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Sourcing and bioprocessing of brown seaweed for maximizing glucose release

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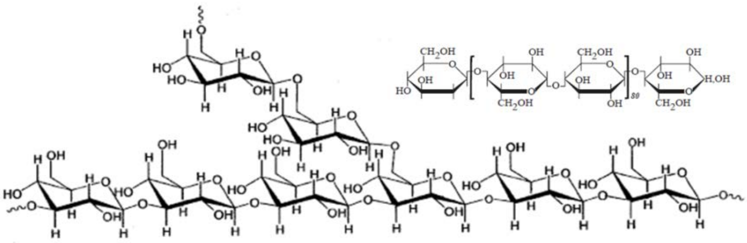

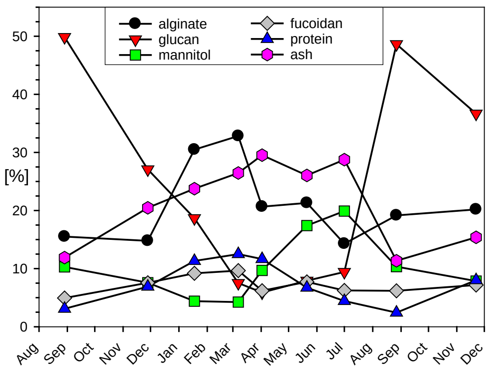

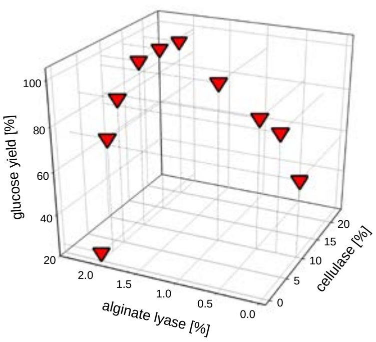

Dirk Manns

February 2016

Center for BioProcess Engineering
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Technical University of Denmark

Sourcing and bioprocessing of brown seaweed for maximizing glucose release

What is nature? How can we form a picture of it as it was before the intervention of humans with their ravaging tools? Even the powerful myth of nature is being transformed into a mere fiction, a negative utopia: nature is now seen as merely the raw material out of which the productive forces of a variety of social systems have forged their particular spaces. True, nature is resistant, and infinite in its depth, but it has been defeated, and now waits only for its ultimate voidance and destruction.

(Henri Lefebvre: The production of space, 1974)

PREFACE

This thesis is submitted in order to fulfill the PhD degree requirements at the Technical University of Denmark. The work was carried out from June 2012 to February 2016, interrupted by a leave of absence from 01.03.-31.08.2015, at the Center for BioProcess Engineering (BioEng), Department of Chemical and Biochemical Engineering supervised by Prof. Anne S. Meyer.

The PhD project was funded by the Danish Council for Strategic Research via the project: 'The MacroAlgaeBiorefinery – sustainable production of third generation (3G) bioenergy carriers and high value aquatic fish feed from macroalgae (MAB3)' as well as supported by a partial PhD scholarship from the Technical University of Denmark. The project was carried out in strong collaboration with the Centre for Wood Science and Technology of the University of Hamburg.

ACKNOWLEDGEMENTS

First, I would like to acknowledge Prof. Anne Meyer for her supervision and the opportunity to participate in the MAB3 project. Likewise, I acknowledge the MAB3 project and DTU for the financial support which allowed me the opportunity to do this PhD project.

Secondly, I would like to thank the Centre for Wood Science and Technology for welcoming me for so many months. I am particularly thankful to Professor Bodo Saake and his group for their great support not only during our collaboration, but above and beyond it. Thanks also to my colleagues at BioEng for the challenging discussions and sharing my moods.

Furthermore, thanks to Christian Nyffenegger (former BioEng researcher), Stinus Andersen (former BioEng MSc student), Alexander Deutschle (former PhD student at the group of Prof. Saake) and Andreas Baum (Post Doc at BioEng) for the fruitful and encouraging time during their contribution to the scientific output of this project.

A big "Danke!" to my bro Ron for the constructive comments on this thesis and the proofread. "Danke!" to my lad Eoin for the final proofread and the advice on the English language. And Andreas K., "Tak for hjaelpen" with the Danish abstract.

For the steady support throughout all my life and especially while studying I want to thank my family. Grandparents, parents, brother and sisters: You always keep me grounded!

Last, but not least, big thanks to my friends for love, support, distraction and hospitality – stay colorful!

Dirk Manns

Lyngby, February 2016

ABSTRACT

BACKGROUND: The research undertaken for this PhD thesis has been part of a larger research program "The MacroAlgaeBiorefinery – sustainable production of third generation (3G) bioenergy carriers and high value aquatic fish feed from macroalgae (MAB3)". The research has been based on the overall hypothesis that brown seaweeds represent a huge unexploited bioresource of the sea which can be upgraded to energy carriers via degradation to fermentable sugars. The research in the PhD thesis has aimed at optimizing pretreatment and enzymatic saccharification of *Saccharina latissima* and *Laminaria digitata* to release maximum levels of glucose.

RESULTS: The first requirement was to develop a robust methodology, including acid hydrolysis and analytical composition analysis, to quantitatively estimate the carbohydrate composition of the brown seaweeds. The monosaccharide composition of four different samples of brown seaweeds *Laminaria digitata* and *Saccharina latissima* were compared by different high performance anion exchange chromatography (HPAEC) methods after 3 different acid hydrolysis treatments or a cellulase treatment. HPAEC analysis with pulsed amperometric detection (PAD) preceded 2-step pretreatment with 72 % sulfuric acid (H_2SO_4) for 1 h at 30 °C and followed by 4 % H_2SO_4 at 120 °C for 40 min allowed quantitative determination of the carbohydrate composition of brown seaweed. The use of guluronic, glucuronic and galacturonic acid standards enabled quantification of the uronic acids. The variation in the biochemical composition of four populations of *Saccharina latissima* and *Laminaria digitata* from three different locations from Danish waters was documented. The chemical composition of brown seaweed varied mainly in regard to the season but differed also with respect to species, location, between the years and even within the population. Concentrations of ash and protein levels varied inversely to the carbohydrate levels, and total carbohydrate concentration varied seasonally, in particular through the storage of carbohydrates glucose and mannitol. Generally, alginate was the most abundant carbohydrate at all sites from December to summer with up to 36 % w/w_{DM} by weight before glucose levels were at least at the same magnitude. Total alginate concentration was relatively independent of seasonal changes but mannuronic (M) and guluronic acid (G) differed strongly throughout the year. M/G ratios varied regarding season, species or location from 1.3 to 3.6 but without a general pattern. The highest concentrations of glucan were found in August for wild growing *L. digitata* from the North Sea, with the glucose potential lying >50 % w/w_{DM} for three sequential years (2012-2014) accompanied by mannitol levels of about 10 % w/w_{DM} and low ash levels of 10-11 % w/w_{DM}. Generally spoken, glucose levels of *L. digitata* appeared to be superior to those of *S. latissima*. Cultivation of *S. latissima* in the Limfjorden, Denmark to obtain high glucan levels was not possible due to the incidence of biofouling in the summer. The average N-to-protein conversion factor was 3.7 but ranged from 2.1 to 5.9. Hence, application of a common factor cannot be recommended since total nitrogen content was more variable than the protein content. Post washing *L. digitata* harvested from the Danish North Sea in August 2012 had a total organic matter of 84 % mostly accounted for glucose (51 % w/w_{DM}), including a smaller contribution of mannitol (8 % w/w_{DM}), making this material an ideal feedstock for biocatalytical processing to achieve maximum glucose release.

The influence of milling as pretreatment to enhance enzymatic degradation was studied on the glucan rich *L. digitata* (North Sea, August 2012). Wet refiner milling, using rotating disc distances of 0.1-2 mm, generated differently sized particle populations with particles having decreasing average surface area (100-0.1 mm^2) with increased milling severity. Milling with disc distances below the thickness of the algae

(≤1 mm) increased the particle volume of the milled seaweed slurries and higher milling severity (lower rotating disc distance) also induced higher carbohydrate solubilization from the material, particularly for glucan and mannitol. However, particle size diminution did not improve the enzymatic glucose release. Milling was thus not required for enzymatic saccharification because all available glucose was released even from unmilled material during the combined treatment of alginate lyase and the cellulase preparation Cellic®CTec2. Apparently, the alginate lyase (Sigma Aldrich) activity catalyzed the cleavage of alginate on the substrate, which both decreases the viscosity of the substrate alginate and catalytically solubilizes the alginate to provide access to the glucan in the brown seaweed cell wall matrix.

The impact of alginate lyase in addition to cellulase on the brown seaweed degradation was studied further for *L. digitata* degradation. Therefore, two bacterial alginate endo-lyases (EC 4.2.2.-) from *Sphingomonas* sp. (SALy) and *Flavobacterium* sp. (FALy) were selected for heterologous, monocomponent expression in *Escherichia coli*. The optimal pH range for SALy was pH 5.5-7.0 with optimum at pH 6. The optimum for FALy and the commercially available alginate lyase from Sigma Aldrich (SigmALy) was pH 7.5. The investigated reaction temperatures of 30-50 °C had no influence on the activity. The thermal stability was reduced above 50 °C, for SigmALy above 40 °C. The FALy preferred poly-mannuronic acid as substrate, but also exhibited activity on poly-guluronic acid, whereas SALy had higher activity on poly-guluronic acid and SigmALy was only active on poly-guluronic acid. Subsequently, the alginate lyases were applied together with the commercial, fungally derived cellulase preparation Cellic®CTec2 at pH 6 and 40 °C on the glucan rich *L. digitata*. A decrease in viscosity decrease ensued in the initial minutes while alginate degradation occurred primarily within the first 1-2 hours of reaction. The level of released mannuronic acid blocks was inversely proportional to the glucose release indicating that the degradation of mannuronic acid blocks inhibited the cellulase catalyzed glucose release from *L. digitata.* Only the selective activity of SigmALy on guluronic acid enabled a 90 % glucose release within 8 hours by the cellulase preparation Cellic®CTec2. Nevertheless, combined alginate lyase and cellulase treatment for 24 hours released all potential glucose regardless of the applied lyase. Treatment with a mixture of 1 % w/w_{DM} SigmALy and 10 % v/w_{DM} Cellic®CTec2 at pH 5 and 40 °C released the available glucose during 8 hours. Two-thirds of the glucose was released with lower enzyme loading. Simple application of only the cellulase preparation enabled the release of only half of the present glucose after 8 h. Analysis after the enzymatic treatment indicated a potential extraction of proteins from the solid residue and the sulfated polysaccharide fucoidan solubilized in the saccharified liquid.

KEYWORDS: Laminaria digitata, Saccharina latissima, biochemical composition, compositional variation, milling, enzymatic glucose release, alginate lyases, combined cellulase-lyase treatment

CONCLUSION: The results of this PhD study demonstrated that brown seaweed can be completely degraded enzymatically by combined cellulase and alginate lyase treatment after milling. The work also showed, that biorefining of brown seaweed with current state of art technology is highly dependent on the cultivation, in particular growth site and season, of a suitable feedstock for achieving maximal glucan content and in turn allowing maximum glucose release.

DANSK SAMMENFATNING

BAGGRUND: Forskningen der ligger til grund for denne afhandling er del af et større forskningsprogram " The MacroAlgaeBiorefinery – sustainable production of third generation (3G) bioenergy carriers and high value aquatic fish feed from macroalgae (MAB3)". Forskningen er baseret på den overordnede tese at brunalger udgør en enorm, uudnyttet biologisk havresource, som kan opgraderes til energibærere ved hjælp af nedbrydning til forgærbare sukre. Forskningen i denne afhandling har fokuseret på at optimere forbehandling og enzymatisk forsukring af *Saccharina latissima* og *Laminaria digitata* med henblik på maksimal glukose-udskillelse.

