-Contreras et al., 2000). For the preparation of pre-cultures, spores were heat-shocked and placed into CM2 medium, composed of, per liter; yeast extract, 2.5 g, KH_2PO_4, 1 g, $K_2HPO_4.3H_2O$, 0.8 g, NH_4Ac, 2.9 g, p-aminobenzoic acid. 0.1 g, $MgSO_4{\cdot}7H_20$, 1 g, and $FeSO_4{\cdot}7H_20$, 6.6 mg. As carbon sources, stock solutions of glucose, xylose, rhamnose or their mixtures were sterilized separately and added to the medium at the indicated concentrations. The pH of the control media was 6.1–6.2. The pH of the hydrolysate based-media varied between 5.3 and 5.8. For media for *C. beijerinckii*, the starting pH of the cultures was adjusted to 6.0–6.4 with 1 M NaOH, while for *C. acetobutylicum* cultures the pH was not adjusted.

2.5. Analysis of metabolites and fermentation products

Sugars and fermentation products were determined in clear culture supernatants from samples taken during the growth experiments and stored at −20 °C. Organic acids, solvents and sugars were analyzed by high performance liquid chromatography (HPLC). Samples were diluted 1:1 with internal standard solution (250 mM propionic acid in 1 M H_2SO_4) and centrifuged for 5 min at approx. 16,000*g* (Centrifuge 5417 C Eppendorf desktop). The supernatant was filtered through a 13-mm GHP acrodisc 0.2-μm filter (Spartan 13/0.2 RC) and transferred to HPLC vials. Separation was carried out in a Waters HPLC system equipped with an autosampler (Waters model 717) and an HPLC column Shodex KC-311 (Shodex, Tokyo, Japan). The column was kept at 80 °C, with 3 mM H_2SO_4 as eluent at a flow rate of 1 mL/min. A refractive index detector (Waters model 2414) and a UV absorbance detector (Waters model 2487) were used in series. The concentrations of most metabolites were determined from the refractive index chromatograms with the exception of butyric acid, which was determined from the UV chromatograms at 210 nm. For analysis of 1,2-propanediol-containing samples, the internal standard was valeric acid at 100 mM instead of propionic acid.

3. Results and discussion

3.1. Composition of U. lactuca

The total solvent extractives (ethanol and ethanol/toluene mix as solvents) were 3.4% (w/w) of the total *U. lactuca* biomass (Table 1), which can be attributed to the lipid fraction in *Ulva* species. The lipid fraction for green seaweed species ranges between 0.3% and 5% of dry matter (Wong and Cheung, 2000; McDermid and Stuercke, 2003; Ortiz et al., 2006). The hot water extractives comprised 37% (w/w) of the *Ulva* dry matter. About 40% of the total sugars of *Ulva* were water extractable and contained rhamnose, xylose and uronic acids, the main constituents of ulvan (Ray and Lahaye, 1995).

Table 2
Amino acid composition of *Ulva lactuca*. Values represent mg per gram dry matter of sample ± SD (*n* = 4).

	Aminoacids (mg/g)	
	Soluble aminoacids	Total aminoacids
Asparagine acid + asparagine		26.3 ± 1.4
Hydroxyproline	nd	2.8 ± 0.1
Threonine	0.76	12.9 ± 0,3
Serine	0.8 ± 0.1	12.9 ± 0.3
Asparagine	0.5	
Glutamic acid	3.9 ± 0.2	–
Glutamine	4.1 ± 0.5	
Glutamic acid + glutamine	–	42.6 ± 2.3
Proline	5.3 ± 0.3	14.4 ± 0.3
Glycine	0.7 ± 0,1	14.2 ± 0.2
Alanine	2.1 ± 0.1	18.8 ± 0.4
Valine	0.7 ± 0.1	13.1 ± 0.3
Methionine	0.7 ± 0.1	nd
Isoleucine	0.5 ± 0.1	8.3 ± 0.1
Leucine	1.4 ± 0.2	14.1 ± 0.2
Tyrosine	1 ± 0.1	7.8 ± 0.1
Phenylalanine	1.0 ± 0.1	10.0 ± 0.1
Hydroxylysine	nd	0.6 ± 0.1
Ornithine	nd	<0.5
Lysine	0.6 ± 0.1	10.1 ± 0.2
Histidine	0.2	4.1 ± 0.1
Arginine	nd	24.9 ± 1.5
Total	24.2 ± 2	238.2 ± 8.1

The total sugar content of *U. lactuca* sample was approx. 20% (w/w) of dry weight, which is in agreement with previous results (Bobin Dubigeon et al., 1997). In general, the sugar content of green seaweeds is lower than that in other seaweeds and in other biomass types, such as lignocellulosic feedstocks.

