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Biorefinery of the brown seaweed *Saccharina latissima* for fuels and chemicals

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Seaweeds (also called macroalgae) are considered a potential biomass feedstock for biorefineries for production of energy and chemicals. In this study, a biorefinery strategy for the brown seaweed *Saccharina latissima* is described. Fresh *S. latissima* harvested at the Irish coast contained glucose and mannitol as most abundant fermentable sugars. The fresh biomass was chopped and pressed in order to obtain a liquid fraction (press juice), which contained 16 g/L of mannitol as main sugar component, and an insoluble fraction referred to a "press cake". The mannitol in the press liquid has been extracted and purified to serve as a substrate for chemical conversions. The use of the press juice and hydrolysed press cake as substrates for production of acetone, butanol and ethanol by anaerobic fermentation has been evaluated. While the press juice was easily fermentable after addition of nutrients, the press cake was toxic for the microorganisms. When the press cake hydrolysate was diluted, fermentation was possible. The toxicity of the hydrolysate might be associated to the high salt concentrations determined in it. The use of the residue after enzymatic hydrolysis of the press cake as fertilizer has been evaluated.

INTRODUCTION

Seaweeds have been used in East Asia since ancient times as vegetables. Currently, seaweeds are worldwide used as food and as source of chemicals (i.e. thickening agents, gelling agents and phycolloids). Annually, 7-8 million tonnes seaweeds are harvested, with an estimated total value of the products of US$ 5-6 billion [1], with an increasing market and production

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capacity in the lasts years[2]. Because of the special chemical composition (wide range in sugars, polymers, etc) of seaweeds and the possibility of cultivating them at large scale in the ocean with high yields, they are potential feedstocks for production of renewable chemicals and fuels [3].

Brown seaweeds, in particular Kelps, are already used as source of alginates and are rich in fermentable sugars, mainly mannitol and glucose. Several brown species have been studied already as feedstock for production of ethanol and butanol [4]. Some species of *Clostridium* produce acetone, butanol and ethanol (ABE) by anaerobic fermentation of sugars, a process known as the ABE fermentation. These species utilize sugars (both C5 and C6) in a variety of substrates, including (lignocellulosic) hydrolysates derived from plant biomass. Since seaweeds are composed by of a mix of different sugars, these organisms are expected to efficiently convert most sugars into an ABE mixture. The ABE process is nowadays 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 [5].

Brown seaweeds are rich in mannitol, which is a sugar-alcohol with a variety of industrial applications, including medical uses and as sweetener in food products. In addition, mannitol can be a precursor of compounds with uses as polymer components, which have a high value.

In this study, a biorefinery route has been assessed for the brown seaweed *Saccharina latissima*, a native specie of the North Sea. The major products envisaged in this biorefinery are acetone, butanol and ethanol formed by fermentation of the sugars in the seaweed fraction, pure mannitol to be used as a precursor of products with a higher market value, and fertilizer from the side stream after solubilisation of sugars for fermentation.

MATERIALS AND METHODS

Source of seaweed sample. *S. latissima* was harvested at the Irish coast at the Sppidal coast in June and transported directly to Wageningen under cooled transport. The biomass was processed immediately upon arrival.

Size reduction and pressing. Size reduction was performed with a Pierret guillotine chopper. Seaweed was fed lengthwise, and the length of the 1 cm-wide pieces was

determined by the width of the blade. Chopped seaweed was pressed in an expeller (oil press). The expeller configuration consisted of transport elements and an obstruction element (hump) in order to increase the resistance and pressing force. Before the hump, filter elements were placed. Screw speed was 20 rpm.

Preparation of hydrolysate from press cake of *S. latissima*. The press cake (PC) was defrosted and milled under liquid N_2 to obtain small pieces. For the solubilisation of sugar polymers in the milled PC, a hydrolysis step was carried out by incubating the material with GC220 enzyme preparation (Genencor) at a concentration of 0.28 mL GC220 suspension per gram of dry matter of PC at 50 °C for 24 h. The soluble and insoluble fractions were separated by centrifugation at 15100 x g for 15 min at RT. The hydrolysate was stored at -20°C until further use. The pellet was freeze dried and stored at RT.