RESULTATER: Den første betingelse var at udvikle en robust metode, inklusive syrehydrolyse og kompositionsanalyse, for at kunne skønne brunalgernes kulhydratsammensætning kvantitativt. Monosakkaridsammensætningen i fire forskellige prøver af brunalgerne *Laminaria digitata* og *Saccharina latissima* blev sammenlignet i forskellige "high performance anion exchange chromatography" (HPAEC)-metoder efter tre forskellige syrehydrolysebehandlinger eller en cellulasebehandling. HPAEC-analyse med "pulsed amperometric detection" (PAD) forud for to-trinsforbehandling med 72% svovlsyre (H_2SO_4) i en time ved 30 °C efterfulgt 4% H_2SO_4 ved 120 °C i 40 minutter tillod at fastlægge brunalgers kulhydratsammensætning kvantitativt. Brugen af guluronic, glucuronic og galacturonic syrestandarder tillod kvantificering af uronic-syrer. Variationen i biokemisk sammensætning i fire populationer af *Saccharina latissima* og *Laminaria digitata* fra tre forskellige lokationer i danske farvande blev dokumenteret. Den kemiske sammensætning i brunalger varierede primært med hensyn til sæson, men også med hensyn til art, lokation, mellem år og tilmed inden for population. Aske- og proteinkoncentrationer varierede inverst med kulhydratkoncentrationer, og total kulhydratkoncentration varierede fra sæson til sæson, særligt via lagring af kulhydraterne glukose og mannitol. Generalt var alginat det mest forekommende kulhydrat på alle lokationer fra december til sommer med op til 36% w/w_{DM} på vægtbasis før glukosemængden nåede mindst samme størrelsesorden. Samlet alginatkoncentration var relativ fri for sæsonudsving mens munnuronic (M) og guluronic syre (G) havde store udsving henover året. M/G-forhold varierede efter sæson, art eller lokation fra 1,3 til 3,6 men uden tydeligt mønster. De højeste glucan-koncentrationer var i Autust for vildtvoksende *L. digitata* fra Nordsøen, med glukosepotentiale >50% w/w_{DM} for tre fortløbende år (2012-2014) fulgt af mannitol-koncentrationer på omkring 10% w/w_{DM} og lave aske-koncentrationer på 10-11% w/w_{DM}. Samlet set fremstår glukose-koncentrationerne i *L. digitata* overlegne i forhold til *S. latissima*. Dyrkning af *S. latissima* i Limfjorden, Danmark, med henblik på at opnå høje glucan-koncentrationer var ikke mulig på grund af biologisk forurening over sommeren. Den gennemsnitlige N-protein omregningsfaktor var 3,7 men spænder fra 2,1 til 5,9. En fast omregningsfaktor kan derfor ikke anbefales eftersom det samlede kvælstofindhold var mere variabelt end proteinindholdet. Vasket *L. digitata* høstet fra den danske del af Nordsøen i august 2012 indeholdt samlet organisk materiale på 84%, primært glukose (51% w/w_{DM}), inklusive en mindre andel mannitol (8% w/w_{DM}), hvilket gør dette materiale til et ideelt råmateriale til biokatalytisk processering for at opnå maksimal udskillelse af glukose.

Betydningen af formaling som forbehandling for at øge enzymatisk nedbrydelse blev studeret på den glucanrige *L. digitata* (Nordsøen, august 2012). Metoden våd raffineringsformaling, med afstand mellem roterende skiver på 0,1-2 mm, genererede populationer med forskellige partikelstørrelser, hvor højere formalingsgrad førte til mindre, gennemsnitlig overfladeareal (100-0,1 mm^2). Formaling med skiveafstand mindre en algens tykkelse (≤1 mm) øgede partikelvolumen af det formalede algeslam mens højere

formalingsgrad (mindre skiveafstand) også medførte højere kulhydrat-solubilisering fra material, særligt for glucan og mannitol. Mindre partikelstørrelse forbedrede dog ikke den enzymatiske glukoseudskillelse. Formaling var derfor ikke nødvendig for enzymatisk forsukring fordi al tilgængelig glukose blev udskilt fra selv ikke-formalet materiale under kombineret behandling med alginatlyase og cellulase-præparatet Cellic®CTec2. Tilsyneladende katalyserer alginatlyase (Sigma Aldrich) spaltningen af alginat på substratet, hvilket både sænker viskositeten af substratets alginat og solubiliserer alginatet katalytisk så der opstår adgang til glucanet i brunalgens cellevægsmatrix.

Betydningen af alginatlyase som supplement til cellulase-behandling for nedbrydning af brunalger blev studeret yderligere på *L. digitata*. To bakterie-alginatendolyaser (EC 4.2.2.-) fra *Sphingomonas* sp. (SALy) og *Flavobacterium* sp. (FALy) blev udvalgt til heterolog, mono-komponent-ekspression. i *Escherichia coli*. Det optimale pH-spænd for SALy var pH 5,5-7,5 med optimum ved pH 6. Optimum for FALy og den kommercielt tilgængelige alginatlyase fra Sigma Aldrich (SigmALy) var pH 7,5. De undersøgte reaktionstemperaturer på 30-50 °C påvirkede ikke aktiviteten. Termisk stabilitet reduceredes ved temperature over 50 °C, for SigmALy's vedkommende 40 °C. FALy foretrak poly-mannuronic syre som substrat, men udtrykte også aktivitet på poly-guluronic syre, mens SALy viste større aktivitet på poly-guluronic syre og SigmALy kun var aktiv på poly-guluronic syre. Efterfølgende blev alginatlyase anvendt sammen med det i handel tilgængelige cellulasepræparat Cellic®CTec2, udvundet af svampe, ved pH 6 og 40 °C på glucanrige *L. digitata*. Et fald i viskositet blev observeret i de første minutter mens alginatnedbrydelse primært foregik inden for de første 1-2 timers reaktion. Niveauet af udskilt mannuronic syreblokke var omvendt proportionalt med glukoseudskillelse hvilket tyder på at nedbrydelsen af mannuronic syreblokke hæmmede cellulase-katalyseret glukoseudskillelse fra *L. digitata*. Alene den selektive aktivitet af SigmALy på guluronic syre medførte en 90% glukoseudskillelse inden for 8 timer af cellulasepræparatet Cellic®CTec2. Ikke desto mindre, så førte kombineret alginatlyase og cellulasebehandling i 24 timer til udskillelse af al tilgængelig glucose uanset valgt lyase. Behandling med en blanding af 1% w/w_{DM} SigmALy og 10 % v/w_{DM} Cellic®CTec2 ved pH 5 og 40 °C udskilte den tilgængelige glukose i løbet af 8 timer. To tredjedele af glukosen blev udskilt ved lavere enzymdosering. Simpel anvendelse af blot glucanase-præparat medførte udskillelse af kun halvdelen af den tilstedeværende glukose efter 8 timer. Analyse efter enzymatisk behandling indikerede en potential udvinding af proteiner fra restproduktet og den sulfaterede polysakkarid fucoidan opløst i væsken.

KONKLUSION: Resultaterne i denne PhD demonstrerer at brunalger kan fuldstændig degraderes enzymatisk med kombineret cellulase- og alginatlyasebehandling efter formaling. Arbejdet viser tilmed at bioraffinering af brunalger med eksisterende state-of-the-art teknologi er meget afhængig af dyrkningen, især dyrkningssted og –sæson, af et egnet råmateriale til opnåelse af maksimal glucanindhold og dermed maksimal glukoseudskillelse.

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PAPER II

Manns, D., Nielsen, M.M., Bruhn, A., Saake, B., Meyer, A.S. Compositional variations of brown seaweeds *Laminaria digitata* and *Saccharina latissima* in Danish waters. Prepared for submission to *Journal of Applied Phycology* in March 2016.

PAPER III

Manns, D., Andersen, S., Saake, B., Meyer, A.S. 2015. Brown seaweed processing: enzymatic saccharification of *Laminaria digitata* requires no pre-treatment. *Journal of Applied Phycology*, 1-8, published online.

PAPER IV

Manns, D., Nyffenegger, C., Saake, B., Meyer, A.S. Impact of different microbial alginate lyases on combined cellulase-lyase saccharification of brown seaweed. Prepared for submission to *RSC Advances* in March 2016.

Additional contribution (not defended within this thesis)

Nielsen, M.M., Manns, D., D'Este, M., Krause-Jensen, D., Rasmussen, M.B., Larsen, M.M., Alvarado-Morales, M., Angelidaki, I., Bruhn, A. 2016. Variation in biochemical composition of *Saccharina latissima* and *Laminaria digitata* along an estuarine salinity gradient in inner Danish waters. *Algal Research*, **13**, 235-245.

0. HYPOTHESES AND OBJECTIVES

This PhD study has dealt with the evaluation of the following concept:

Glucose from glucan rich brown seaweed can be biocatalytically released given the right enzymatic treatment post refiner milling

For this concept to work, the potential of this new biomass resource must be evaluated. Furthermore, laboratory technologies for the pretreatment and enzymatic deconstruction of brown seaweed must lead to a maximum release of glucose. The evaluation of these requirements is based on the following posed hypotheses:

a. site specific and seasonal factors influence the glucose potential of brown seaweeds,
b. brown seaweed harvested in late summer is a rich glucan source for glucose based biorefinery concepts,
c. only minor pretreatment is required for enzymatic seaweed saccharification since brown seaweed plant tissue is soft and free of lignin,
d. enzymatic decomposition of brown seaweed can be achieved by alginate and glucan degrading enzymes,
e. brown seaweed glucan is accessible for expansive glucose release over a short period of enzymatic treatment,
f. alginate lyase induced viscosity reduction supports enzymatic release of glucose, and
g. by-products mannitol, proteins and fucoidan remain after the saccharification of the brown seaweed.

In order to investigate the validity of these hypotheses, the following specific objectives were set:

1. develop a fast and reliable method for characterization of the biomass, notably with respect to the carbohydrate composition, in order to assess the carbohydrate potential of brown seaweeds,
2. evaluate the effect of milling pretreatment on the subsequent enzymatic glucose release,
3. a) assess the potential of cellulases for degradation of brown seaweed glucans, b) evaluate the application of alginate lyase as a tool to support of enzymatic glucose release,
4. optimize enzyme dosages and the period of time for the enzymatic treatment,
5. assess the liquefaction of brown seaweed during the application of alginate lyase and compare the induced viscosity reduction to the enzymatic saccharification, and
6. investigate the saccharified brown seaweed for potential by-products.

1. INTRODUCTION

On the 12th of December 2015, all 195 states at the United Nations Climate Change Conference, COP 21, in Paris, France negotiated a global agreement to achieve zero net greenhouse gas emissions and pursue efforts to limit the temperature increase to 1.5 °C during the 21st century (Sutter et al., 2015). Even though the legal binding of the agreement is pending the need for alternative sources for substitution of oil derived products and energy is behind any doubt.

Nowadays, first generation biofuels are produced from food crops, such as corn and sugar cane which requires arable land and freshwater and thus resulting in land use competition with food crops or indirect land use changes which can exacerbate climate change. While biofuels from lignocellulosic materials (2nd generation) still occupy land producing 3rd generation biofuels in the sea could eliminate many of the problems associated with conventional biofuels.

Macroalgae, or seaweed, is a robust crop that requires zero fresh water, arable land, pesticides or fertilizers (Aitken et al., 2014). The biomass potential with an average photosynthetic efficiency of 6-8 % is much higher than of land based crops with 1.8-2.2 % (Aresta et al., 2005). Macroalgae therefore, serve as a sink to assimilate carbon dioxide and nitrogen, minimizing their influence to the environment and converting them back into valuable carbohydrates and proteins for biofuels, food application or highly valuable feedstocks for the pharmaceutical and cosmetic industries (Singh et al., 2011; Wijesinghe and Jeon, 2012; Kraan, 2013). However, the biochemical composition of brown seaweeds varies profoundly especially with regard to the biofuel potential (Adams et al., 2011; Schiener et al., 2015). In this respect, identification of the right time and place for harvest of seaweed for bioenergy is indispensable (Kerrison et al., 2015).

In "The MacroAlgaeBiorefinery (MAB3)" project, two brown macroalgae (*Saccharina latissima* and *Laminaria digitata*), naturally growing around the Danish coast line were investigated for an integrated biorefinery concept into energy carriers and extraction of proteins from the energy conversion processes. While the MAB3 project considers all aspects from cultivation, harvesting, and conversion of biomass up to sustainability and feasibility studies, the main task of the present PhD study was the development of pretreatment and enzymatic deconstruction technologies to release in particular maximal glucose from brown seaweed.