The ash content of *U. lactuca* was 15.9% (w/w). An important component in the ash is sulfate derived from the sulfated polysaccharides. Elemental analysis of *U. lactuca* showed a 2% (w/w) sulfur content, indicating that the maximum amount of sulfate ions in this *U. lactuca* sample was 5.2% (w/w). This relatively low amount of sulfate explains the lower amount of ash determined compared to that reported earlier (Bobin Dubigeon et al., 1997). The amount of total proteins present in the material was estimated by multiplying the total nitrogen content found (4.4%, w/w) by 5.59 (Lourenço et al., 2002), resulting in 25.6% (w/w), which is closely related to the total amount of amino acids determined independently (Table 2) of approx. 24% (w/w). The carbon, hydrogen and phosphorus contents were 33.5% (w/w), 5.9% (w/w) and 1.3% (w/w), respectively.

Table 2 shows that *U. lactuca* had a high protein content (approx., 25% of its dry matter), similar to that of legumes and grains (Ortiz et al., 2006), justifying its traditional use in human or animal nutrition. Of the total amino acids, only 10% were present as free amino acids (Table 2). Tryptophan could not be determined. The amino acid profile found in *U. lactuca* is consistent with those from other *Ulva* species, with glutamic acid and glutamine, asparagine and aspartic acid and arginine, the most abundant amino acids (Wong and Cheung, 2000; Ortiz et al., 2006).

Table 1
Biochemical composition of *Ulva lactuca*, as weight percentage of the dry weight in whole material. The sugars arabinose and fucose were not detected in any of the samples.

	Extractives			Neutral sugars					Uronic ac.	Protein	Ash
	EtOH	EtOH/toluene	Water	Rha	Gal	Glu	Xyl	Man			
Ulva[1]	–	–	–	7.0	1.0	8.2	4.5	0	3.2	24.6	15.9
Ulva[2]	2.9	0.5	37	3.3	0.3	6.4	3	0	1.4	–	–

[1] Sugar composition of whole *Ulva lactuca* biomass.
[2] Sugar composition of residue after determination of extractives.

3.2. Pre-treatment and enzymatic hydrolysis of U. lactuca

Preliminary tests carried out by re-suspending *U. lactuca* in water or alkali at 85 °C resulted in very viscous suspensions, which were difficult to mix to ensure efficient heat transfer throughout the sample. The viscosity increase observed can be attributed to the solubilization of water-extractable polysaccharides (mainly ulvan) (Lahaye and Axelos, 1993). At 150 °C, there was no significant increase in the viscosity of the suspension, indicating that the water extractable polysaccharides were degraded.

Fig. 1 shows the percentage of sugars in *U. lactuca* that were solubilized by the different pre-treatments. During pre-treatment at 150 °C, approx., 20% more sugars were solubilized compared to at 85 °C. At both temperatures, the addition of sodium hydroxide or sulfuric acid did not result in additional solubilization of total sugars from the material. The addition of sulfuric acid resulted in an increase in the amount of monomeric sugars in the hydrolysate, as also observed by Potts et al. (2012) for increasing concentrations of sulfuric acid, due to hydrolysis of soluble sugar oligomers and to the acid-catalyzed removal of sulfate groups from rhamnose (Lahaye and Axelos, 1993) (Table 3). It is not expected that the relatively high sulfuric acid concentration (7.5% w/w) will lead to significant degradation of sugars because of the mild temperature and pH (2.0) and short time for an acid pretreatment.

