ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/324843245

Acid preservation of Saccharina latissima for application as a carbon source for fermentation to biofuels and chemicals

Article *in* Journal of Applied Phycology · December 2018

DOI: 10.1007/s10811-018-1489-z

CITATIONS
15

READS
133

4 authors, including:

Ingrid Schafroth Sandbakken
Norwegian University of Science and Technology
4 PUBLICATIONS **87** CITATIONS
SEE PROFILE

Funderud Jon
5 PUBLICATIONS **167** CITATIONS
SEE PROFILE

Inga Marie Aasen
SINTEF
46 PUBLICATIONS **2,009** CITATIONS
SEE PROFILE

Some of the authors of this publication are also working on these related projects:

Foods of Norway - A Centre for Research-based Innovation, where we develop novel feed resources from renewable natural resources such as trees and seaweed with new technology. View project

All content following this page was uploaded by Ingrid Schafroth Sandbakken on 14 September 2018.

The user has requested enhancement of the downloaded file.

Journal of Applied Phycology
https://doi.org/10.1007/s10811-018-1489-z

Acid preservation of *Saccharina latissima* for application as a carbon source for fermentation to biofuels and chemicals

Ingrid S. Sandbakken[1] · Maren Sæther[2] · Jon Funderud[2] · Inga Marie Aasen[1]

Received: 3 March 2018 / Revised and accepted: 18 April 2018
© Springer Science+Business Media B.V., part of Springer Nature 2018

Abstract

Cultivated kelps (brown algae) are a sustainable biomass resource and a potential feedstock for conversion to biofuels and chemicals. Due to seasonal variations, and a short period with optimal biomass composition, preservation and storage of the biomass is required for a year-round operation of a seaweed processing plant. For use of the biomass as a carbon source for fermentation to biofuels, only low-cost preservation methods are feasible. Preservation of *Saccharina latissima* (sugar kelp) by sulphuric and formic acid has been evaluated as a method to maintain the fermentable carbohydrates laminaran and mannitol. In milled biomass, stored anaerobically for up to 6.5 months at different pH values, laminaran and mannitol were efficiently preserved in samples stored at pH below 3.7, obtained by addition of sulphuric acid. When a combination of sulphuric and formic acid was used, no sugar loss could be detected up to pH 4. The content of free glucose increased during the storage period in the well-preserved samples without loss of sugars. The free glucose levels were highest at the highest storage pH, providing strong evidence for the presence of endogenous β-glucanases that hydrolyse laminaran to glucose. Our work was primarily aimed at preservation of the biomass for application as a carbon source for fermentation. However, the method will be equally suited for other applications of the biomass, such as extraction of valuable compounds for use in functional food, feed or other areas.

Keywords Macroalgae · Seaweed · Preservation · pH · Carbohydrates · Fermentation

Introduction

Cultivated macroalgae, or seaweed, are a sustainable biomass resource with potential application for conversion to biofuels (Marquez et al. 2015; Milledge and Harvey 2016b) and other bulk chemicals. Cultivation is performed at sea and does not require cultivable land, freshwater or fertilisers. Brown algae in the order Laminariales (kelp species) are among the fastest growing plants in the world. They prefer temperate waters and in Europe they can be found from Portugal to northern Norway (Kraan 2013). Large-scale cultivation of seaweed in Europe has therefore targeted the kelp species, particularly *Saccharina latissima* (Stévant et al. 2017b).

S. latissima accumulates the storage carbohydrates laminaran (1–15% of dry weight (dw)) and mannitol (10–25% of dw) during the spring and summer season (Schiener et al. 2015). These carbohydrates are consumed as an energy source during late autumn and winter. For application as a carbon source for fermentation, the glucose polymer laminaran and the sugar alcohol mannitol are most easily solubilised and fermented (Horn et al. 2000), but with proper pretreatment and appropriate microbial strains, also the structural polysaccharides cellulose and alginate may be utilised, resulting in a total carbohydrate content of 65–70% of dry weight. The biomass should be harvested when the dry weight and the content of the storage carbohydrates are at the highest. This requires efficient preservation and storage of the biomass to allow whole-year processing for a sustainable seaweed industry. Commonly used preservation methods like drying or freezing are energy demanding and expensive, and profitable only for high-value products. Sun drying is applied in Asia and South America, but is not feasible in Northern Europe due to the climatic conditions. Short-term storage (24 h) of whole

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10811-018-1489-z) contains supplementary material, which is available to authorized users.

✉ Inga Marie Aasen
inga.m.aasen@sintef.no

[1] Department of Biotechnology and Nanomedicine, SINTEF Industry, 7465 Trondheim, Norway

[2] Seaweed Energy Solutions AS, Bynesveien 48, 7018 Trondheim, Norway

Published online: 30 April 2018

kelp in seawater maintains most of the components (Stévant et al. 2017a), but the biomass will decay quickly unless the seawater is exchanged often; thus, it may not be used for long-term storage. The cheapest and most feasible method for preserving large volumes of seaweed biomass is acid preservation, either by adding acid, or by microbial acid production by lactic acid bacteria (ensiling). In order to maintain the macroalgae carbohydrates, acid addition should be used, since microbial fermentation will consume the sugars. Only a very limited number of reports on acid preservation, or ensiling, of seaweed exist. The first well-documented study describes ensiling of brown seaweeds for animal feed applications (Black 1955). More recently, ensiling has been applied for preservation of brown algae for anaerobic digestion to methane gas (Milledge and Harvey 2016a; Herrmann et al. 2015) and investigation of the energy loss during ensiling, determined as the 'higher heating value' (Redden et al. 2016). Methods for preservation of the carbohydrates as a pre-processing for liquid biofuel production have not been reported.