Whereas enzymatic hydrolysis of lignocellulosic feedstocks is inefficient without a preceding hydrothermal or other physicochemical biomass pretreatment (Alvira et al., 2010), such harsh pretreatment may not be required for enzymatic seaweed saccharification because the plant tissue of brown seaweed is soft and free of lignin (Roesijadi et al., 2010; John et al., 2011). Another difference from terrestrial biomass is that in brown seaweeds the main matrix polysaccharide is alginate, which constitutes a key component of the seaweed cell walls (Deniaud-Bouët et al., 2014). This suggested the potential of employing alginate lyases for pretreatment of macroalgae for biofuel production (Kim et al., 2011a; Kraan, 2013).

The objective of this PhD study was to assess the significance of pretreatment on enzymatic saccharification of brown seaweed biomass. In particular the impact of alginate lyase treatment for enhancing the enzymatic glucose release was assessed. To evaluate the carbohydrate potential, another major objective was to develop a fast and reliable method for quantitative determination of the carbohydrate composition of brown seaweeds.

2. BROWN SEAWEED
Laminaria digitata and *Saccharina latissima*

2.1. Perspective background

Phylogenetically, the "kelp" type brown seaweeds (Phaeophyceae) belong to a major line of Eukaryotes, with the stramenopiles and the sublittoral zone forming the typical habitat for kelps. In contrast to most species of this domain, brown seaweed evolved complex multicellularity and plant-like structures (Michel et al., 2010a; Deniaud-Bouët et al., 2014).

Brown seaweeds with over 1500 species worldwide are widely distributed in marine temperate and polar waters and are the most complex and largest of the macroalgae. They are common on rocky shorelines, and their olive green to dark brown color derives from yellow-brown pigments. The large "kelp forests" with lengths of up to 45 m (*Macrocystis pyrifera*) serve as ecological habitat and refuge for many marine organisms. Temperatures between 5 and 15 °C guarantee optimal growth but some species can tolerate up to 25 °C and others temperatures of -1.5 °C. Tolerances to salinity are similar and most suitable salinities are of 25-35 practical salinity units (PSU). Further requirements are adequate water movement in order to deliver nutrients and carbon dioxide. In addition, sufficient light must be available to allow photosynthesis (Dean and Jacobsen, 1984; Bold and Wynne, 1985; Kerrison et al., 2015).

For Danish marine waters 26 species of brown algae were reported (Middelboe et al., 1998). Among these species *Saccharina latissima* and *Laminaria digitata* are the most common species which may be suitable for large-scale offshore cultivation (Kerrison et al., 2015). Phytomorphologically, the root-like holdfast of brown seaweeds, which anchors to the fixed substrates (bedrocks, boulders, *etc.*, or cultivation lines), joints the flexible stipe with the large blades. The sugar kelp *S. latissima* consists of a long undivided blade of up to 3-4 m (Figure 1A). *L. digitata* is commonly called finger kelp attributed to its finger like segments of the large blade (Figure 1B). Finger kelps grow commonly to about 2 m of length.

Figure 1: Phytomorphological appearance of the brown seaweeds (**A**) *Saccharina latissima* and (**B**) *Laminaria digitata*.

Recent genome annotation has revealed the unique carbohydrate metabolism of brown seaweed that converts the photoassimilate D-fructose-6-phosphate into D-mannitol. Mannitol and laminarin represent the unique storage carbohydrates in brown seaweeds. Unlike red and green macroalgae, pathways for sucrose, starch and glycogen synthesis are absent in this type of seaweed. Instead, long term carbon storage is based on the soluble vacuolar glucan laminarin (Michel et al., 2010b). The deposits of mannitol and laminarin carbohydrates in sieve elements of the lumina can be easily mobilized and transported to the rapidly growing parts of the kelp (Michel et al. 2010b). Like terrestrial plants brown algae consists of cellulose but lignin is absent (Michel et al., 2010a). Further structural carbohydrates of the brown seaweeds are alginate and fucoidan.

The major polysaccharide in brown seaweed cell walls is alginate, whereby, especially the Ca^{2+} crosslinked regions are designated to strength the cell wall. Fucose-containing sulfated polysaccharides (fucoidan) are localized mainly among the cellulose microfibrils and serve for binding alginate with cellulose, which forms the 3-D structure of the cell wall (Figure 2). Notably, it is hypothesized that hemicellulose might bridge the fucoidan with the cellulose (Deniaud-Bouët et al., 2014). However, the occurrence of hemicellulose in seaweed was only confirmed for green macroalgae, also only in minor amounts (Mikkelsen et al., 2014). Furthermore, fucoidan are also associated with proteins in the cell wall (Figure 2). It has been suggested that while cross-linkages of alginate with phenols regulate the strengthening of the cell wall in the amorphous sections, sulfated polysaccharides (fucoidan) may also play a role in the adaption to osmotic stress (Michel et al., 2010a, Deniaud-Bouët et al., 2014).

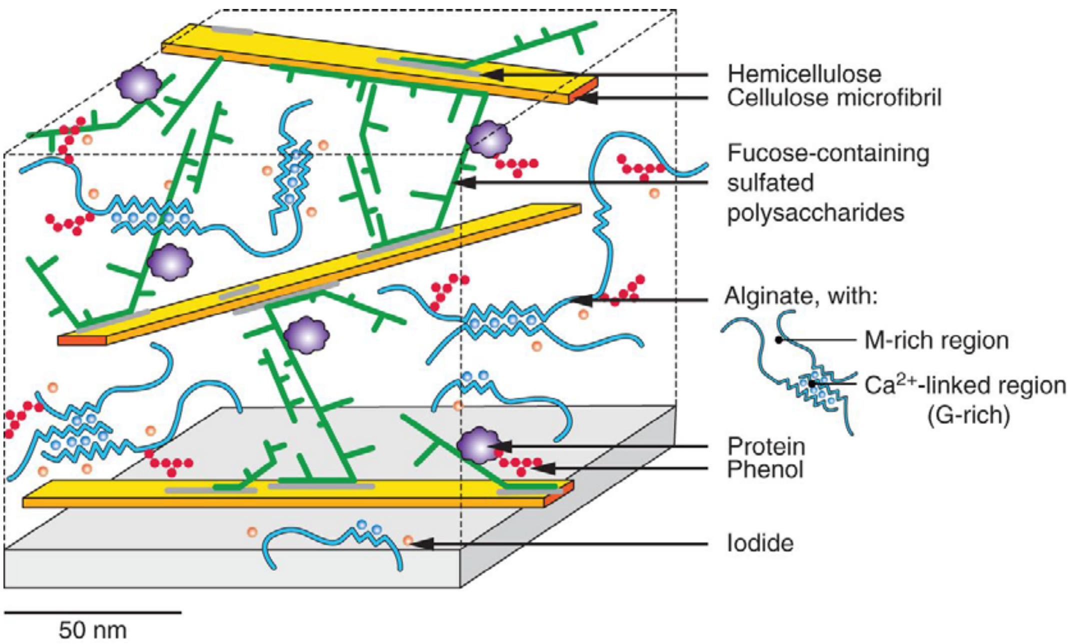

Figure 2: Cell wall model for brown algae from the order Fucales (Deniaud-Bouët et al., 2014). Remark: *Fucus* spp. usually contain of relative high fucoidan concentration (Obluchinskaya, 2008).

2.2. Biochemical composition (*PAPER I*)

Brown seaweeds are highly heterogeneous in their carbohydrate composition and their polysaccharides differ profoundly to those in terrestrial plants. Brown seaweed biomass is mainly composed of β-linked polysaccharides of neutral sugars, the sugar alcohol mannitol and uronic acids. Apart from the carbohydrates brown seaweed also possess significant amounts of proteins and high ash contents. As emphasized for iodide in Figure 2, p. 12 also the heavy metals are being absorbed by the cell wall constituents. Furthermore, minor quantities of lipids, vitamins, pigments, phenols, essential minerals and halogens like iodine and chlorine are present.

In the past, several extraction and determination methods for analyzing the compounds of brown seaweed have been developed but no method existed for qualification and quantification of all carbohydrates existed. Hence, the first objective of this PhD study was to establish a "*Methodology for quantitative determination of the carbohydrate composition of brown seaweeds (Laminariaceae)"* (*PAPER I*).

2.2.1. Carbohydrates

In *PAPER I* the monosaccharide composition of brown seaweeds *Laminaria digitata* and *Saccharina latissima* were compared by different high performance anion exchange chromatography (HPAEC) methods after different hydrolysis treatments. A conclusive database of brown seaweed compounds was generated by adding quantification of proteins, minerals (ash) and lipids (*PAPER I*).

The optimal type of acid hydrolysis treatment depends on the type of plant material, and no universal method exists. For pectinaceous plant materials, rich in uronic acid contents, treatment with hydrochloric acid (HCl) or trifluoroacetic acid (TFA) is usually favored (Arnous and Meyer, 2008) whereas for lignocellulosic biomass acid hydrolysis with sulfuric acid (H_2SO_4) is generally preferred (Willför et al., 2009; Sluiter et al., 2011). Analogously, for brown seaweed a two-step H_2SO_4 hydrolysis (1. step: 72 % H_2SO_4 at 30 °C for 1 h; 2. step: 4% H_2SO_4 at 120 °C for 40 min) performed best (Table 1; H_2SO_4, method A), while TFA hydrolysis was unable to decompose the seaweed biomass sufficiently (*PAPER I*). The sulfuric acid treatment was slightly modified (40 min instead of 60 min) from the original NREL protocol for determination of structural carbohydrates in biomass (Sluiter et al., 2011).

HPAEC-Borate has been established as an optimal analytical method for analysis of lignocellulosic carbohydrates (Willför et al., 2009). Accordingly, for determination of typically lignocellulosic carbohydrates such as glucose, xylose and mannose in acid hydrolysates of brown seaweed, the HPAEC-Borate method produced highly reproducible results (Table 1). In contrast, the high heterogeneity in the type of monomeric compounds and the high amounts of β-bonds in the polysaccharides in the brown seaweed along with high ion load challenged the analysis and could cause elevated deviations. However, it was only possible to detect all carbohydrates especially mannitol and uronic acids by HPAEC with pulsed amperometric detection (PAD) in NaOH solution (Table 1). Based on the results presented in *PAPER I* the HPAEC-PAD method for determination of all carbohydrate monomers from one hydrolysate of brown seaweed was established (*PAPER I*).

Table 1: Hydrated monomeric carbohydrate yields ($w/w_{dry\ matter}$) of brown seaweeds after different hydrolysis treatments of glucose after HPAEC-Borate and HPAEC-PAD analysis and uronic acids (for a more detailed table including the statistical analysis see *PAPER I*).

Sample	Hydrolysis treatment[1]	glucose [% w/w_{DM}]		uronic acids[2] [% w/w_{DM}]
		HPAEC-Borate	HPAEC-PAD	HPAEC-PAD
L. digitata (Apr'12)	$HClO_4$	1.1 ± >0.1	1.3 ± 0.1	7.6 ± 0.9
	H_2SO_4 (method A)	7.9 ± 0.2	7.8 ± 0.2	32.5 ± 3.5
	H_2SO_4 (method B)	7.4 ± 0.2	6.4 ± 0.2	26.0 ± 1.1
	enzym. glc release	8.7 ± 0.1	10.7 ± 0.4	n.d.
S. latissima (Apr'12)	$HClO_4$	0.8 ± 0.1	0.9 ± 0.1	7.2 ± 0.9
	H_2SO_4 (method A)	6.5 ± >0.1	6.8 ± 1.2	31.8 ± 5.4
	H_2SO_4 (method B)	5.9 ± 0.4	4.6 ± 0.2	21.8 ± 0.9
	enzym. glc release	8.5 ± 0.1	13.1 ± 3.4	n.d.
L. digitata (Aug'12; washed)	$HClO_4$	44.9 ± 2.3	53.3 ± 1.7	19.3 ± 0.5
	H_2SO_4 (method A)	56.6 ± 1.2	57.1 ± 3.9	24.4 ± 0.7
	H_2SO_4 (method B)	55.0 ± 0.2	43.9 ± 4.9	18.7 ± 2.6
	enzym. glc release	63.7 ± 5.2	68.2 ± 0.3	n.d.
L. digitata (Aug'12)	$HClO_4$	49.4 ± 4.4	53.7 ± 1.7	14.2 ± 0.8
	H_2SO_4 (method A)	57.5 ± 0.8	56.5 ± 9.2	17.2 ± 2.5
	H_2SO_4 (method B)	55.3 ± 0.1	43.6 ± 2.8	13.9 ± 1.0
	enzym. glc release	72.5 ± 0.4	77.0 ± 0.7	n.d.