The enzymatic digestibility of the pellets remaining after the pre-treatment was high for all pre-treatment conditions, resulting in increased solubilization of sugars after enzymatic hydrolysis to almost 100% (w/w) of the total sugars (Fig. 1). After pre-treatment at 150 °C, almost all rhamnose was solubilized, with only glucose and xylose remaining as polysaccharides in the insoluble fraction. These insoluble sugar polymers were readily hydrolyzed by the commercial cellulase GC220. The enzymatic degradability of the pellets resulting from pre-treatments at 85 °C was somewhat lower (85% of total neutral sugars). This difference may be due to a more efficient removal of non-cellulosic sugars from the biomass at 150 °C compared to that at 85 °C, leaving the remaining cellulose more accessible to hydrolysis by the cellulases.

The enzymatic hydrolysis of the sugar polymers in the pre-treated material was not optimized in the current study. A commercial cellulase preparation generally applied in the hydrolysis of lignocellulosic feedstocks (GC220) was used at a relatively high load to ensure polysaccharide degradation. Enzyme loadings can be reduced when a more specific enzyme mixture is used for *U. lactuca*, however, such a specific enzyme mixture is not commercially available. The solid residue remaining after the enzymatic hydrolysis step is expected to be enriched in proteins, since the pre-treatment and hydrolysis conditions were milder than those used for protein extraction (Fleurence et al., 1995). This residue could be used for other applications (food, feed, for example) however protein content in this residue was not determined and will be subject of other studies.

Although the sugar content in this seaweed is relatively low when compared to lignocellulosic feedstocks, the solubilization of the monomeric sugars in the material does not require stringent pre-treatment conditions (high temperatures, strong acidic or alkaline conditions, etc.) as those needed for lignocelluloses. A

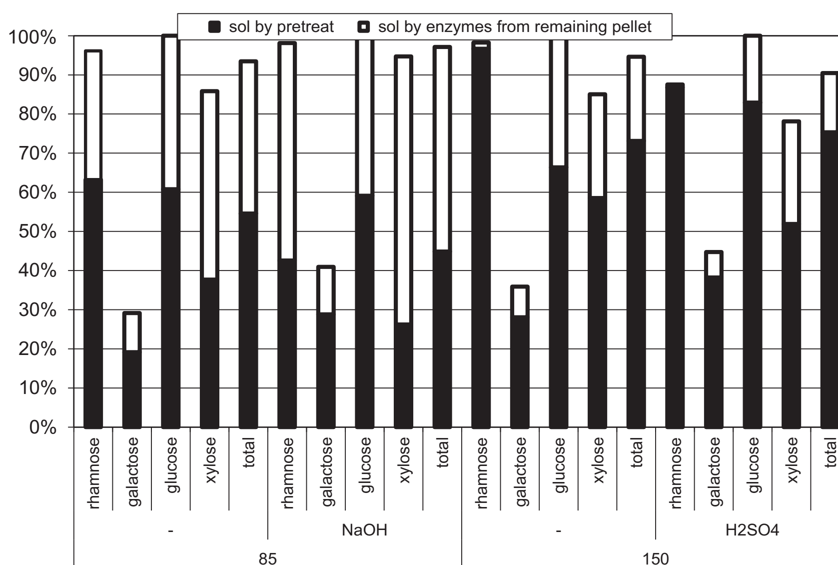

Fig. 1. Percentage of total sugars present in *Ulva lactuca* solubilized during the different protocols followed during this study. The percentage of the total sugars solubilized by the different pre-treatments, indicated in the *X* axis (the temperature was 85 or 100 °C, with or without addition of NaOH or H_2SO_4) is shown as black bars. The percentage of total sugars (remaining in the insoluble fraction after the pre-treatment) solubilized by enzymatic hydrolysis by cellulase GC220 is shown as white bars.

Table 3
Total sugar content (in g/L) in *Ulva lactuca* hydrolysates prepared by different pre-treatments (see Section 2.3.1), determined after hydrolysis with 1 M H_2SO_4. In brackets, the concentration in g/L of the corresponding monomeric sugar before acid hydrolysis is indicated. The hydrolysate for fermentation was prepared by pre-treatment in water at 150 °C followed by enzymatic hydrolysis by GC220 cellulase, as described in Section 2.3.2.