Since laminaran is a storage polysaccharide that is utilised as energy source during the wintertime, it is likely that the kelp species contain enzymes for hydrolysis of laminaran to glucose ('laminaranases'). Such activity was observed by Hou et al. (2015), where glucose was formed in control samples without enzyme addition, in studies of enzymatic laminaran hydrolysis. Depending on the level of activity and its pH dependency, laminaran hydrolysis during the storage period could reduce the need for hydrolytic processing steps prior to fermentation.

The aim of the present work was to develop a preservation method for macroalgae that maintained the fermentable sugars for further fermentation to fuels or chemicals. For this purpose, ensiling by use of lactic acid bacteria is not feasible, since the desired sugars would be consumed. The chosen strategy was therefore to use a cheap mineral acid to lower the pH. As part of the work, the presence of laminaranases and the formation of free glucose as a function of storage pH was investigated.

Materials and methods

Raw materials

The brown alga *S. latissima* was collected at the coast of Trøndelag, Norway (Table 1). Seawater was drained, and small stones and other impurities were removed manually. The biomass was stored at 4 °C in plastic bags until further processing, which was performed within 3 and 6 days after harvesting of the November and December batches, respectively. The biomass was milled using a laboratory scale hammer mill (Schutte Buffalo) (4500 rpm, 10 mm gaps). A part of the milled biomass was packed in plastic bags and frozen at − 20 °C.

Preservation experiments

Fresh, milled *S. latissima* was distributed in anaerobic culture bottles (100 or 300 mL, 80–90 g wet weight (ww) per bottle). Sulphuric acid was added to obtain desired pH (0–120 mM final concentration). When combinations of sulphuric and formic acid were used, formic acid (100 mM final concentration) was first added, and pH adjusted to the desired value with sulphuric acid. The approximate quantity of sulphuric acid needed had been pre-determined (Supplementary Material, Fig. S1). In one experiment, when fresh *S. latissima* was not available, frozen biomass harvested in December was used. Initial experiments with frozen biomass showed that freezing reduced the microbial activity. Therefore, fresh, milled, decaying seaweed was used as an 'inoculum' of a viable microbial population. One gram was added per bottle. The added liquid volume was adjusted with water to obtain the same liquid addition for all pH values and unpreserved controls (0.17 mL g^{-1} ww fresh November and December biomass, and 0.18 mL g^{-1} ww frozen December biomass). All the bottles were flushed with N_2 gas to ensure anaerobic conditions, before incubation at 13 °C for up to 6.5 months.

The pH was measured every fourth day until stable. Sampling was performed regularly by collecting a representative fraction of liquid and solids (~ 10 g) from the bottles. The samples were freeze-dried before analyses of laminaran and mannitol. Liquid phase (0.1 mL) was collected for analysis of organic acids and other fermentation products. After each sampling, the bottles were flushed with N_2 gas and shaken to ensure proper mixing.

Three independent storage experiments with different seaweed batches and two replicates were conducted. Data from all experiments and replicates have been considered together and are plotted in the graphs to illustrate the robustness of the results. Standard deviations are calculated for replicate samples collected the same day.

Table 1 *S. latissima* batches used in the work

Harvesting date	Harvesting location (N, E)	Total solids (dw) (% of wet weight)	Laminaran (% of dw)	Mannitol (% of dw)
18 Nov 2013	63.4534, 10.2586	16.2	12.8	11.2
6 Dec 2013	63.8165, 8.6788	13.1	8.4	9.2

Dissolved solids and laminaran in preserved samples

An exact mass (30–40 g) of preserved biomass was centrifuged at 3200×*g* for 15 min. The supernatant was removed, and the pellet was washed with the same volume of deionised water. The masses of supernatant and pellet after each of the two centrifugations were recorded. Total solids (dw) and laminaran in the remaining pellet were determined. The dissolved fraction was calculated as the difference between the initial dw and laminaran content, and the remaining content in the washed pellets. The reported values provide a comparison of samples stored at different pH, but will not represent total dissolved components, since some soluble components will remain in the pellet after one wash.

Growth of yeast with preserved seaweed as carbon source

Selected samples, stored at different pH values from 2.6 to 3.7, were used as carbon source in growth tests with the yeast *Pichia angophorae* CBS 5830. For comparison, frozen, fresh seaweed from the same feedstock batch (December 2013) was adjusted to pH 3.5 in order to reduce the viscosity caused by fucoidan. The preserved and fresh samples were suspended in a basic growth medium containing KH_2PO_4 (3.6 g L^{-1}) and $(NH_4)_2SO_4$ (8.9 g L^{-1}) and pH was adjusted to 5.5 with NH_3. The final concentrations of KH_2PO_4 and $(NH_4)_2SO_4$ were 1.2 and 3.0 g L^{-1}, respectively. The seaweed provided 6.0–6.7 g L^{-1} laminaran and 6.6–7.3 g L^{-1} mannitol as carbon sources.