[1] $HClO_4$: perchloric acid hydrolysis; H_2SO_4: sulfuric acid hydrolysis where post-hydrolysis with 4 % H_2SO_4 labelled as method A and 2 % H_2SO_4 as method B; enym. glc release: hydrolysis with cellulase preparation Cellic®CTec2; n.d. = not detected
[2] uronic acids determined as galacturonic acid equivalents

Using HPAEC-PAD, for brown seaweed *Laminaria digitata* harvested from the Danish North Sea in August 2012 a total carbohydrate content of 80.0 % $w/w_{dry\ matter}$ (w/w_{DM}) was determined. The quantitation of 50.9 % w/w_{DM} dehydrated glucose moieties represent the highest concentration has been reported in literature for brown seaweed. The high glucose content along with 10.4 % w/w_{DM} mannitol and a low ash content (11.9 % w/w_{DM}) indicates that *L. digitata* is a predestinated candidate for e.g. biofuels (*PAPER I*) and was subjected to further investigations in this PhD study (see section 3.).

The carbohydrates in brown seaweed can be distinguished as structural carbohydrates and storage carbohydrates (see section 2.1., p. 12). The structural polymers in brown seaweed are:

- alginate, or alginic acid, composed of guluronic acid and mannuronic acid,
- fucoidan, a heterogeneous branched and sulfated polysaccharide; composed of fucose along with other monosaccharides and uronic acids, and
- cellulose, composed of glucose.

Storage carbohydrates are built up during times of high photosynthetic activity and simultaneous restriction of bioavailable nitrogen. Therefore, the storage carbohydrates undergo particularly seasonal but also spatial variations. The storage carbohydrates in brown seaweeds are:

- laminarin, a reserve polymer composed of glucose with residual branches, and
- mannitol, present in monomeric form or as termination of a laminarin chain at the reducing end.

Structural carbohydrates

ALGINATE. The linear chains of alginic acids consist of 1,4-glycosidically linked α-L-guluronic acid (G) and β-D-mannuronic acid (M) in varying proportions (Figure 3). Normally the M/G ratio ranges between 1.2 to 2.1 (Percival and McDowell, 1967; Aarstad et al., 2011), however in case of *L. digitata* (harvested North Sea, August 2012) high contents of mannuronic acid and thus high M/G ratio of 2.8-3.0 were found (*PAPER I*).

The chains of alginic acid are made up of different blocks of guluronic and mannuronic acids, which are C-5 epimers (Percival and McDowell, 1967). The blocks are referred to as MM blocks or GG blocks, but less crystalline MG/GM blocks may also occur (Figure 3). The degree of polymerization (DP) of MM- and GG-blocks is compositionally homogenous with a DP ≥90. In contrast, the DP of the alternating sequences (MG/GM-blocks) are usually much smaller blocks and highly diverse (Aarstad et al., 2011). Alginate is the salt of alginic acid and is water soluble with monovalent ions, e.g. K^+, Na^+, and insoluble with di-/polyvalent ions (except Mg^{2+}). In the presence of Ca^{2+} the GG blocks form ionic complexes to generate a stacked, folded and rigid structure known as the ''egg-box model'' (Figure 4), responsible for hard gel formation that ensures the stiffness in the polymer chain (Percival and McDowell, 1967; Rhein-Knudsen et al., 2015).

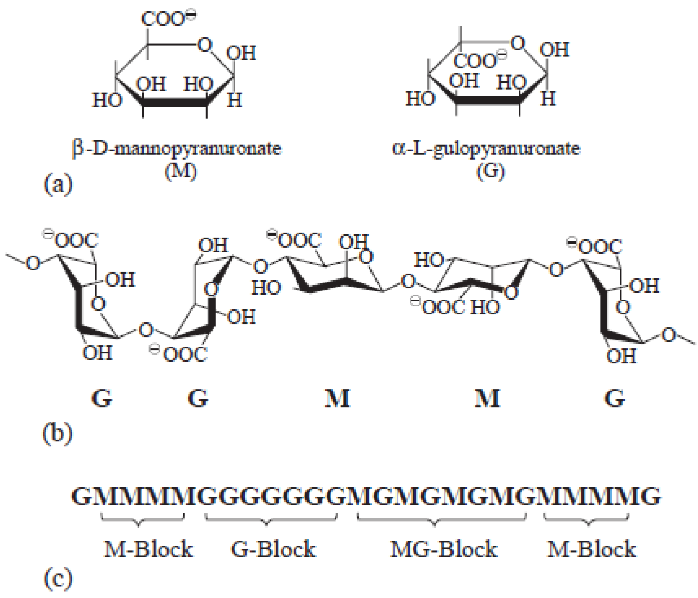

Figure 3: Alginate structural data: (a) alginate monomers (M vs. G); (b) the alginate polymer; (c) chain sequences of the alginate polymer (Davis et al., 2003).

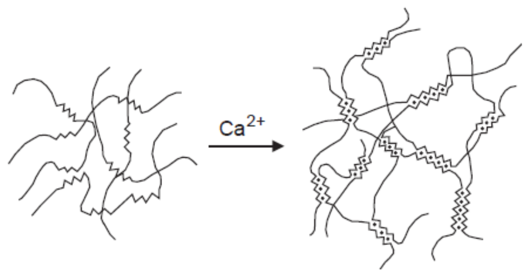

Figure 4: Schematic representation of the calcium-induced gelation of alginate in accordance with the "egg-box" structure (Davis et al., 2003).

The intense hydrolysis condition during lignocellulosic biomass treatment with acids, such as sulfuric acid, is known to cause decarboxylation of uronic acids. However, acid hydrolysis is required for degrading the crystalline structures of polysaccharides (Willför et al., 2009). In *PAPER I*, surprisingly, the highest monosaccharide levels of brown seaweed were generally achieved with H_2SO_4 hydrolysis, notably with regard to the detection of uronic acids. However, this finding was in agreement with the report of Percival and McDowell (1967), that polysaccharides containing high levels of uronic acids, like alginate, need drastic hydrolysis conditions to achieve a satisfactory decomposition into their carbohydrate monomers.

Nowadays, high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) is commonly used for the analysis of uronic acids (Willför et al., 2009). Therefore, the uronic acid monomers of the brown seaweed samples namely mannuronic, guluronic and glucuronic acid were also effectively separated and detected by HPAEC-PAD. However, due to the lack of a pure commercially available standard mannuronic acid was likely quantified as galacturonic acid equivalents (*PAPER I*).

In literature the alginate is noted with levels differing from 17 to 45 % (Østgaard et al., 1993; Schiener et al., 2015; Holdt and Kraan 2011). Similarly, amounts of 17-31 % w/w_{DM} of hydrated alginic acid monomers were detected in the study of *PAPER I* for *S. latissima* and *L. digitata* collected in April. Here, alginic acid was the predominant component along with high levels of ash. Furthermore, changes in the M/G ratio from 2 in April 2012 to 3 in August 2012 (e.g. the washed *L. digitata* from August consisted of 17.2 % mannuronic acid and 5.7 % w/w guluronic acid w/w_{DM}) indicated different structures in the composition of alginate (*PAPER I*).

Another uronic acid, glucuronic acid, was detected in relative small amounts of 1 % w/w_{DM} (*PAPER I*). The presence of the glucuronic acid and also galacturonic acid in brown seaweeds was mentioned by studies focused on the structure of brown seaweed fucoidans (Cumashi et al., 2007, Rioux et al., 2010).

FUCOIDAN. The fucose-containing sulfated polysaccharide fucoidan constitute another unique type of brown seaweed polysaccharide. The chemical structure and the abundance of the sulfated fucans making up fucoidan in brown seaweeds are heterogenic and represent the mixtures of structurally related polysaccharides with certain variations of the content of carbohydrate units (Ale and Meyer, 2013). Primarily, fucoidan from the Laminariaceae are composed of a backbone of α-1,3-linked- L -fucopyranose residues (Figure 5) with sulfate substitutions at C-4 (Figure 6 - A) and occasionally a second at the C-2 position (Figure 6 - B). Additionally, the fucose backbone some carries 2-*O*-α-L-fucopyranosyl substituents at the C-2 position (Figure 6 - C) (Cumashi et al., 2007).

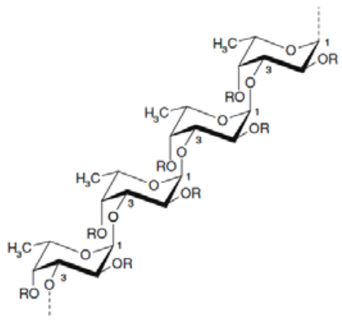

Figure 5: Homofucose backbone constructed of repeating fucose residues. R depicts the places of potential attachments of carbohydrate (α-L-fucopyranose, α-D-glucuronic acid) and non-carbohydrate (sulfate and acetyl groups) substituents (Cumashi et al., 2007).

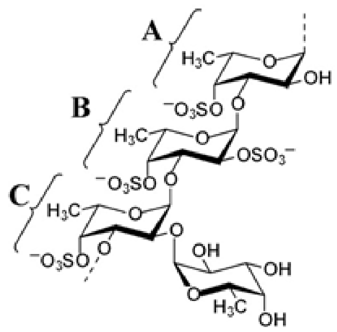

Figure 6: Structural motifs for fucoidan isolated from the brown seaweeds *S. latissima*. A: 4-sulfated; B: additionally 2-sulfated; C: 2-*O*-α-L-fucopyranosyl substituent (Usov et al., 1998; Cumashi et al., 2007).

Hence, fucoidan of the Laminariaceae *Saccharina latissima* and *Laminaria digitata* are primarily made of fucose and SO_3Na. In addition, other glycosyl such as galactose, xylose, mannose, glucose, glucuronic acid and galacturonic acid occur in different amounts and may even have acetate substitutions (Bilan et al., 2010; Rioux et al., 2010). For example, Cumashi et al. (2007) studied *S latissima* and *L. digitata* and found the composition of fucoidan as presented in Table 2.

Table 2: Biochemical composition of fucoidans (in %, w/w_{DM}) of *S. latissima and L. digitata* (Cumashi et al., 2007).

seaweed source	fucose	xylose	mannose	glucose	galactose	uronic acids	SO_3Na
S. latissima	36.7	1.2	1.0	2.2	4.6	4.8	29.6
L. digitata	30.1	1.9	1.7	1.4	6.3	7.0	27.5

While brown seaweeds, especially of the genus *Fucus* sp., fucoidan concentrations can reach 15 % *S. latissima* consisted of comparable lower levels of 9 % w/w_{DM} (Obluchinskaya, 2008). Considering that the monosaccharide composition presented in Table 2 represents approx. 10 % of the total seaweed biomass the finding of fucose of 2-4 % w/w_{DM} along with 1-2 % w/w_{DM} of glucuronic acid and 1.3-2.2 % w/w_{DM} of other carbohydrates such as xylose and mannose reflected the expectation with regard to composition and portions of fucoidan related monosaccharides in *S. latissima* and *L. digitata* (*PAPER I*).

***CELLULOSE*.** Cellulose is the most abundant organic substance in terrestrial plant materials. Brown seaweed, however, contains 10-15 % w/w_{DM} cellulose (Siddhanta et al., 2005; Schiener et al., 2015). It is composed of β-1,4-glycosidically linked chains of D-glucose, congregated to fibrils by extramolecular hydrogen bondings. The elementary fibrils are either ordered (crystalline structure) or less ordered (amorphous structure), both structure present in brown seaweed (Siddhanta et al., 2005).