Analysis of biomass fractions: sugars, ash, elements, protein. Total sugars were determined in the different fractions after acid hydrolysis of the milled material by incubation with 72% w/w H_2SO_4 at 30ºC for 45 min, followed by dilution to a 1M H_2SO_4 concentration and hydrolysis for 1 h at 100ºC. The hydrolysate was neutralized with Na_2CO_3 and analyzed for neutral sugars on a HPAEC equipped with a CarboPac PA1 column with a CarboPac PA1 guard-column (Dionex), and pulsed amperometric detection.

The total ash content in the solid samples was determined by combustion at 550 °C. The N elemental composition was measured with an elemental analyzer (Carlo Erba Instruments FLASH EA 1112, Wigan, UK). The freely available ions available in the samples (chlorine, fluorine, bromine, iodine, sulfate, phosphate) were determined by water washing and subsequent analysis by ion chromatography. Protein content in the samples was estimated by multiplying the content in N by the factor 5.38, according to Lourenco et al [6].

The inorganic elemental composition was measured using inductively coupled plasma atomic emission spectroscopy (ICP-AES) (Thermo ICAP 6000). The freeze-dried solid samples were first finely milled using a tungsten–carbide vibratory ring pulveriser. The milled solid was digested using HNO_3/$HClO_4$/HF before ICP analysis. Sulfate, K^+ and Na^+ in the hydrolysate and in the fermentation medium were determined using element analysis by ICP-IAS technique.

Extraction of mannitol from *S. latissima* press juice. Isolation of mannitol was accomplished by a simplified version of the method reported by Porter [7]. *S. latissima* press

juice was centrifuged (4000 rpm, 10 min.) to remove insoluble impurities. The clear liquor was subsequently evaporated to dryness on a rotary evaporator. The solid residue was subjected to Soxhlet extraction with methanol for approximately 48 h. Upon standing at room temperature, white needles formed, which were isolated by filtration and drying. The mother liquor was concentrated *in vacuo* and left standing overnight, yielding a second fraction of white crystals. These were isolated. ^{1}H and ^{13}C NMR spectra in DMSO-d_6 showed the presence of mannitol as a pure product, i.e. no signals of other organic compounds were detected. The isolated amount of mannitol corresponds to 71% of the amount of mannitol present in the press juice.

Strains and growth conditions. *C. acetobutylicum* ATCC 824 and *C. beijerinckii* NCIMB 8052 are laboratory strains, and were stored as spore suspensions in glycerol at -20°C. For the preparation of pre-cultures, spores were heat-shocked and placed into CM2 medium, as described previously [8]. As carbon sources, stock solutions of glucose, mannitol or their mixes were prepared and sterilized separately and added to the medium at the indicated concentrations. The pH of the seaweed based-media varied between 5.3 and 5.8, and was adjusted to 6.0-6.4 with 1 M NaOH prior fermentation. The *S. latissima* fractions were tested for fermentation as such, or diluted, as indicated in the text. To reduce viscosity of the press liquid, GC220 was added at a concentration of 0.3 mL GC220/g dry matter at pH 6 and incubated during 17 h at 50°C.

Analysis of metabolites. 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) as described previously[8].

RESULTS

Biorefinery

Our vision for a biorefinery for *S.latissima* is presented in Figure 1. It is based on the use of fresh seaweed and the avoidance of energy intensive methods like drying, grinding and extensive washing as much as possible. The process starts with a chopping and pressing stage where the first fractionation takes place. By pressing, the mannitol can be separated from the remainder of the seaweed carbohydrates. The press cake can then be hydrolysed under mild acidic conditions to obtain fermentable sugars originating from laminarin and fucoidan. As

alginate is not hydrolysed under these conditions an alginate residue remains which could be used for extraction of sodium alginate.

The intermediate products mannitol, sugars and alginate can be converted by chemical or biochemical methods to chemical building blocks like isomannide and 2,5-FDCA (Furan Di-Carboxylic Acid) or acetone-butanol-ethanol (ABE). Residues from all processing steps could be collected for anaerobic digestion to biogas, or some may be used as fertilizer, since seaweeds are rich in minerals.