Precultures of *P. angophorae* were grown over night in a yeast medium, pH 5.5: mannitol (16 g L^{-1}), glucose (4 g L^{-1}), yeast extract (2 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g L^{-1}), KH_2PO_4 (0.8 g L^{-1}) and $(NH_4)_2SO_4$ (2 g L^{-1}). The preculture (50 mL) were concentrated by centrifugation (3220×*g*, 15 min) and resuspended in the basic medium (5 mL). Concentrated preculture was added to the *S. latissima* slurry medium to achieve an initial optical density of 0.5 (600 nm). Cultivation was carried out with 24 g medium in 125 mL shake flasks at 25 °C, 225 rpm for 40 h. Sampling of liquid (0.5–1.5 mL) from the cultures was performed regularly for analyses of the sugar consumption.

Analytical methods

Dry weight

Dry weight of the raw materials was determined by drying of approximately 5 g wet weight at 105 °C for 18–24 h, until constant weight.

Laminaran, mannitol and fermentation products

Laminaran was analysed as glucose after acid hydrolysis. Combined extraction and hydrolysis was performed as described by Schiener et al. (2015). Freeze-dried samples (600 ± 60 mg dw) were added sulphuric acid (3 mL, 0.5 M) in thick-walled glass tubes and heated to 121 °C for 15 min in an autoclave. Deionised water was added to dilute the samples, before centrifugation (3220×*g*, 10 min). Hydrolysis of laminaran in the liquid phase was performed by adding 2.5 M H_2SO_4 (0.1 mL) to 0.4 mL sample and incubation for 2.5 h at 105 °C in sealed vials. The samples were diluted and filtered (0.2 μm) before analyses by HPLC.

Glucose, mannitol, organic acids and ethanol in unhydrolysed or acid-hydrolysed samples were quantified by HPLC (Shimadzu) on an Aminex HPX-87-H (BioRad Laboratories) column at 45 °C, and refractive index detection (RID-6A, Shimadzu). Five millimolar H_2SO_4 was used as mobile phase at 0.6 mL min^{-1}. The standards used were glucose, mannitol, formic acid, acetic acid, lactic acid and ethanol.

Results

Storage of unpreserved biomass

In unpreserved *S. latissima* stored under anaerobic conditions, pH decreased to below 5 the first 15–20 days due to acid production by fermentative microorganisms, before stabilising at 4.0–4.2 for the period from 50 to 200 days (Fig. 1). Laminaran was almost completely consumed during

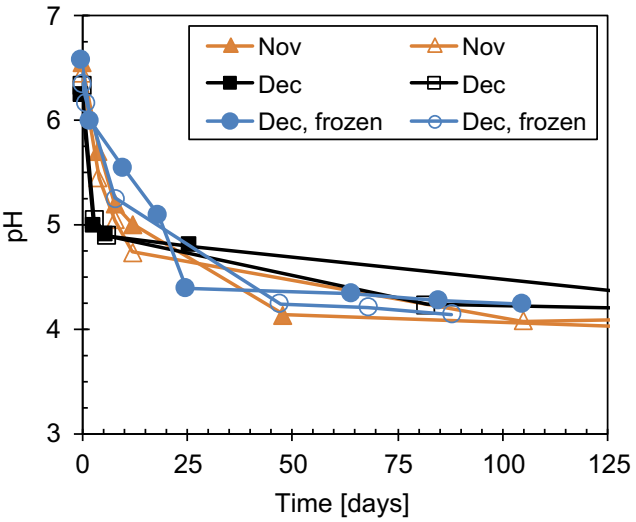

Fig. 1 pH decrease in unpreserved *S. latissima* stored at 13 °C for up to 200 days. Data from three independent experiments with different biomass batches (Nov and Dec), and two replicates. For the frozen biomass, an inoculum containing bacteria from decaying seaweed was added

the first 20–50 days (Fig. 2a), in parallel with the pH decrease. Some more laminaran (~ 30% after 100 days) remained in the experiment where frozen biomass was used, either due to a less efficient microbiota or due to changes in the laminaran availability caused by the freezing. More than 80% of the mannitol remained after 100 days, and approximately 50% after 170–200 days (24–28 weeks) (Fig. 2b).

The dominating fermentation product from both feedstock batches was lactic acid. Acetic acid constituted a minor fraction, while the ethanol level varied between the November and December batches (Fig. 2c, d). A relatively strong gas production was also observed. The total consumed sugars (100% of the laminaran and ~ 50% of the mannitol) corresponds to 9.4 and 5.2 g L^{-1} on C-basis. The sum of fermentation products, *exclusive* CO_2, constituted 11 and 6 g L^{-1}on a C-basis. This indicates that other components than the analysed sugars had been fermented, such as soluble protein, fucoidan or alginate.