Glucose is present not only in cellulose but also in laminarin (for laminarin see next page). This agrees with the experimental findings that enzymatic liberation of glucose from brown seaweeds is effectively accomplished by enzyme cocktails harboring β-1,3-glucanases and cellulases (Adams et al., 2009, 2011; Kim et al., 2011b; Yanagisawa et al., 2011). Hence, qquantification of the cellulose content by HPAEC analysis of monomeric glucose requires distinguishing between glucose from the structural cellulose and the non-structural laminarin. Schiener et al. (2015) quantified the total glucose of brown seaweed after two-step sulfuric acid hydrolysis and HPLC analysis similarly to *PAPER I*. In another method they applied a weaker single step acid hydrolysis for extraction and hydrolysis of laminarin and mannitol. Finally, the cellulose content was determined by subtracting the laminarin derived amount of glucose (identified by the soft hydrolysis) from the total glucose content (analyzed after two-step sulfuric acid hydrolysis).

L. digitata collected in August 2010 by Schiener et al. (2015) consisted of a total glucose of approx. 28 % w/w$_{DM}$. By subtracting the glucose derived from laminarin the cellulose content was attributed to approx. 11 % w/w$_{DM}$, similar to the sample from 21/03/2011 where the laminarin level was depleted (Schiener et al., 2015). By comparison, *L. digitata* collected in April 2012 consisted of similar total glucose of 7.9 % w/w$_{DM}$, whereas, *L. digitata* collected from the North Sea in August 2012 contained 57 % w/w$_{DM}$ glucose (i.e. approx. 51 % w/w$_{DM}$ dehydrated glucose), a much higher concentration (*PAPER I*).

Storage carbohydrates

Mannitol and laminarin represents the storage carbohydrates of brown seaweeds. Mannitol is present in monomeric form and as termination residue on laminarin chains (Østgaard et al., 1993; Rioux et al., 2010). The laminarin backbone consists of β-1,3 bonded glucose carrying occasional β-1,6 branched glucose substituents (Figure 7A) (Torosantucci et al., 2005). A typical chain is presumed to be made up of about 25 units that may be terminated at the reducing end with D-mannitol (M-chain; Figure 7B) or glucose (G-chain; Figure 7C), i.e. in different ratios (Percival and McDowell, 1967; Rioux et al., 2010). Hence, mannitol makes up to 5 % (w/w$_{DM\ laminarin}$) of the total laminarin carbohydrates (Rioux et al., 2010).

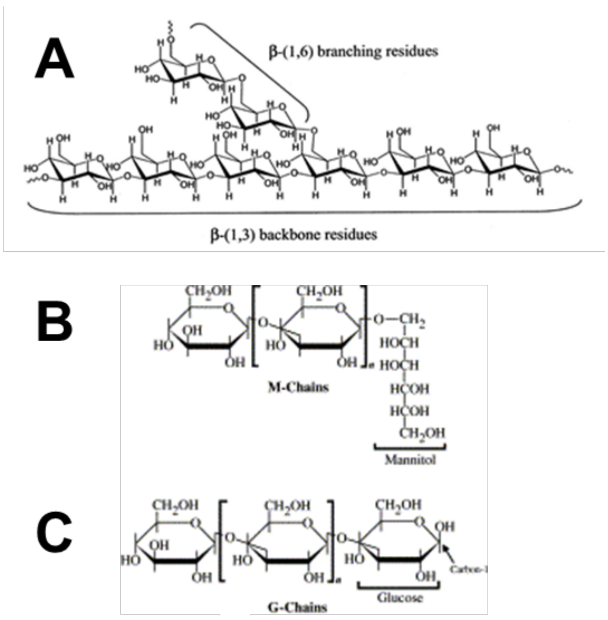

Figure 7: Laminarin structure: (**A**) β-1,3-linked glucan chain; (**B**) and (**C**) mannitol or glucose attached to the reducing end (Davis et al., 2003; Torosantucci et al., 2005).

Liberation of mannitol and glucose from laminarin can be achieved by acid treatment (0.5 M H_2SO_4) and specific enzymes (laminarinase) (Adams et al., 2011; Schiener et al., 2015). In both investigations, laminarin contents below 5 % w/w_{DM} were analyzed when *L. digitata* and *S. latissima* were collected in the first quarter of the year. At this time of the year, total mannitol levels of 5-10 % w/w_{DM} for *L. digitata* and 10-15 % w/w_{DM} for *S. latissima* were measured (Adams et al., 2011; Schiener et al., 2015).

In *PAPER I*, both species collected from the Kattegat (Baltic Sea) in April 2012 had slightly lower mannitol levels of 4 % and 6 % w/w_{DM} for *L. digitata* and *S. latissimi*, respectively. However, North Sea *L. digitata* from August 2012 consisted of approx. 10.4 % w/w_{DM}. Washing of the seaweed prior to analysis reduced the mannitol content to 8.0 % w/w_{DM}. Total glucose determination for the seaweeds from April 2012 revealed levels of 6.5 % and 7.9 % (*PAPER I*). Since the storage laminarin not typically present early in the year, these glucose contents presumably attribute to the cellulose content alone. Contrarily, the high glucose level of 57 % w/w_{DM} of *L. digitata* (North Sea, August 2012) indicated the presence of laminarin.

2.2.2. Proteins and minerals (ash)

Ash content and mineral composition of brown seaweeds differ highly from terrestrial plants and vary seasonally (Indergaard and Minsaas, 1991; Morrissey et al., 2001). In general, brown seaweeds have higher ash contents than other seaweed types (Ruperez, 2002). The investigated seaweed samples from April 2012 had an ash content of over 30 % w/w_{DM}. In contrast, when storage carbohydrate contents of glucose and mannitol were high, *L. digitata* possessed an ash content of just 11.9 % w/w_{DM}. By applying washing as pretreatment the ash content was lowered to 7.9 % (*PAPER I*). Similarly, ash of approx. 10 to 40 % w/w_{DM} was reported in literature, for *L. digitata* and *S. latissima* (Ross et al., 2008; Adams et al., 2011; Schiener et al., 2015). Together with sodium and potassium, calcium, phosphorus and sulfur were the major minerals in brown seaweed (*PAPER I*).

Protein content is known to range from 3-21 % w/w_{DM} for *L. digitata* and *S. latissima* (Morrisey et al., 2001; Holdt and Kraan, 2011). The differences are primarily being due to the source and season, but are also affected by the application of different nitrogen-to-protein factors. The most commonly used factor for calculating the protein content of plant material from the nitrogen content is 6.25. Generally, conversion factors are higher by Kjeldahl than for total nitrogen measured by elemental analysis (Gonzalez et al., 2010; Slocombe et al., 2013). Furthermore, the presence of non-protein nitrogenous substances such as pigments and dissolved inorganic nitrogen affect the factor (Lourenco et al., 2002). The levels of inorganic nitrogen in seawater differ seasonally (Zimmerman and Kremer, 1986; Carstensen et al., 2006). Lourenco et al. (2002) performed amino acid analysis and elemental analysis for total nitrogen and calculated N-to-protein conversion factors with amino acid residues divided by nitrogen. The investigation of four brown seaweeds species revealed a nitrogen-to-protein factor of 5.4 ± 0.5. Furthermore, a literature study based on all available protein concentrations for brown, red and green seaweed suggested a global N-to-protein factor for seaweed of 5 (Angell et al., 2015).

Compared to 3 % total amino acids present in *L. digitata* from August 2012, relatively high levels of 9-10 % w/w_{DM} of total amino acids (protein) were found in the brown seaweed samples from April 2012. The determined N-to-protein factor was 4.0; in particular 3.4 for *L. digitata* (April 2012), 4.4 for *L. digitata* (August 2012), and 3.8 for *S. latissima*. This indicated that application of any nitrogen-to-protein factor, such as 6.25, 5.4, 5.0 or 4.0, should be used carefully in order to avoid a potential risk of misestimating (*PAPER I*).

2.2.3. Overall database of compounds

Multiple extraction and quantification methods are commonly applied to generate a database of all particular seaweed compounds (Obluchinskaya, 2008; Schiener et al., 2015). However, application of multiple methods influences in particular the accuracy of the total mass balance, through the introduction of impurities, as well as losses. For example, Rioux et al. (2007) analyzed all compounds from brown seaweed. A sum-up of all extracted fractions of carbohydrate including proteins and lipids led to a maximum yield of 2/3 of the expected carbohydrate yield calculated as the subtraction of ash, protein and lipids content from the total mass (Rioux et al., 2007).

Table 3 displays a conclusive map of the major brown seaweed compounds such as structural and non-structural carbohydrates, proteins and ash (proxy for minerals). Furthermore, the total lipid concentration was determined but did not exceed levels of 1 % (w/w_{DM}). The quantification of all carbohydrate monomers from one hydrolysate of brown seaweed was achieved HPAEC-PAD analysis (see section 2.2.1., p. 13f). Performance of amino acid analysis for total protein and incineration for ash contents completed the mass balance. Through the use of these three methods total yields (for ash, protein and carbohydrates) between 86.7 % and 95.5 % w/w_{DM} were detected for brown seaweed samples in Table 3. Additionally, the mass of total organic compounds (carbohydrates, protein and lipids) was successfully cross-verified with the sum of C, H, N and O as total organic compounds received from elemental analysis (*PAPER I*).

The brown seaweeds *Laminaria digitata* and *Saccharina latissima* collected in April in the Danish Kattegat (Baltic Sea) showed only minor differences in their composition (Table 3). Here their total detected organic matters were approximately 56 % w/w_{DM} and the ash content ranged between 31-35 % w/w_{DM}. In contrast, August collected *L. digitata* from the Danish North Sea had a much higher organic matter of 84 % dominated by glucan with 51 % w/w_{DM} (Table 3). Therefore, *L. digitata* from the Danish North Sea is predestinated for biofuel production in biorefineries (*PAPER I*).

Table 3: Mass balance [% w/w_{DM}] of analyzed *L. digitata* and *S. latissima* from the Kattegat of April 2012 and *L. digitata* from the North Sea of August 2012 after and prior washing. Each data represents the average (± SD) of individual triplicates (for more details and the statistical analysis see *PAPER I*).

sample	ash	protein	N	ManA[1, 2]	GulA[1, 2]	glucose[1]	mannitol[1]	fucose[1]	others[1, 3]	total[4]
	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]	[%]
L. digitata (Apr'12)	31.0 ± 0.1	9.3 ± 0.4	2.7 ± >0.1	18.7 ± 2.0	9.5 ± 1.0	7.0 ± 0.2	4.1 ± 0.4	3.6 ± 0.4	3.5 ± 0.5	86.7 ± 5.0
S. latissima (Apr'12)	34.6 ± 0.2	10.1 ± 0.1	2.6 ± >0.1	19.5 ± 3.3	8.2 ± 1.5	6.1 ± 1.1	6.5 ± 1.1	2.6 ± 0.5	2.9 ± 0.6	90.5 ± 8.4
L. digitata (Aug'12; washed)	7.9 ± >0.1	3.1 ± 0.4	0.7 ± >0.1	15.7 ± 0.5	5.2 ± >0.1	51.4 ± 3.5	8.0 ± 0.3	2.1 ± 0.1	2.1 ± 0.7	95.5 ± 5.6
L. digitata (Aug'12)	11.9 ± 0.1	3.1 ± 0.2	0.5 ± >0.1	11.1 ± 1.6	2.3 ± 0.6	50.9 ± 7.4	10.4 ± 1.8	1.7 ± 0.4	1.8 ± 0.4	93.2 ± 12.5

[1]all values are given from hydrated monomers (conversion factors for dehydration on polymerization: uronic acids = 0.91; glc, gal, man = 0.90; fuc, rha = 0.89; xyl, ara = 0.88); [2]GulA = guluronic acid, ManA = mannuronic acid (given as galacturonic acid equivalents); [3]total of arabinose, rhamnose, galactose, xylose, mannose and glucuronic acid; [4]as sum of all detected compounds exclusive nitrogen

2.3. Compositional variation (*PAPER II*)

In the waters of the relatively cold Northern hemisphere, such as the European, North American, and Canadian waters, the biochemical composition of brown seaweeds varies throughout the year. At the beginning of the spring, when the storage carbohydrates are at minimum, contents of especially ash and protein are at their maximum. In autumn, conversely, the storage carbohydrates mannitol and laminarin are at their maximum (Black, 1950; Schiener et al., 2015). Danish marine waters are subjected to large temporal and spatial variations influencing environmental growth parameters such as salinity, temperature, nutrients, exposure and light among many others (Conley et al., 2000; Nielsen et al., 2016). Accordingly, high differences concerning biomass yields and biochemical composition of Danish brown seaweed can be observed (Marinho et al., 2015; Nielsen et al., 2016).