T (°C)	Chemical	Fraction	Rha	Gal	Glu	Xyl
85	–	Supernatant	4.1 (0)	0.4 (0)	6.6 (0)	1.6 (0)
	NaOH	Supernatant	6.2 (0)	0.3 (0.03)	6.9 (1)	2.4 (0)
150	–	Supernatant	8.4 (0)	0.3 (0)	6.7 (3.1)	3.3 (0)
	H_2SO_4	Supernatant	7.5 (2.4)	0.5 (0.3)	8.4 (4)	2.9 (1.8)
150	–	Supernatant for fermentation	8.1 (0.8)	0.7 (0.1)	8.4 (8.4)	5.2 (1.2)

hot-water pre-treatment followed by enzymatic hydrolysis resulted in the solubilization of more than 90% (w/w) of the sugars present in the biomass (Fig. 1). Similar conditions were found to be suitable for the solubilization of sugars in the brown seaweed *Saccharina latissima* (Adams et al., 2009).

3.3. Preparation of hydrolysate of U. lactuca for fermentation

As shown in Fig. 1, the enzymatic degradability of the sugar polymers in the pellet of the small-scale tests at 150 °C, both with and without added sulfuric acid, was very efficient, resulting in the solubilization of approx. 90% of the total sugars. Since the addition of sulfuric acid during pre-treatment of cellulosic materials may lead to the production of fermentation inhibitors like 5-hydroxymethyl furfural and furfural (Mussatto and Roberto, 2004), pre-treatment at 150 °C without the addition of sulfuric acid followed by enzymatic hydrolysis was chosen as the methodology for the preparation of a hydrolysate for fermentation at a larger scale. The sugar content in the hydrolysate is shown in Table 3. Glucose, rhamnose and xylose were present at similar concentrations. During preparation of the hydrolysate for fermentation, the yield of solubilized sugars was 75% (w/w) of the sugars in the feedstock, compared to 93% (w/w) solubilized in the tests at small scale. This lower yield may be due to the shorter incubation time for enzymatic hydrolysis compared to that for the small-scale tests, and to different heat and mass transfer characteristics of the larger pretreatment reactor used and/or to the scale-up conditions.

3.4. Fermentation of U. lactuca hydrolysate by C. acetobutylicum and C. beijerinckii

The *U. lactuca* hydrolysate was used as substrate for fermentation by *C. acetobutylicum* ATCC824 and *C. beijerinckii* NCIMB 8052, two of the best characterized ABE-producing strains. The pH of the hydrolysate was 5.8, which is close to the one of the control cultures (pH 6.1–6.2). Hydrolysate-based media were fermentable by *C. acetobutylicum* without pH adjustment, while media for *C. beijerinckii* cultures required adjustment to a pH above 6 (6.0–6.4) in order to obtain solventogenic fermentations. This difference in pH conditions requirements between strains has been observed before (Shaheen et al., 2000).

In order to determine nutrient requirements and to detect possible toxicity effects during fermentation, the hydrolysate cultures were supplemented with glucose and/or nutrients, as indicated in the legend of Table 4. Two different control media were used, one with glucose and xylose as carbon sources, and one in which rhamnose was added, to study sugar utilization by the strains. Xylose was added at a low concentration to the control cultures in order to mimic the concentration of this sugar in the hydrolysate. Both strains produced ABE in the control media, however, *C. acetobutylicum* did not utilize rhamnose, resulting in lower product levels on the mixed sugar (glucose, xylose and rhamose) control cultures (Table 4).

On hydrolysate without any supplementation (culture H, Table 4), *C. beijerinckii* utilized the majority of the sugars, leaving approx 50% of the rhamnose unused, and ABE were produced. In cultures of *C. acetobutylicum*, the fermentation of hydrolysate resulted in acidogenic fermentation, and low levels of ABE were produced. Fig. 2 shows the levels of sugars and products during the fermentation on hydrolysate for both strains. *C. beijerinckii* started consuming rhamnose only after the other sugars were almost depleted (Fig. 2C).