Preservation of the biomass by acid addition

The pH reduction required for an efficient preservation of *S. latissima* was investigated in three independent storage experiments, where pH was adjusted to different values ranging from 2.4 to 4.5 by addition of sulphuric acid or combinations of sulphuric and formic acid. Immediately after the acid addition, pH was 0.2–1 unit below the final pH, before equilibrating to the desired value within 24 h. The slow equilibration is due to the buffering capacity of alginate. During the further storage, pH kept constant until the experiment was ended, except for samples stored at pH 4.5 and at pH 4.0 without formic acid added, where pH decreased to 3.7. Laminaran and mannitol were completely maintained for 25–30 weeks in all experiments at pH 3.7 or lower, independent of the presence of formic acid, or not (Fig. 3). Some variability between experiments and between replicate bottles was observed, most likely due to inhomogeneous sampling, since there was no decreasing trend with time. The recovery of laminaran and mannitol seemed to be lower at pH < 3, perhaps due to precipitation of alginate, as discussed below. Only negligible amounts of fermentation products (lactic acid and acetic acid) were detected in samples with pH below 3.7.

Higher pH values in the range 3.7 to 4.5 were tested in order to determine the maximum allowable pH for a stable biomass, and for verification of endogenous laminaranase activity, see below. Mannitol was properly preserved during the storage period of 90 days in all samples that had pH between 3.7 and 4.5. However, a significant loss of laminaran (20%) occurred, and fermentation products were formed, in the samples at pH 4 and 4.5 without formic acid (Fig. 4). When 0.1 M formic acid was included, the preservation was efficient also at

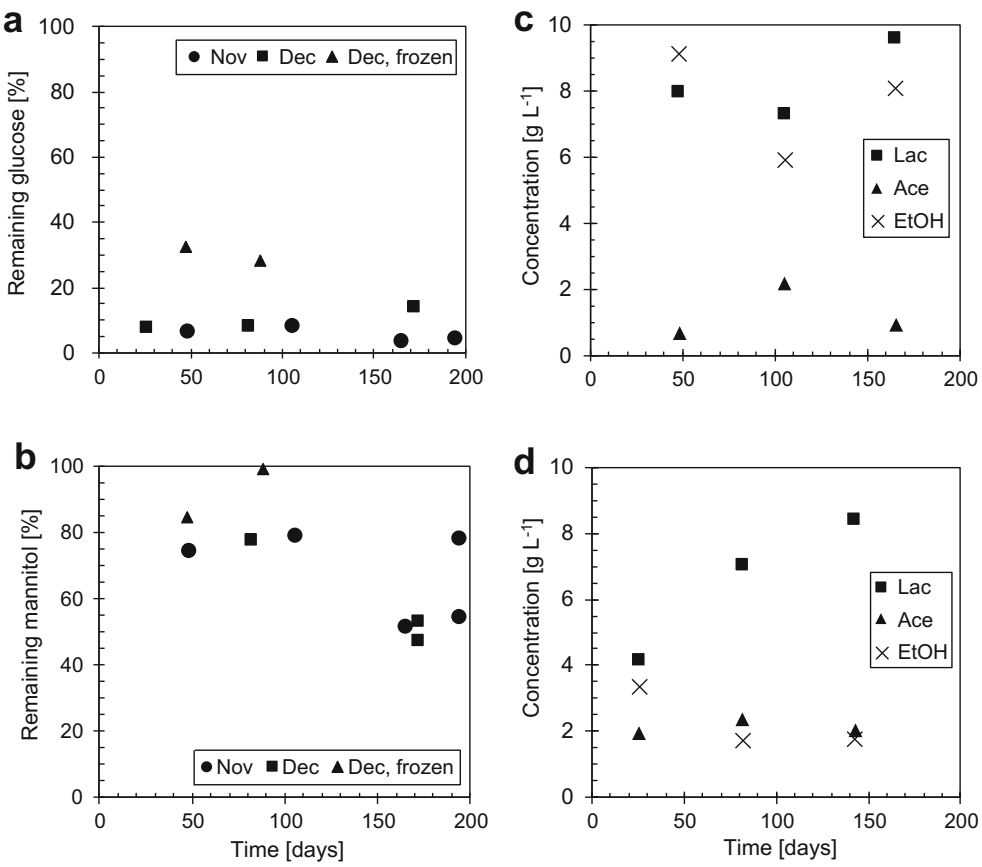

Fig. 2 Consumption of laminaran and mannitol, and formation of fermentation products in unpreserved *S. latissima* stored at 13 °C. Remaining laminaran, as glucose (**a**), remaining mannitol (**b**), fermentation products in the November biomass (**c**), fermentation products in the fresh December biomass (**d**). The initial concentrations of laminaran and mannitol (defined as 100%) were 17.8 and 15.5 g kg^{-1} total mass in the experiment with biomass from November, and 9.4 and 10.3 g kg^{-1} total mass in the experiment with biomass from December