While seasonal and spatial variations in composition of brown seaweed biomass have been known for long time in Europe (Black, 1950), compositional seasonal variations in macroalgae is in revision due to their potential use as biofuel feedstock (Adams et al., 2011; Schiener et al., 2015). Furthermore, in Denmark there is a need for a thorough analysis of the parameters important for the growth of locally grown and cultivated *S. latissima* and *L. digitata* in order to understand which areas could be suited for future cultivation practices.

Therefore, the carbohydrate potential of Danish brown seaweeds was assessed with the method developed in *PAPER I*. The study was a collaboration with work package one (WP 1) of the MAB3 project (responsible for seaweed cultivation and harvesting). In collaboration with WP 1 two approaches for analyzing the influence of environmental factors on the growth of brown seaweeds were investigated. One study focuses on the *"Variation in biochemical composition of Saccharina latissima and Laminaria digitata along an estuarine salinity gradient in inner Danish waters"* (Nielsen et al., 2016). In the other study, seasonal and spatial influences of *L. digitata* from the North Sea and two other wild seaweed populations from the Kattegat at the Bay of Aarhus as well as one cultivation in the Limfjorden, Denmark were investigated for *"Compositional variations of brown seaweeds Laminaria digitata and Saccharina latissima in Danish waters"* (*PAPER II*).

The high glucose yields from *Laminaria digitata* from the North Sea of August 2012 discovered as a result of the research undertaken for *PAPER I*, was confirmed for the two sequential harvests of August 2013 and 2014 by the investigations of *PAPER II*. Therefore, the North Sea *L. digitata* seaweed represented the most suitable biomass for application as resource for bioenergy. For three sequential years (2012-2014) glucose potential was >50 % w/w_{DM} accompanied with mannitol of about 10 % w/w_{DM} and low ash levels of 10-11 % w/w_{DM}. *L. digitata* from the North Sea was more exposed to elaborated water movements than the other populations from the Danish waters. Among the site-specific physicochemical variables, temperature was found to influence the chemical composition. The optimal temperature conditions at the exposed site of the North Sea appeared to be optimal for high production of brown seaweed carbohydrates (*PAPER II*).

2.3.1. Variation in the composition of carbohydrates

The carbohydrate composition of the four brown seaweed populations varied with respect to location, season, species and environmental conditions. Variations were more evident in *Laminaria digitata* than in *Saccharina latissima*. Generally, alginate was the most abundant carbohydrate polymer but in time of high accumulation of storage carbohydrates glucose levels were at least of the same magnitude (Figure 8) (*PAPER II*).

The cultivated seaweed at Limfjorden (Figure 8C) differed from the three natural populations. Firstly, after placement of the seedling lines in the cultivation site in September 2012, sampling was first possible the following February 2013. Secondly, from May onwards growth was increasingly hampered by settling and growth of various biofouling organisms on the *S. latissima* fronds. In August 2013, the biofouling caused massive losses and the last sample obtained in August showed only small amounts of remaining carbohydrates (*PAPER II*).

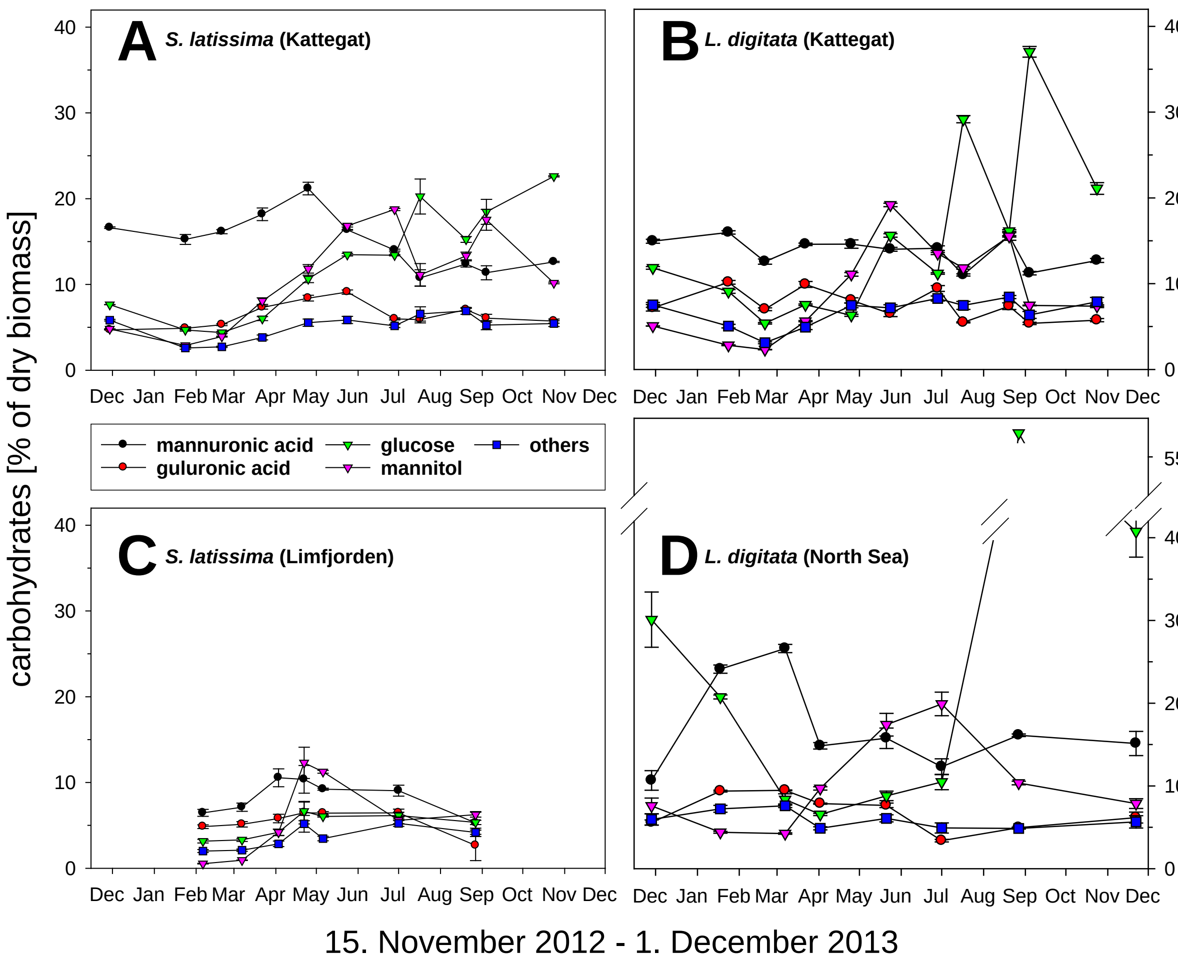

Figure 8: Seasonal variation from of the carbohydrate compositions of (**A**) *S. latissima* and (**B**) *L. digitata* from Danish Kattegat, (**C**) *S. latissima* from the cultivation in Danish Limfjorden and (**D**) *L. digitata* from the Danish North Sea. Each data point represents average values of independent triplicates; error bars indicate the standard deviation. All values are given as hydrated monomers; others: fucose, galactose, arabinose, rhamnose, mannose, xylose and glucuronic acid.

The commercial extraction of high value compounds is currently dominated by the hydrocolloid alginate preferably of seaweeds with high portions of guluronnate in alginate (McHugh, 2003; Rhein-Knudsen et al., 2015). For Northern Europe in particular *Laminaria hyperborea* with M/G ratios of >1 is used for alginate production (Fertah et al., 2014). Yields of fucose, the backbone of the hydrocolloid fucoidan, is presented in Figure 8 together with other carbohydrates such as xylose, mannose, arabinose and some more monosaccharides. However, these sugar monosaccharides contributed only minor quantities (2.6-8.4 % w/w_{DM}) towards the overall carbohydrate contents of the four investigated brown seaweed populations (*PAPER II*).

The total alginate content as the sum of its monomers mannuronic acid (M) and guluronic acid (G) underwent less seasonal variation than their relative proportion to each other, which differed strongly throughout the year with M/G ratios from 1.3 to 3.6 (mostly around 2.0). Lowest alginate levels of about 16 % w/w_{DM} were found for samples in July. Conversely, levels were highest in spring up to 30 % and 36 % w/w_{DM} for species in the Kattegat and North Sea, respectively (Figure 8A/B/D) (*PAPER II*).

The M/G ratio of the cultivated *S. latissima* (Limfjorden) differed between 1.3 and 1.8 without correlation to harvest time. Likewise, the M/G ratio of *L. digitata* of the Kattegat varied between 1.5 and 2.2 regardless the time of measurement with the total alginate concentrations (M+G) of 15-25 % w/w_{DM} (Figure 8B). In contrast, *S. latissima* from the Kattegat exhibited a strong correlation to harvesting time from November 2012 (M/G ratio 3.5) to May 2012 with M/G ratio of 1.8. In April 8.4 % w/w_{DM} (G) and 21.2 % (M) was present in *S. latissima*, while in July there was less than 10 % w/w_{DM} (M) (Figure 8A). The M/G ratio of *L. digitata* from the North Sea was the opposite, high during the summer, e.g. in July 2013 of 3.6 ((M): 12.3 % w/w_{DM}; (G): 3.4 % w/w_{DM}). In November the population contained similar amounts of (M)+(G) but differences between (G) (5.6 % w/w_{DM}) and (M) (10.7 % w/w_{DM}) were less pronounced and thus the M/G ratio decreased to 1.9 (Figure 8D) (*PAPER II*).

Overall, no general seasonal pattern for M/G ratios could be observed. The variation was apparently due to specific location and individual population parameters. Factors in the process of epimerization of ß-D-mannuronic acid to α-L-guluronic acid were possibly different between location and/or species (Indergaard et al., 1990). The seasonal variation of the total alginate content of the wild brown seaweeds (16-36 % w/w_{DM}) corresponded to what has been reported elsewhere being ≈ 15-30 % w/w_{DM} (Schiener et al. 2015).

High contents of glucose, the basic unit of laminarin and cellulose in brown seaweeds, are essential for application of brown seaweed in sugar based biorefineries. However, glucose, i.e. laminarin, is strongly subjected to seasonal variation of 0-33 % w/w_{DM}. Also, the level of the second reserve substance mannitol, an alcohol form of mannose, differs widely due to seasonal variation from 5-26 % w/w_{DM} (Holdt and Kraan, 2011; Adams et al., 2011).

Expected fluctuations in concentration of storage carbohydrates mannitol and laminarin (i.e. glucose) with seasonal variation were verified. Glucose was strongly affected by seasonal variation in all samples, followed by mannitol (Figure 8) (*PAPER II*). Mannitol always peaked before glucose due to the metabolic process where mannitol is produced first (Percival and McDowell, 1967).

In general, *L. digitata* accumulated more glucose with lower ash contents than *S. latissima*. For the two populations of the Kattegat (Bay of Aarhus) highest mannitol contents of approx. 19 % w/w_{DM} were found in May/June (Figure 8A/B). Glucose levels increased steeply from the beginning of spring until July. Against expectations of the seasonal trend, values dropped in August. For example the glucose content of *L. digitata* dropped drastically in August. Potentially, this could be a consequence of the sudden nutrient impulse. The total nitrogen in the seaweed rose from 1.4 % w/w dry matter in July to 2.2 % in August. Later on, the glucose concentration increased once again to 37 % w/w_{DM} in September 2013 (Figure 8B). In contrast, *L. digitata* collected from the North Sea had its maximum of mannitol (20 % w/w_{DM}) and glucose (54 % w/w_{DM}) in July and August (Figure 8C) (*PAPER II*).

Thus, the glucose yield from the virulent North Sea exceeded levels of the calm site of the Kattegat (Bay of Aarhus). At the peak for *L. digitata* (North Sea) the differences compared to the seaweed samples from the Kattegat were 17 % compared to *L. digitata*, respectively 31 % compared to *S. latissima* (compare Figure 8C to 8A/B). The cultivated seaweed *S. latissima* exhibited glucose levels not exceeding 7 % w/w_{DM} and mannitol reached its maximum in April (12 % w/w_{DM}). Biofouling was found to be most important influencing factor in low exposed sites (Buck and Buchholz, 2004). This has also been observed by another investigation for the Limfjorden (Marinho et al., 2015). Most likely seaweed cultivation to obtain high glucan levels is incompatible at shallow-sheltered locations, such as the Limfjorden (*PAPER II*).