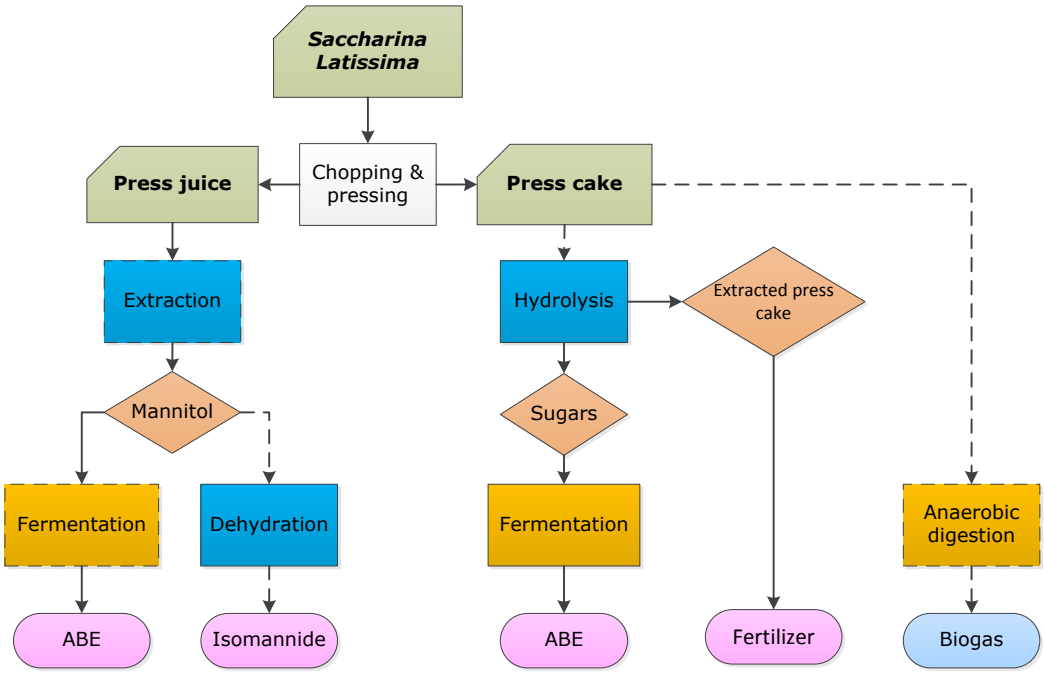

Figure 1: Biorefinery scheme of processing steps described in this study. Solid lines represent processes described in this study, while the dashed lines represent possible processes.

Pressing of fresh *S. latissima*

Properties of press cake

The amount of press cake corresponded to 72 wt % of the total weight (in dry matter) of the fresh seaweed. Dry matter content of the seaweed as received was 15.5 ± 0.2 wt% and after chopping and pressing the dry matter content was slightly increased to 16.7 ± 0.1 wt%. The total sugar content in the untreated seaweed was 25.7 wt% of the dry matter, while in the press cake the amount of sugars determined was of 28.9 wt% of the dry matter (Table 1). The

weight reduction of 20% obtained after pressing was mainly caused by to the removal of mannitol and glucose (originating from laminarin). Sugars originating from fucoidan (galactose, xylose, mannose and fucose) were hardly found in both the seaweed as well as the press cake.

Properties of press juice

The amount of press juice was 28 wt% of the total fresh seaweed. Dry matter content of the press juice was 7.8 wt%, divided in dissolved material (7.2 wt%) and solids (0.6 wt%). The press juice was analysed for sugar content by an additional hydrolysis in order to hydrolyse oligomeric sugars to monomeric sugars. The most abundant sugar in the press juice was mannitol (16 g/L) followed by glucose (1 g/L) and fucose (1 g/L). Based on these data the total sugar content in the press juice was 18.5 g/L.

Hydrolysis of sugar polymers in the *S. latissima* press cake

The pressed cake was subjected to a short thermal treatment followed by enzymatic hydrolysis using commercial cellulases (GC220) at standard loading, since no specific enzymes could be found in the market for hydrolysis of brown seaweed biomass. The enzyme loading was not optimised.

The hydrolysate obtained after the enzymatic hydrolysis contained 17.9 g/L of glucose and 42.8 g/L of mannitol. This corresponded to a solubilisation yield of 89% of the total sugars present in the press cake. The major glucose polysaccharide in *S. latissima*, laminarin, is easily degraded by cellulases, and mannitol is free in the biomass, which explains the high yield obtained of hydrolysis of the press cake. Of the total proteins in the pressed cake, 23% were solubilised and 77% remained in the insoluble fraction.

Table 1. Composition of *S. latissima* and its fractions, in % of dry matter.