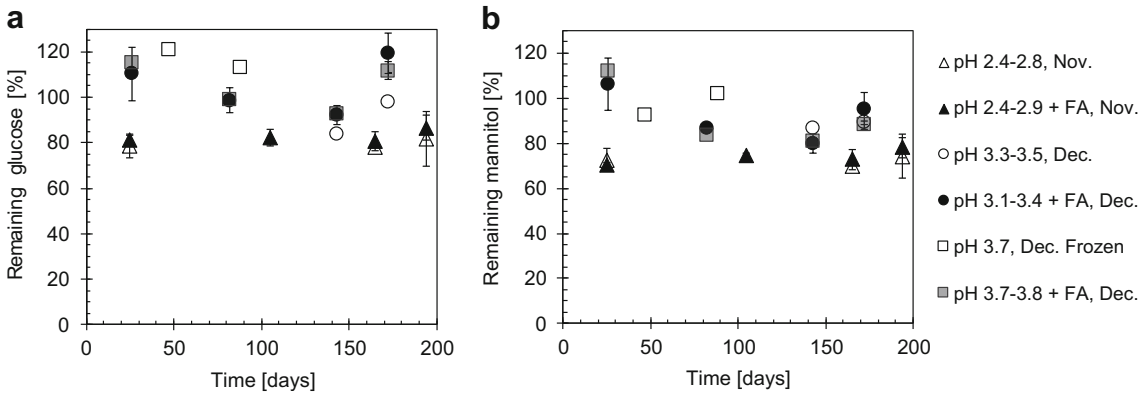

Fig. 3 Remaining laminaran, as glucose (**a**) and mannitol (**b**) in *S. latissima* preserved by acid addition and stored at 13 °C. FA, formic acid added. Data from three independent experiments with different biomass batches (Nov and Dec), and two replicates. For the frozen biomass, an inoculum containing bacteria from decaying macroalgae was added. The error bars indicate standard deviation from the mean of $n = 3–8$ samples, except for Nov. seaweed after 25 days, where mean ± range of two samples is presented.

pH 4.0, while lactic acid was formed at pH 4.5, although no reduction in laminaran was detected.

Endogenous laminaran hydrolysis

Increasing concentrations of free glucose were observed during storage of all the well-preserved samples, and the fraction of free glucose of total glucose increased with increasing pH, indicating enzymatic hydrolysis of laminaran (Fig. 5). As much as 80% of the remaining laminaran was present as free glucose in samples at pH 4 and pH 4.5 added formic acid. Control experiments were carried out in order to verify that the hydrolysis was enzymatic, not chemical, and caused by the macroalgae enzymes, not microbial enzymes. When laminaran dissolved in buffers with pH 4.5, 3.5 and 2.5 was stored at 13 °C, no increase in free glucose was observed after more than 5 months, indicating no chemical autolysis. In seaweed extracts stored at 25 °C, with pH adjusted to 4.6, 3.5 and 2.5 and antibiotics added to prevent microbial growth, the amount of free glucose increased over time in the extracts with highest pH (Supplementary Materials, Fig. S2). This confirmed the results from the preservation experiments and supports the theory that kelps contain laminaran-degrading enzymes.

Effect of storage at low pH on solubility and fermentability of *S. latissima* sugars

Long-term storage at low pH can contribute to solubilisation of some compounds, while others may precipitate and be less

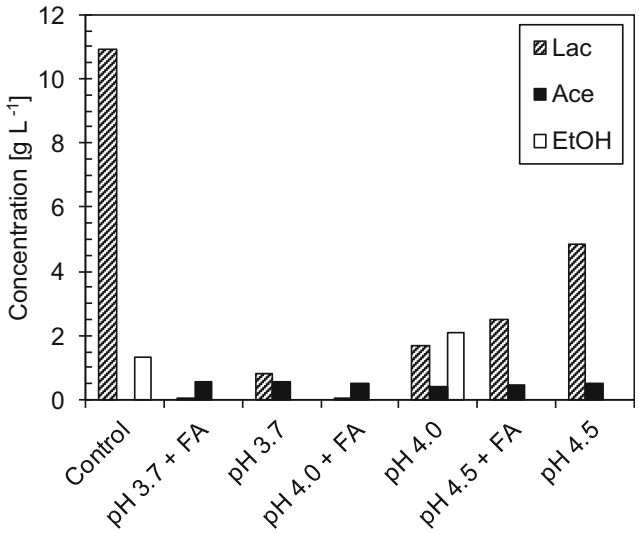

Fig. 4 Fermentation products formed in *S. latissima* preserved by acid addition and stored for 88 days at 13 °C (Dec, frozen, with inoculum), measured concentrations in the liquid phase. FA, formic acid added. The control was unpreserved *S. latissima* with a final pH of 4.15. Initial concentrations of laminaran (as glucose) and mannitol, 9.3 and 10.2 g kg^{-1} total mass. Lac, lactic acid; Ace, acetic acid; EtOH, ethanol

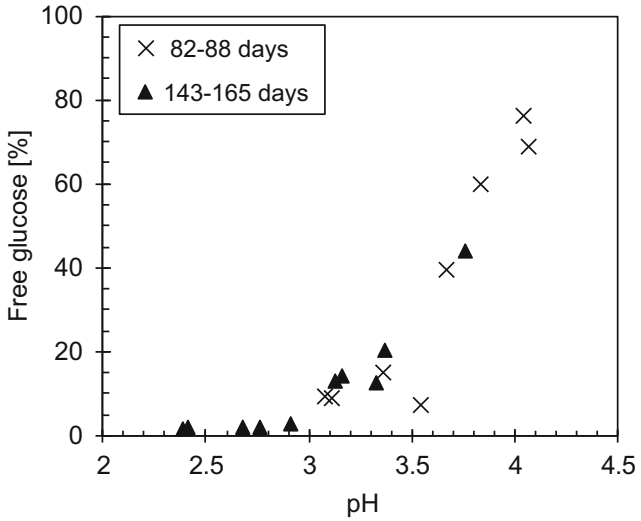

Fig. 5 Free glucose formed in samples without loss of laminaran (total glucose) during storage of *S. latissima*, as a function of the storage pH. The fraction of free glucose was calculated as the concentration of glucose in the liquid phase as % of theoretical, total concentration of laminaran in the total sample volume. The samples had been stored for 82–165 days