Higher glucose level for *L. digitata* growing in open-sea rather than in an inlet was contradictive to the results of Black (1950). However, Black (1950) only determined the glucose extracted from laminarin. For *L. digitata* populated in the virulent North Sea extraordinary glucan contents were found for three sequential years during August. The glucose averages for the population's individuals presented in Figure 9 were of 55 % w/w_{DM} for 2012, 54 % w/w_{DM} for 2013 and in 2014 53 % w/w_{DM} (*PAPER II*).

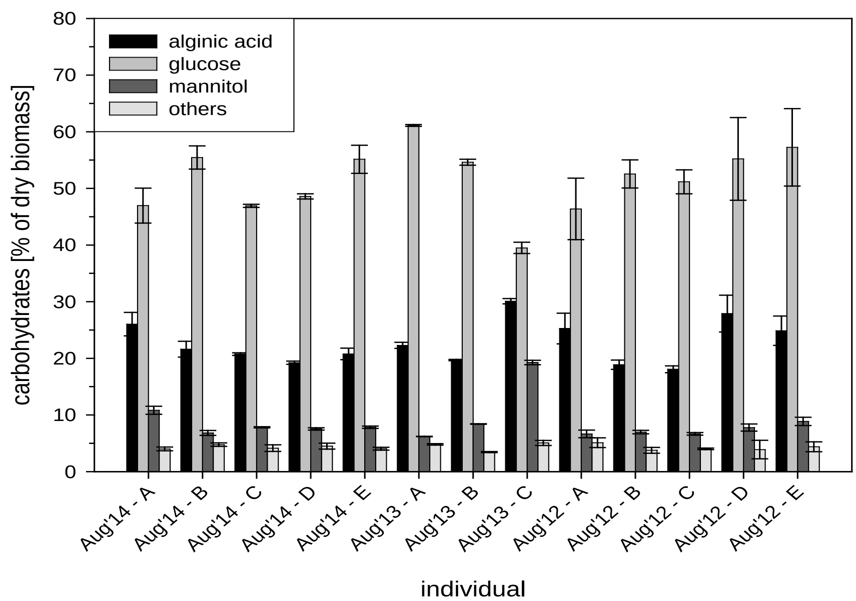

Figure 9: Variation of carbohydrate composition of seaweed individuals within the population of *L. digitata* from the Danish North Sea of August 2014, 2013 and 2012. Each data point represents average values of independent triplicates; error bars indicate the standard deviation. All values are given as hydrated monomers; alginic acid: sum of mannuronic acid and guluronic acid monomers; other: fucose, galactose, mannose, xylose and glucuronic acid.

L. digitata from sheltered shores investigated by Schiener et al. (2015) contained glucose values not exceeding 25-30 % w/w_{DM} between August 2010 and October 2011. However, mannitol reached higher values (up to 28 % w/w_{DM}) than *L. digitata* presented in *PAPER II* with max 20 % w/w_{DM} (Schiener et al., 2015).

Analytical carbohydrate analysis of the populations presented in Figure 8 was performed on three randomly pooled samples of seaweed individuals. However, variances occurred also within a population. Individuals of *L. digitata* from the North Sea showed significant differences not only between the seasons 2012 to 2014 but also from individual to individual of the same sampling date (Figure 9). For example in August 2012 the glucose varied within the five samples of individuals A-E from 46 to 57 % w/w_{DM} (Figure 9). Samples A, B and C from 2013 contained of 61.1, 54.6, 39.5 % w/w_{DM} glucose; 6.2, 8.4, 19.3 % w/w_{DM} mannitol and 22.3, 19.7, 30.1 % w/w_{DM} alginate. In August 2014 the difference was less severe but still significant and varied for example for glucose from 46.9 to 55.4 % w/w_{DM} or alginate from 19.2 to 26.0 % w/w_{DM} (Figure 9) (*PAPER II*).

2.3.2. Variation in the concentration of protein to nitrogen

Amino acids analysis and elemental analysis for nitrogen (N) of the investigated brown seaweeds in *PAPER I* revealed an average N-to-protein factor of 4.0. The application of this factor in *PAPER II* is presented in Table 4 (N-to-protein × 4) for the times of maximum and minimum concentrations of the tissue N concentration of the four seaweed populations. Consequently, highest protein concentrations were suggested for February of about 20 % w/w_{DM} for the brown seaweeds of the Kattegat and the cultivation in Limfjorden, respectively for March with 15 % w/w_{DM} for *L. digitata* in the North Sea. Oppositely, lowest potential protein amounts were calculated for the months of July and August from 2.3 up to 8.4 % w/w_{DM} (*PAPER II*).

However, amino acid analysis of these extreme points yielded in significant different protein concentrations (Table 4, total AA). Exemplary, *S. latissima* of the Kattegat was assumed to contain 21.4 % w/w_{DM} but found to have only 11.1 % w/w_{DM} (Table 4). In contrast, for the samples of *S. latissima* collected in July the N-to-protein factor of 4.0 underestimated the values found by amino acid analysis. *L. digitata* of the North Sea consisted of total amino acid concentrations of 12.5 % w/w_{DM} in March and 2.4 % w/w_{DM} in August. Here, conversion factors of 4.1 and 3.4 in Table 4 were in accordance with an N-to-protein factor of 4.0 (*PAPER II*).

Overall, the average conversion factor nitrogen to protein from Table 4 was calculated as 3.7 ± 1.3. The high variation was in accordance with conversion factors of 2.1 to 6.25 with average of 4.6 of a literature study of 459 brown seaweed samples (Angell et al., 2015). In seawaters the levels of inorganic nitrogen is seasonally affected and so is the concentration of dissolved inorganic nitrogen in the brown seaweed tissue. Hence, algal total nitrogen contents are known to be more variable compared to the protein content over season (Zimmerman and Kremer, 1986; Marinho et al., 2015). This revealed also the comparison of the $N_{non\text{-}protein}$ and the $N_{protein}$ data of the investigated brown seaweed samples in Table 4. Non-protein related nitrogen made up 2-4 % w/w_{DM} in the samples of early 2013, equally to more than 50 % of the total nitrogen (Table 4). Later in the year the $N_{non\text{-}protein}$ concentrations decreased strongly to values between 0.3-0.6 % w/w_{DM}. Thus, the reduction of the total $N_{biomass}$ in the summer samples was to a major part attributed to the decrease of $N_{non\text{-}protein}$. Therefore, also the conversion factor of 3.7 was affected by high standard deviation of ± 1.3 (*PAPER II*).

In general, season, species and sampling site appeared to influence the protein content and non-protein related nitrogen concentrations. Furthermore, the amino acid profile was dominated by aspartic and glutamic acid at the beginning of the year, and glutamic acid and alanine during the summer. Conclusively, an application of a common N-to-protein factor for determination of protein concentration of the brown seaweeds was not applicable. Instead, for quantification of the protein concentration amino acid analysis is recommended (*PAPER II*).

Table 4: Distribution of nitrogen, total protein after application of N-to-protein factor of 4, total protein after amino acid analysis (total AA) [all in % w/w_{DM}] and determination of actual of nitrogen-to-protein factors [-] of brown seaweeds from Danish Waters containing the highest and the lowest level of nitrogen within the period of sampling between 15. November 2012 and 1. December 2013 (for more details and the statistical analysis see *PAPER II*).

		S. latissima Kattegat Feb'13	*S. latissima* Kattegat Jul'13	*L. digitata* Kattegat Feb'13	*L. digitata* Kattegat Jul'13	*L. digitata* North Sea Mar'13	*L. digitata* North Sea Aug'13	*S. latissima* Cultivation Feb'13	*S. latissima* Cultivation Jul'13
$N_{biomass}$	[%]	5.3	1.0	4.6	1.4	3.7	0.6	4.7	2.4
N-to-protein × 4	[%]	21.4	4.1	18.3	5.4	14.8	2.3	18.6	9.6
$N_{non\text{-}protein}$	[%]	3.9	0.4[d]	2.9	0.6	2.1	0.3	3.2	0.5
$N_{protein}$	[%]	1.5	0.6	1.7	0.7	1.6	0.3	1.5	1.9
total AA	[%]	11.1	4.8	12.7	5.5	12.5	2.4	10.9	14.2
$N_{biomass}$-to-protein factor		2.1	4.7	2.8	4.0	3.4	4.1	2.3	5.9

3. BIOCATALYTICAL PROCESSING OF BROWN SEAWEED FOR GLUCOSE RELEASE

3.1. Perspective background

Glucose serves as feedstock for (biological) fermentation processes. Nowadays, traditional fermentation products, such as ethanol and lactic acid, still predominate the increasing market but modern biotechnology targets previously abandoned (e.g. butanol) and new innovative fermentation products (e.g. succinic acid, isoprene, glutamic acid, etc.) (Jong et al., 2012). Glucose for the fermentation products is currently predominantly derived from food crops such as corn and wheat, which has caused a controversial *'fuel or food discussion'* (Balat and Balat, 2009; Jong et al., 2012). Due to the abundance and sustainability of feedstocks, a lot of research efforts are placed on commercializing the production of biofuels from terrestrial non-food crops and agricultural and forestry residues. Marine plant biomass such as macroalgae and microalgae is considered as another source for biofuels (Jiang et al., 2016; Wei et al., 2013).

Glucose for 1st generation biofuel production originates from starch and sugar containing crops. Cellulose, the major carbohydrate in lignocellulosic biomass provides glucose for 2nd generation biofuels. Fermentation to e.g. ethanol is fundamentally based on glucose converting organisms with the most commonly used fermenter being *Saccharomyces cerevisiae* (Wei et al., 2013). Among macroalgae brown seaweeds are the most widely studied for ethanol fermentation using typically *S. cerevisiae* as yeast species (Jiang et al., 2016).

The MacroAlgaeBiorefinery (MAB3) aims to convert the carbohydrates of brown seaweed into energy carriers such as ethanol, butanol or methane. Glucan from brown seaweed can be enzymatically hydrolyzed to glucose (Adams et al., 2009; Kim et al., 2011b). However, higher yields were achieved using microbes possessing the additional ability to metabolize mannitol (Horn et al., 2000a/b; Kim et al., 2011b; Wang et al., 2013). Although, the glucose from laminarin and mannitol can be easily extracted (Kim et al., 2011b; Adams et al., 2009), the full potential of biofuel production from brown macroalgae has not yet been realized because industrial microbes do not have the capacity to metabolize the major component alginate (Takeda et al., 2011; Wargacki et al., 2012).

In addition to production of biorefinery feedstocks, the cultivation of macroalgae offers valuable bioremediation services, through absorption of nitrogen, phosphorus (limiting water eutrophication) and heavy metals (Nielsen et al., 2016). Biofuels are an interesting energy source but challenges in terms of the composition of the biomass and the resulting energy efficiencies has to be compensated to make the biofuel prices competitive in replacing fossil fuel (Balat and Balat, 2009). Since it is difficult to increase the yield of the single biorefinery, the overall system productivity can be improved producing high value added products (Jong et al., 2012; Wei et al., 2013).

Regardless of the final fermentation product, the degradation of glucan to glucose is a crucial prerequisite and was subject to *PAPER III* and *PAPER IV* within this PhD study. Here, the investigations of *PAPER I* and *PAPER II* outlined brown seaweed *L. digitata* harvested from the North Sea as a potential feedstock of high concentration of glucan. Following, the harvest of August 2012, consisting of 51 % w/w_{DM} glucan was subject to physical pretreatment and subsequent biocatalytical processing.

3.2. Milling pretreatment of glucan rich *L. digitata* (*PAPER III*)

Enzymatic hydrolysis of lignocellulosic feedstocks is inefficient without a preceding hydrothermal or other physicochemical biomass pre-treatment to expose the cellulose (Alvira et al., 2010; Kumar et al., 2009). A such harsh pre-treatment may not be required for enzymatic brown seaweed saccharification compared to lignocellulosic biomass as its soft, flat plant tissue does not contain recalcitrant lignin and few cellulose crystalline structures (John et al., 2011; Roesijadi et al., 2010).