	Sugars				
Sample	Glucose	Mannitol	Fucose	Protein	Ash
S. latissima (fresh)	9.1*	15.6*	1*	8.6	31
Press cake	11.6	16.4	0.9	8.6	25
Extracted Press cake	4.8	6.1	0.8	16.1	22

* Estimated values from the composition of the press juice and the press cake

Isolation of mannitol from the press liquid

The press liquid resulting after the chopping and pressing of the biomass contained 16 g/L of mannitol. Practically pure mannitol was isolated from the press liquid by evaporating the liquid to dryness and subsequently performing a Soxhlet extraction with methanol on the residue. More than 70% of the mannitol present in the press juice could be isolated this way as white crystals.

Fermentation of *S. latissima* fractions to acetone, butanol and ethanol

In this study, two ABE-producing strains were selected, *C. acetobutylicum* and *C. beijerinckii*, since they are both well studied and show different fermentation and sugar use profile. The utilisation of mannitol/glucose mixtures by these strains has not been characterized extensively, therefore tests on control medium containing glucose and mannitol were carried out. Both strains fermented glucose, mannitol and mixes of glucose and mannitol to ABE. In Figure 1, the profile of consumption of glucose and mannitol in a medium containing a mix of both sugars by both strains is shown. It is observed that, although both sugars a consumed, both strains show a preference for glucose, being mannitol consumed only after glucose is depleted in the medium. On the glucose/mannitol mixture, *C. acetobutylicum* produced 1.1 g/L of ABE and 3.2 g/L of butyric acid as major products, while on cultures on 20 g/L of glucose the ABE and butyric acid levels were 3.4 g/L and 2.5 g/L, respectively. These data are in agreement with our experience in the cultivation of this strain on the requirement of high concentrations of sugars in the medium for an efficient solventogenic fermentation. In the case of the cultures of *C. beijerinckii* on the glucose/mannitol mixtures, the ABE

production after 163 h of cultivation was 6.6 g/L and the butyric acid level was 1 g/L. On media with glucose at 20 g/L as carbon source this strain produced 7.8 g/L of ABE and 0.2 g/L of butyric acid, indicating efficient solventogenic fermentations at low sugar concentrations. These results illustrate the big difference in fermentation performance between solventogenic strains, and the importance of selecting the most suitable strain according to the properties of the feedstock.

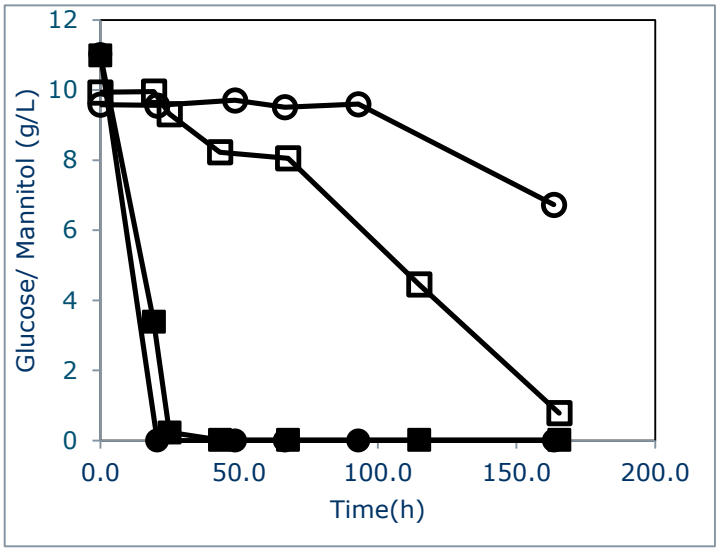

Figure 2. Sugar consumption by *C. beijerinckii* (Cb) and *C. acetobutylicum* (Ca) in cultures with a mix of glucose and mannitol as carbon source. Legend: ■= glucose Cb ; □= mannitol Cb ; ●= glucose Ca ; o= mannitol Ca.

The press juice obtained after pressing the fresh *S. latissima* and the hydrolysate from the press cake were used as substrate for the ABE fermentation. In both cases, the major fermentable sugar present in the fractions was mannitol.

The press liquid contained 18.5 g/L of total sugars and presented a high viscosity. Therefore it was incubated with the cellulase GC220 before being used as fermentation feedstock. This treated press liquid was not fermentable as such, but after addition of nutrients to reach the same levels as in the control medium, sugars were consumed and products, mostly butyric acid at a concentration of approx. 4g/L, were formed by *C. acetobutylicum*, indicating that the press liquid is too poor in nutrients to support growth. In all fermentation experiments, the utilization of fucose was not studied, since little is known about the fermentation of this sugar by the strains used.