available. For use of the biomass as a carbon source for fermentation, the carbohydrates should be easily available for enzymatic hydrolysis and microbial conversion, implying that the release or solubilisation should not be rate limiting. However, polysaccharides may be accessible for enzymatic hydrolysis even if they are only partly dissolved, as may apply to laminaran. A simple solubility test, carried out by centrifugation and determination of the distribution of total solids (dry weight) and laminaran between supernatant and pellet, indicated reduced solubility below pH 3–3.5 (Fig. 6). The reported values do not represent the total amounts of soluble solids and laminaran, since dissolved components will remain in the pellet after one wash, but provide a comparison of samples stored at different pH.

As a measure for the availability of the sugars for enzymatic hydrolysis, the rate of sugar consumption by the yeast *P. angophorae* was determined for preserved and fresh pH-adjusted biomass. *P. angophorae* produces extracellular laminaranases and can utilise both laminaran and mannitol as carbon sources. Not surprisingly, the consumption rates were highest in the samples that had the highest content of free glucose (preserved samples stored at pH 3.3–3.7, data not shown). The unpreserved controls and the samples that had been stored at pH 2.6 did not contain significant amounts of free glucose. The consumption profiles for these two were quite similar (Supplementary Material, Fig. S3); however, in the pH 2.6 samples, a significant proportion of the laminaran (2 g L^{-1}, or 30% of the initial laminaran concentration) remained unconsumed, indicating resistance towards hydrolysis. This is in accordance with the solubility tests (Fig. 6), which indicates reduced solubility if pH becomes too low.

Discussion

Our aim was to develop a method that preserves the fermentable carbohydrates of *S. latissima* for use as a carbon source in fermentation to biofuels and bulk chemicals. Wild seaweed may be harvested in autumn, with optimal biomass composition. In Norway, cultivated seaweed is currently harvested in April–June, due to rapid onset of biofouling and degradation of the biomass in late summer (Førde et al. 2016), and the content of storage carbohydrates in available, cultivated seaweed is low. In our work, we therefore used wild biomass. The fouling problems need to be solved before cultivated *S. latissima* can be used for biofuels or other bulk fermentation products. However, cultivated seaweed has a market for a range of other applications, such as food, animal feed and extraction of valuable compounds. In any case, a preservation method will be needed.

Since sugars for fermentation is a low-value product, only low-cost preservation methods will be feasible. Our choice was to use the cheapest available acid—sulphuric acid—to lower the pH for prevention of microbial growth. Combinations of sulphuric and formic acid were also tested, since the small, organic acids, such as formic, acetic and lactic acid in their acid form, have an antimicrobial effect that adds to the effect of low pH and reduces the total amount of acid needed (Theron and Lues 2010). Formic acid is commonly used in ensiling of grass and has also been evaluated in combinations with sulphuric acid (Kennedy 1990; Mayne 1993). In the present work, efficient preservation of the macroalgae biomass, with no reduction in sugar content after 6.5 months storage, was obtained when pH was reduced to 3.7 or lower by addition of sulphuric acid. With a combination of sulphuric acid and formic acid, pH up to 4.0 could be applied without detectable loss of laminaran. These results are in agreement with results obtained for grass ensilage, where pH reduction to 4.3–4.5 by sulphuric or formic acid was not sufficient for stability, as pH continued to decrease to 3.5–3.7 due to production of organic acids (Mayne 1993). Laminaran was more easily degraded than mannitol under anaerobic conditions, as can be seen in the results from unpreserved samples. Mannitol is more reduced than glucose, and an additional NADH is formed when mannitol is oxidised to fructose by the enzyme mannitol dehydrogenase. Without oxygen or another electron acceptor present, the NADH cannot be regenerated to NAD^+. In the period between 100 and 165 days, some mannitol was

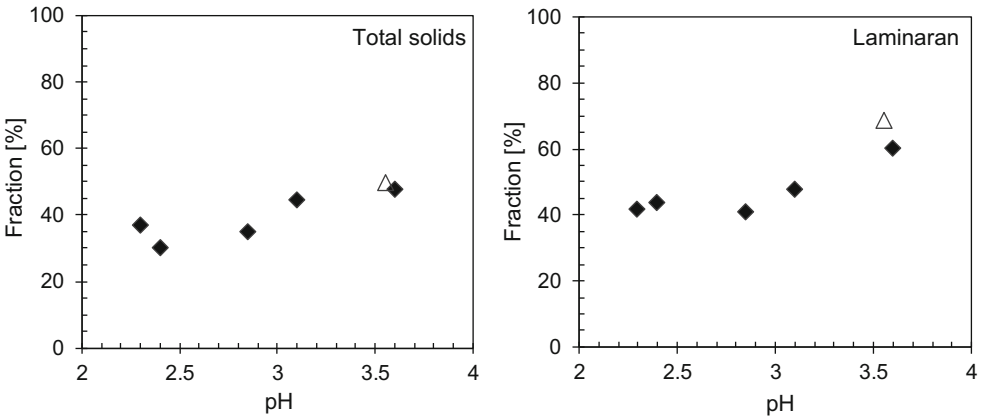

Fig. 6 Soluble solids and laminaran as fraction of total in samples stored for 3 months at different pH (closed symbols) and a sample of fresh biomass (open symbol) where pH was reduced to 3.5. The soluble fraction was calculated as the difference between the initial solids and laminaran content of the respective samples, and the remaining content in washed pellets, see 'Materials and methods'