When the hydrolysate was supplemented with glucose (cultures encoded HS in Table 4), the production of ABE was higher for both strains compared to that in cultures on hydrolysate (H cultures, Table 4). This indicates that the *U. lactuca* hydrolysate contained enough nutrients (nitrogen sources, minerals, etc.) to support utilization of a high concentration of sugars and production of ABE by both strains, making the use of external nutrient supplements (such as yeast extract or corn steep liquor) unnecessary. *C acetobutylicum* produced ABE up to a total concentration of approximately 18 g/L in HS cultures, mostly due to glucose fermentation, since xylose was only partially utilized.

Table 4
Fermentation of *Ulva lactuca* hydrolysates by ABE-producing strains. Culture codes for media: control, CM2 medium with glucose as major carbon source; control G/R, CM2 medium with glucose and rhamnose as major carbon sources; H, *Ulva lactuca* hydrolysate; HS, H supplemented with glucose and xylose; HN, H supplemented with nutrients as in CM2 medium; HSN, H supplemented with glucose, xylose and nutrients as in CM2 medium.

	C. beijerinckii cultures						*C. acetobutylicum* cultures					
	Control	Control G/R	H	HS	HN	HSN	Control	Control G/R	H	HS	HN	HSN
Sugars at t = 0 h (g/L)												
Glucose	54.8	19.2	7.8	52.0	7.9	52.2	55.6	19.2	8.1	54.3	7.9	51.9
Xylose	1.9	0.8	2.4	4.4	2.4	3.9	2.0	0.8	2.5	3.9	2.4	3.9
Rhamnose		36.7	5.5	5.6	5.5	5.6		35.9	5.6	5.5	5.4	5.4
Total	56.6	56.6	15.7	62.0	15.8	61.7	57.6	55.8	16.2	63.6	15.6	61.1
Sugars at t = 140 h (g/L)												
Glucose	17.1	0.0	0.0	31.4	0.0	22.4	3.1	0.0	0.5	0.7	0.1	24.6
Xylose	0.0	0.0	0.0	1.3	0.0	1.3	0.1	0.0	1.3	1.8	0.8	2.5
Rhamnose		10.2	2.8	5.7	0.0	5.4		34.0	5.2	4.7	5.1	5.0
Total	17.1	10.2	2.8	38.4	0.0	29.1	3.1	34.0	7.0	7.2	5.9	32.1
Products at t = 140 h (g/L)												
Acetic acid	0.4	0.4	0.3	0.2	0.6	0.4	1.3	2.4	1.7	0.7	3.7	4.1
Butyric acid	0.0	0.4	1.3	0.0	2.5	0.0	0.7	2.2	2.4	0.0	3.5	1.3
1,2-Propanediol	nd	9.7	0.2	nd	0.2	0.3	nd	nd	nd	nd	nd	nd
Acetone	3.7	4.1	1.3	2.3	1.9	4.1	5.1	1.6	0.0	4.9	0.0	2.4
Ethanol	0.5	0.3	0.2	0.1	0.3	0.5	1.3	0.5	0.0	1.9	0.0	0.6
Butanol	10.7	6.9	3.0	6.8	3.3	8.4	10.9	2.9	0.8	11.4	0.3	4.8
Total ABE	14.8	11.3	4.5	9.2	5.5	13.0	17.3	4.9	0.8	18.1	0.3	7.8
Yield (g ABE/g sugar)	0.37	0.24	0.35	0.39	0.35	0.40	0.32	0.22	0.08	0.32	0.03	0.27

nd, not detected.