The hydrolysate from the press cake had a high sugar concentration, 60 g/L of total sugars. However it was not fermentable by *C. beijerinckii*. In cultures where the hydrolysate was diluted 2-fold, the bacteria grew and produced solvents to the same extent as in control medium with the similar amount of sugars, 3.8 g /L of total ABE and 1.9 g/L of butyric acid. This fermentation was not optimal, and not all sugars were consumed, indicating possible inhibitory effects of medium components on the culture.

The toxicity of the hydrolysate may be caused by salts present in it that originate from the biomass, since seaweeds are known by their high content salts and inorganic material taking part in ash. In the *S. latissima* press cake the amount of ash was 25% (Table 1) of the dry matter content. Therefore the content in salts in the hydrolysate was determined (Table 2). The concentration of sulphate was 3.3 g/L, which is 10-fold higher than that in control medium. High concentrations of sulphate are known to be inhibitory of bacterial growth [9], and the concentration in this hydrolysate is above the toxicity limits. The levels of K^+ and Na^+ were also high, but their effect on microorganisms has not been well characterized yet.

Table 2. Content in K^+, Na^+ and sulphate ions in the hydrolysate of *S. latissima* compared to that of control medium used for fermentation.

	Component (g/L)		
Medium	K^+	Na^+	SO_4^{2-}
S. latissima hydrolysate	14.5	7.3	3.3
Control medium	0.4	nd	0.3

Evaluation of the use of the extracted press cake as fertilizer

The pellet obtained after the enzymatic hydrolysis of the press cake, referred to as "extracted press cake" was chemically characterized (Table 1). The ash content in this fraction was 22% of the dry matter. The extracted press cake showed a relatively high content in the primary inorganic fertilizer components, K and P. Secondary fertilizer components (Mn and Ca and S) are also potentially useful, as are the trace elements Cu, Fe, Mn, Mo, Zn and Ni.

Potentially environmentally harmful elements, which are maximised by law in the Netherlands are listed in the table below. Most of these elements, with the exception of As, are well below the limit set in the Netherlands for compost, the most stringent rule. The high content in As (>3 times the max. allowed for compost) represent an issue for the use of this stream as fertilizer in The Netherlands, and other uses should be evaluated as well. It should be noted that the mineral composition of seaweed is strongly location-dependent, and therefore samples from *S. latissima* or other brown seaweed harvested at other geographical locations may contain lower values in As.

Table 3. Content in potentially harmful elements in the extracted press cake as compared to the max. level allowed for compost in The Netherlands.

Element	Maximum level allowed in The Netherlands [10] (mg/kg)	Amount in extracted press cake (mg/kg)
As	15	51.41
Cd	1	0.76
Cr	50	7.9
Cu	90	9.27
Ni	20	6.78
Pb	100	6.51
Zn	290	64.4

DISCUSSION

Fresh seaweeds have low dry matter content, and have the ability to retain water very well due to the presence of hydrocolloids in their cell structure. Some brown seaweed species, including *S. latissima*, contain the sulphated polysaccharide fucoidan which protects the seaplant from dehydration. During this study, it was seen that increasing the dry matter content in *S. latissima* was very difficult, however, by pressing the biomass a viscous press juice with a high concentration in mannitol was obtained. The mannitol in the press juice was purified to a high degree of purity, allowing its use as a feedstock for chemical conversions.

The fractions made from the seaweed were fermentable by ABE-producing organisms, however, under suboptimal conditions. The press liquid required the addition of nutrients to be fermentable. The hydrolysate from the press cake had a high sugar content, however, it was toxic to the bacteria. The salt content in the hydrolysate was very high, as a result of the high content of salts in the seaweed. This issue could be addressed in future work by removing the salts in the biomass (by washing or by other technique) before the pre-treatment, in this way part of the salts can be removed and be used for other applications.

The residue resulting after the solubilisation of sugars in the press cake has been analysed and its use as fertilizer has been evaluated. This residue had a content in arsenic that is more than three times the current maximum level allowed for compost in The Netherlands, making the use of this fraction as fertilizer not possible. Because the mineral content in seaweeds is highly dependent on the harvesting location and cultivation conditions, this does not mean that all brown seaweeds have a high arsenic content, and the data shown apply to the specific sample used in our work.

This study represents one of the first biorefinery studies for brown seaweeds in which multi-products have been obtained from fresh biomass using a fractionation strategy. Valorisation of all components in the biomass will contribute to the development of sustainable processes for seaweed uses as feedstock for fuels and chemicals.

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