consumed, in parallel with decreasing acetate concentrations. This indicates that acetate was used as an electron acceptor for fermentation of mannitol (McFeeters and Chen 1986). Our results indicate that pH should not be too low. In samples that had been stored at pH 2.6, the solubility of laminaran was lower than in samples stored at higher pH, and a significant proportion of the laminaran remained unconsumed in growth experiments with the yeast *P. angophorae*. The reason is probably precipitation of the alginate as alginic acid, as alginic acid has a pKa value of ~3.5 (Haug 1961). This precipitation can cause co-precipitation of other components.

For *S. latissima* harvested in November–December, 0.35 mol H_2SO_4 per kg dw was required to reduce pH to 3.5 (Supplementary Material, Fig. S1). With 0.1 M formic acid included, the same preservation effect was obtained at pH 3.8, which required 0.2 mol H_2SO_4 per kg dw. With costs of sulphuric acid of 370 € t^{-1} and formic acid 920 € t^{-1} (bulk prices from Norwegian dealers, 2016), the preservation costs will be in the same order for the two cases, approximately 10 € t^{-1} dw seaweed. This corresponds to 0.015–0.025 € kg^{-1} sugar, depending on the seaweed sugar content (40–65%).

In previous studies, ensiling by microbial acid production has been applied for preservation of seaweed for methane production by anaerobic digestion or thermochemical conversion (Herrmann et al. 2015; Milledge and Harvey 2016a; Redden et al. 2016). In these cases, the preservation should preserve the carbon. Maintaining the sugars is not critical, provided that carbon is not lost as CO_2. In the present work, the main fermentation products generated by natural fermentation of unpreserved samples were lactic acid, acetic acid and ethanol. A strong gas production was also observed, but not quantified. These products are typical for heterofermentative lactic acid bacteria (LAB), as well as 'mixed acid fermentation', carried out by several bacterial genera. The ratios between the fermentation products formed from the feedstock batch collected in November (Lac ≈ EtOH >> Ace) were similar to those obtained by Herrman et al. (2015). A much higher fraction of lactic acid in samples from the December batch indicates a higher fraction of homofermentative LAB. Our results clearly indicate that relying on the natural microbiota for pH reduction and preservation will imply loss of carbon as CO_2. This loss can be reduced by using a starter culture of homofermentative LAB, such as *Lactobacillus plantarum*. In addition to a starter culture, it may also be necessary to add more sugars to obtain lower pH values than 4.2, as obtained in the present work. For use of the biomass as fermentation substrate, laminaran must be hydrolysed to glucose, either by added enzymes in a separate or simultaneous saccharification and fermentation, or by using microbes that excrete the required enzymes. As part of the current work, the formation of free glucose during storage at conditions with no microbial activity was investigated. The glucose concentrations increased with increasing pH, and almost 80% of the laminaran was converted to glucose at pH 4–4.5 after 88 days. This provides a strong evidence for the presence of the β-glucanases and β-glucosidase required for hydrolysis of laminaran to glucose. Assays were also conducted at higher pH than those applied in the long-term storage experiments and indicate a pH optimum between 5.5 and 6 (data not shown). The activity measured as the rate of glucose formation was 0.6–0.7 mg g^{-1} dw day^{-1} at 25–30 °C and pH 5.5–6.5. When considering the temperature dependencies of enzyme activities, assuming a doubling of the activity when the temperature is increased by 10 °C ($Q_{10} = 2$), this is well in accordance with values obtained by Hou et al. (2015), who measured 2.5 mg g^{-1} dw day^{-1} at 50 °C, pH 5.0. In an industrial process, the biomass should be stored at pH between 3.5 and 3.8, and at low temperature. In this pH range and 13 °C, 30–60% of the laminaran was hydrolysed to free glucose after storage for 3–6 months. For a complete hydrolysis during the storage period, the endogenous laminaranases can be supplemented with commercial enzymes and possibly eliminate the need for a hydrolysis step prior to fermentation.

The normal pH range for microbial growth is 6–7, although yeast and lactic acid bacteria may grow at pH as low as 4–4.5. Acid-preserved biomass therefore needs to be neutralised before fermentation. Neutralisation with alkali will increase the salt concentration, which already is high in seaweed (more than 30% ash of dw). A high salt concentration may inhibit growth of the microorganisms used for further fermentation. However, the contribution from the pH adjustments was low compared with the salts in the biomass. Reduction of pH to 3.7 and subsequent neutralisation increased the salt concentration with 10%, while the salt content increased with 20% for pH reduction to 2.6 and neutralisation. The osmolarity of the liquid phase after neutralisation was approximately ~1 osmol kg^{-1} liquid, corresponding to 0.5 M NaCl, which is similar to the salt concentration of seawater. Schiener et al. (2016) observed only an insignificant reduction in ethanol production rates by *Saccharomyces cerevisiae* cultivated in a salt concentration like seawater, but a considerable reduction at twice the concentration of seawater.