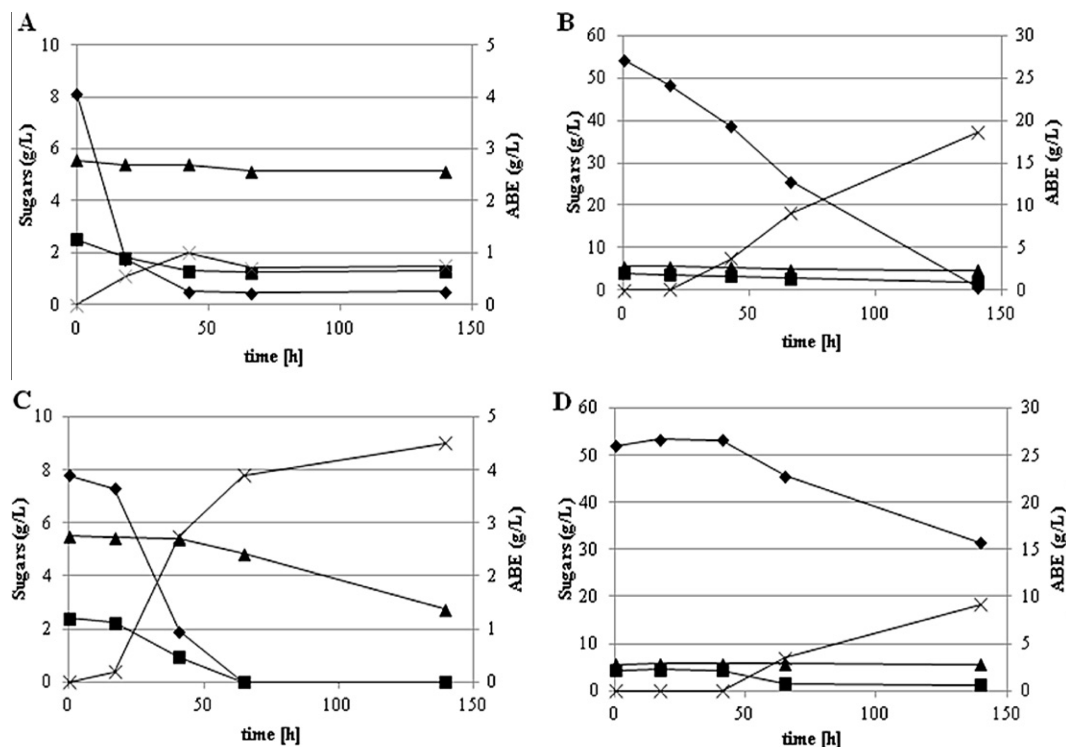

Fig. 2. Concentration profiles of sugars and products (total ABE) in cultures of *C. acetobutylicum* (A, B) and *C. beijerinckii* (C, D) during growth on *Ulva lactuca* hydrolysate-based media. (A) *C. acetobutylicum* cultures on hydrolysate (H); (B) *C. acetobutylicum* cultures on hydrolysate supplemented with glucose (HS); (C) *C. beijerinckii* cultures on hydrolysate (H) and (D) *C. beijerinckii* cultures grown on hydrolysate supplemented with glucose (HS). Symbols: ♦, glucose; ■, xylose; ▲, rhamnose; ×, total ABE.

In general, *C. acetobutylicum* ATCC824 produced mainly acids on the cultivation media with lower total sugar concentrations (Table 4, cultures H and HN), leaving a part of the sugars unused. When grown on media with high sugars concentration, this strain produced ABE as major end-products, with yields of approximately 0.3 g ABE/g sugar consumed. As already observed in other studies, *C. acetobutylicum* showed a strong preference for glucose as carbon source. It utilized xylose poorly and left rhamnose unused.

C. beijerinckii produced ABE as major end-products on all media, including those with low sugar contents (H and HN). The yields of solvents per gram of sugar consumed were generally higher than those found in cultures with the *C. acetobutylicum* strain, indicating a more efficient sugar use (Table 4). In addition, *C. beijerinckii* utilized rhamnose, albeit partially, while the *C. acetobutylicum* strain did not. Although *C. beijerinckii* showed higher yields of solvents per sugar consumed, the total end concentrations of ABE in solventogenic cultures of *C. acetobutylicum* were generally higher (Table 4, Fig 2). Important differences in fermentation performances between different *Clostridium* species have been reported and are considered the result of the wide genetic diversity between the species (Shaheen et al., 2000).