In conclusion, the seaweed carbohydrates were well preserved at pH below 3.7 obtained by addition of sulphuric acid, and at pH below 4.0 using a combination of sulphuric and formic acid. At these upper limits, there was no detectable loss of laminaran, although small amounts of organic acids and ethanol were formed, indicating some microbial activity. The recommended pH values would therefore be somewhat lower, e.g. 3.5 and 3.8, respectively. pH should not be too low, since the solubility of the biomass components was reduced at pH below 3. The storage period can be utilised for hydrolysis of laminaran to glucose, saving costs for the fermentation process. Our work was primarily aimed at preservation of the biomass for application as a carbon source for fermentation into biofuels or chemicals. However, the method will be

equally suited for other applications of the biomass, such as extraction of valuable compounds for use in functional food, feed or other areas. These are more likely applications of cultivated brown algae in a near future, due to yet high biomass costs, and due to biofouling, which necessitates harvesting when the carbohydrate content is low. Future studies should investigate the effect of acid preservation on the other valuable compounds in macroalgae.

References

Black W (1955) The preservation of seaweed by ensiling and bactericides. J Sci Food Agric 6:14–23

Førde H, Forbord S, Handå A, Fossberg J, Arff J, Johnsen G, Reitan KI (2016) Development of bryozoan fouling on cultivated kelp (*Saccharina latissima*) in Norway. J Appl Phycol 28:1225–1234

Haug A (1961) Dissociation of alginic acid. Acta Chem Scand 15:950–952

Herrmann C, Fitz Gerald J, O'Shea R, Xia A, O'Kiely P, Murphy JD (2015) Ensiling of seaweed for a seaweed biofuel industry. Bioresour Technol 196:301–313

Horn S, Aasen I, Østgaard K (2000) Ethanol production from seaweed extract. J Ind Microbiol Biotechnol 25:249–254

Hou X, Hansen JH, Bjerre A-B (2015) Integrated bioethanol and protein production from brown seaweed *Laminaria digitata*. Bioresour Technol 197:310–317

Kennedy SJ (1990) Comparison of the fermentation quality and nutritive value of sulphuric and formic acid-treated silages fed to beef cattle. Grass Forage Sci 45:17–28

Kraan S (2013) Mass-cultivation of carbohydrate rich macroalgae, a possible solution for sustainable biofuel production. Mitig Adapt Strat Glob Chang 18:27–46

Marquez GPB, Santiañez WJE, Trono GC, de la Rama SRB, Takeuchi H, Hasegawa T (2015) Seaweeds: a sustainable fuel source. In: Tiwari B, Troy D (eds) Seaweed sustainability: food and non-food applications, Elsevier, Amsterdam, pp 421–458

Mayne C (1993) The effect of formic acid, sulphuric acid and a bacterial inoculant on silage fermentation and the food intake and milk production of lactating dairy cows. Anim Sci 56:29–42

McFeeters RF, Chen K-H (1986) Utilization of electron acceptors for anaerobic mannitol metabolism by *Lactobacillus plantarum*. Compounds which serve as electron acceptors. Food Microbiol 3:73–81

Milledge JJ, Harvey PJ (2016a) Ensilage and anaerobic digestion of *Sargassum muticum*. J Appl Phycol 28:3021–3030

Milledge JJ, Harvey PJ (2016b) Potential process 'hurdles' in the use of macroalgae as feedstock for biofuel production in the British Isles. J Chem Technol Biotechnol 91:2221–2234

Redden H, Milledge JJ, Greenwell HC, Dyer PW, Harvey PJ (2016) Changes in higher heating value and ash content of seaweed during ensiling. J Appl Phycol 29:1037–1046

Schiener P, Black KD, Stanley MS, Green DH (2015) The seasonal variation in the chemical composition of the kelp species *Laminaria digitata*, *Laminaria hyperborea*, *Saccharina latissima* and *Alaria esculenta*. J Appl Phycol 27:363–373

Schiener P, Stanley MS, Black KD, Green DH (2016) Assessment of saccharification and fermentation of brown seaweeds to identify the seasonal effect on bioethanol production. J Appl Phycol 28:3009–3020

Stévant P, Marfaing H, Duinker A, Fleurence J, Rustad T, Sandbakken I, Chapman A (2017a) Biomass soaking treatments to reduce potentially undesirable compounds in the edible seaweeds sugar kelp (*Saccharina latissima*) and winged kelp (*Alaria esculenta*) and health risk estimation for human consumption. J Appl Phycol. https://doi.org/10.1007/s10811-017-1343-8

Stévant P, Rebours C, Chapman A (2017b) Seaweed aquaculture in Norway: recent industrial developments and future perspectives. Aquac Int 25:1373–1390

Theron MM, Rykers Lues JF (2010) Organic acids and food preservation. CRC Press, Boca Raton, Florida

View publication stats