In cultures of *C. beijerinckii* on hydrolysate were rhamnose was utilized, an extra peak was observed in the RI-chromatogram obtained after analyzing culture samples by HPLC. This peak was identified as 1,2-propanediol, and the concentrations in the cultures were low (<1 g/L, Table 4). 1,2-propanediol is a C3-alcohol that has been reported to be an end-product from anaerobic conversions of rhamnose by several microorganisms, including clostridial species (Saxena et al., 2010). Therefore, control cultures were prepared in which, in addition to glucose, rhamnose was present in high concentrations (approx., 40 g/L, culture named Control G/R, Table 4). In cultures of *C. beijerinckii* on this control medium, approx., 75% of the rhamnose was utilized, and, in addition to ABE, 1,2-propanediol was detected at a concentration of 9.7 g/L, which is one of the highest levels reported so far. This production of 1,2-propanediol corresponded to approximately 0.75 mol of 1,2-propanediol per mol of rhamnose consumed, close to the reported maximum of 1 mol 1,2-propanediol per mol of rhamnose (Saxena et al., 2010). The fermentation of the 6-deoxy-sugar rhamnose to 1,2 propanediol by several solventogenic clostridial strains (including some *C. beijerinckii* and *C. acetobutylicum* strains, although not the ones used in this study) has been described earlier by Forsberg et al. (1987). The metabolic routes from rhamnose to 1,2 propanediol in solventogenic clostridia are considered to be analogous to those described from deoxysugars (L-rhamnose and L-fucose) in strains of *Escherichia coli* and *Salmonella typhimuriumi* (Forsberg et al., 1987; Bennett and San, 2001), where these sugars are used as carbon source. In this route, rhamnose is phosphorylated to rhamnulose-1-phosphate, which is cleaved into L-lactaldehyde and dihydroxyacetonephosphate (DHAP). The L-lactaldehyde is then reduced to L-1,2-propanediol. Another metabolic route from sugars to 1,2 propanediol is that with glycolytic intermediate DHAP as precursor. DHAP is converted into methylglyoxal (MG) by the enzyme methylglyoxal synthase, and subsequently MG can be reduced to 1,2 propanediol via acetol (hydroxyacetone) or lactaldehyde (Bennett and San, 2001), Although enzymes related to the MG metabolic route have been identified in some clostridial species (Bennett and San, 2001), there is no evidence yet of a complete route from DHAP to 1,2 propanediol in *C. acetobutylicum* or *C. beijerinckii*.

A metabolic route for the utilization of rhamnose as carbon source to produce 1,2-propanediol has been proposed for the anaerobic thermophilic bacterium *Caldicellulosiruptor saccharolyticus* (van de Werken et al., 2008). A search for genes homologous to those of *C. saccharolyticus* encoding for enzymes involved in this metabolic route in the published genomes of *C. beijerinckii* NCIMB 8052 or *C. acetobutylicum* 824 (the strains used in this study) in the NCBI database (http://www.ncbi.nlm.nih.gov/) revealed the presence of such genes in the genome of the *C. beijerinckii* strain. In the genome of *C. acetobutylicum*, no clear homologous genes

were found encoding enzymes with rhamnose isomerase or with rhamnulokinase activity, the first two steps in the rhamnose utilization pathway (from rhamnose to rhamnulose-1-P). Lack of these enzymatic activities could explain the inability of this strain to utilize rhamnose,

1,2 propanediol (also called propylene glycol), a commodity chemical with an expanding market, is currently produced from fossil oil (Saxena et al., 2010). The development of a process for the biological production of 1,2-propanediol from deoxysugars relies on the availability of inexpensive feedstocks rich in these sugars. Green seaweeds, including *U. lactuca* sp. have the potential to be such a feedstock given their high content in rhamnose, provided that they could be cultivated at the necessary scale. Many efforts are currently being made in The Netherlands and other countries toward mass scale cultivation of seaweeds, including green seaweeds, both near- and offshore (http://www.noordzeeboerderij.nl (in Dutch) accessed 8th April 2012)(Kraan, 2011) with the objective of using them as source of food, fuels or chemicals in future biorefineries.

4. Conclusions

This study describes the first step toward a biorefinery based on the seaweed *U. lactuca*. Hydrolysates containing 75–93% of the sugars in *U. lactuca* were prepared using pre-treatments that did not rely on chemical catalysts and used as fermentation media for acetone, butanol and ethanol production by clostridial strains. This hydrolysate did not require supplementation with nutrients to support growth and ABE production at high sugar concentrations, which is an economical advance over other substrates. Interestingly, *C. beijerinckii* produced 1,2-propanediol from rhamnose, the second major sugar in *U. lactuca*, opening the possibility of using rhamnose-rich seaweeds as feedstock for 1,2-propanediol production.

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