A positive influence of particle size reduction (i.e. milling) on enzymatic biomass deconstruction has been observed for both cellulose and various types of lignocellulosic biomass (Silva et al., 2012; Yeh et al., 2010). The substrate particle diminution increases the accessible surface area for enzymatic attack as well as a shortening of the entry and exit paths for the enzymes and the hydrolysis (Pedersen and Meyer, 2009).

A comparison of five pre-treatment technologies for processing of the green seaweed *Chaetomorpha linum* for ethanol production showed that a ball milling pretreatment producing particles of 2 mm was superior to classical lignocellulosic pretreatments such as hydrothermal pretreatment or steam explosion (Schultz-Jensen et al., 2013). In fact, some of the classical pretreatments employed for lignocellulose induced significant losses of convertible seaweed biomass (Schultz-Jensen et al., 2013).

Mechanical grinding has also been shown to enhance ethanol yields on *S. latissima* biomass (Adams et al., 2009). Particle size reduction of seaweed biomass by milling has been envisaged to increase the substrate surface area which in turn would enhance the enzymatic processing and fermentation to ethanol (Roesijadi, et al., 2010; Wargacki et al., 2012).

Mechanical size reduction was hypothesized to be sufficient for opening the complex structure of brown seaweed for further enzymatic processing i.e. the release of glucose. The significance of milling concerning substrate particle size diminution for increasing the enzymatic saccharification of glucan rich *Laminaria digitata* biomass was assessed within *PAPER III*.

3.2.1. Particle size reduction

Washing is the first step of seaweed processing to remove foreign objects and debris such as stones, sand, snails, or other litter that may be caught in the biomass (Roesijadi et al., 2010). Hence, prior to processing the *L. digitata* material was washed successively four times with water. The washing removed residual sand and lowered the ash content from 11 to 7 % w/w_{DM} (*PAPER I; III*).

The washed seaweed was subjected to wet refiner milling, using rotating disc distances of 0.1-2 mm. The seaweed material consisted of elongated flat blades with an average thickness about 1 mm. Repeated milling with disc distance above 1.0 mm produced only a two-dimensional disruption of *L. digitata* samples after the refiner milling. A three dimensional disruption due to milling and therefore a significant increase in available surface area of this flat material can only occur via milling or refining at disc distances below the thickness of the seaweed (see *L. digitata* after refiner treatments with distances ≥1.0 mm in Figure 10A/B/C and with distances <1.0 mm in Figure 10D/E/F) (*PAPER III*).

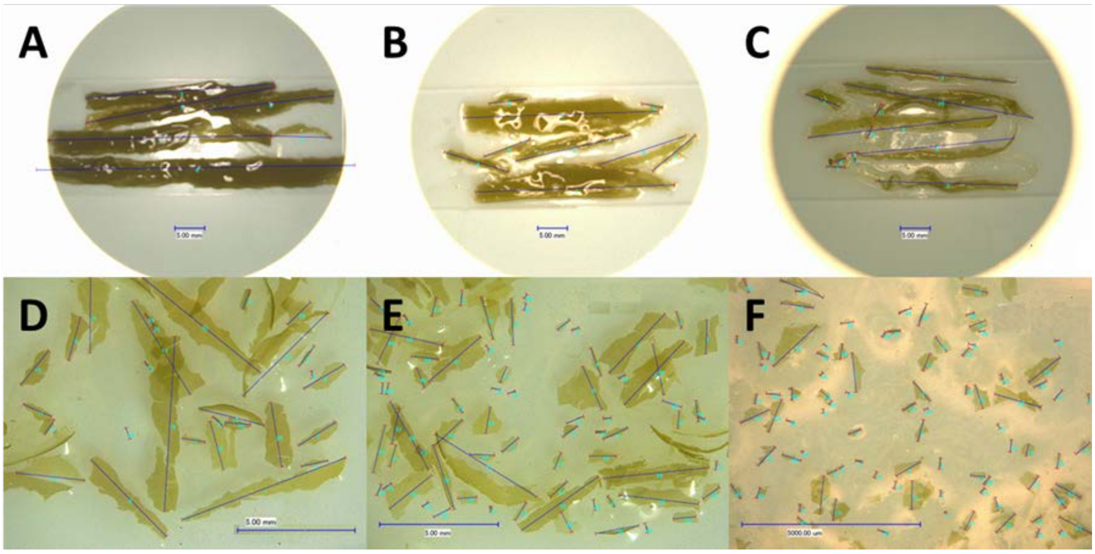

Figure 10: Wet *Laminaria digitata* after refiner milling at disc distances of 2.0 (**A**), 1.5 (**B**), 1.0 (**C**) mm and of 0.6 (**D**), 0.3 (**E**) and 0.1 (**F**) mm. The reference bar in the pictures corresponds to 5.0 mm. Pictures was recorded on a KEYENCE digital microscope (VHX-500FD). Remark: The integrated software was used for evaluation of the surface area of milled particles (*PAPER III*).

Refiner milling with disc distances between 0.1 and 2.0 mm generated differently sized particle fractions with surface areas from 0.1-100 mm^2. The tightest distances produced the smallest surface areas. Furthermore, a large span of particle sizes was obtained within each type of milling severity. The obtained mean particle surface area was thus strongly affected by the bigger particles and was always above the median of 50 % of the particles. The data imply that even though the smaller particles outnumbered the larger ones, the fewer bigger particles dominated the surface area of the particle fraction (Figure 10) (*PAPER III*).

The larger disc distances of 2.0 and 1.5 mm did in particular produce some particles which had large particle size areas with mean particle sizes of 60 and 34 mm^2. Millings at disc distances of 1.0, 0.6, and 0.3 mm, i.e. at disc distances lower or equal than the thickness of the algae blades, produced particles with mean sizes of 7.5, 1.9, and 0.6 mm^2, respectively. At the very tightest distances of 0.2 and 0.1 mm more than 75 % of the particles were below 0.25 mm^2 averaging 0.2 and 0.1 mm^2, respectively (*PAPER III*).

Figure 10 emphasized that refiner milling merely cut the seaweed blades into smaller pieces, and thus that the resulting available surface area may have been much less than what occurs from three-dimensional fibrillation on lignocellulosic materials. In order to achieve a better understanding of the correlation between refiner milling degree, true biomass material disruption, and resulting surface area, an investigation of the viscosity response to the milled *L. digitata* refiner slurries was conducted (Figure 11). In general, the viscosity response to particle size diminution of suspensions of homogenous solid particles is mainly influenced by the so-called particle volume fraction, which is correlated to the particle size; in other words, the viscosity increases with particle size reduction because the particle volume increases (Mueller et al., 2009).

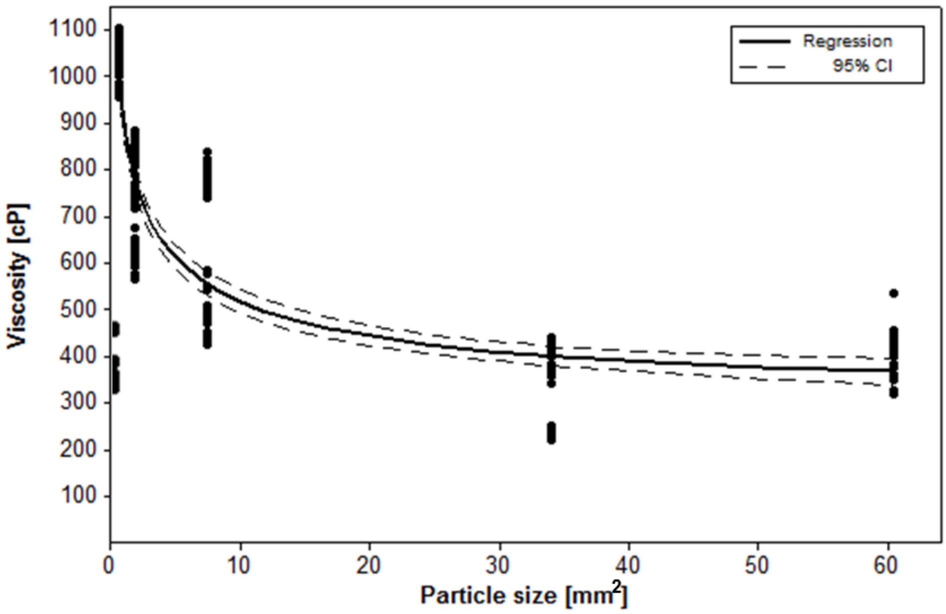

Figure 11: Fitted plot of received viscosities at shear rate of 150 rpm of the 7.5 % w/w_{DM} refiner slurries (●) milled at disc distances of 0.1, 0.2, 0.3, 0.6, 1.0, 1.5 and 2.0 mm over mean particle sizes (polynomial regression analysis with confidential interval (CI) of 95 % excluding the data for 0.1 and 0.2 mm disc distances).

The viscosity response to particle size after milling with disc distances from 2.0 mm down to 0.3 mm followed a steep polynomial function (Figure 11).For the milling slurries with disc distances above the blade thickness (particles sizes of 60 and 34 mm^2) relatively low slurry viscosities averaging approximately 400 cP were achieved (Figure 11). At a disc distance of 1.0 mm, i.e. equal to the seaweed thickness, the average viscosity of the particle volume was of 640 cP. Further on, the viscosity increased at low particle size (1.9, and 0.64 mm^2, Figure 11) and reached 800–1050 cP, highest with tightest disc distance of 0.3 mm (Figure 11). This was in accord with the solid particle volume fraction theory indicating an increased available surface area. The drop in the slurry viscosity to ~320–480 cP with the smallest refiner disc distances, i.e. at intensified milling (Figure 11), was most likely be caused by agglomeration. The high content of minerals in the brown seaweed might have caused the agglomeration between small particles due to ionic exchanges and increased the proportion of the solid fraction of the milling slurry (Figure 12).

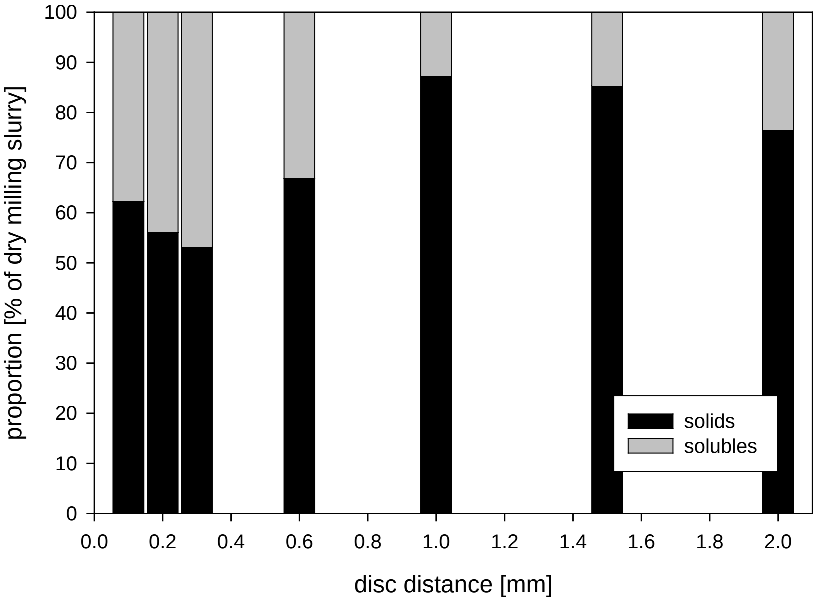

Figure 12: Relative proportions [% w/w_{DM}] of the solid and solubilized material of the received milling slurries fractions over increasing severities (i.e. decreasing disc distances from 2.0 mm to 0.1 mm). Prior freeze drying the solubilized fraction was separated by decanting from the solids after centrifugation for 30 min at 5000×g.

The received slurries after milling of seaweed biomass were separated into a solid and a liquid fraction (Figure 12). While 77 to 87 % w/w_{DM} of the total slurries for refiner millings with disc distances above 0.6 mm remained solid, 47 % w/w_{DM} of the seaweed biomass was solubilized by refiner milling at disc distance of 0.3 mm. The three-dimensional disruption at disc distances ≤ 1 mm changed the morphology of the seaweed and more carbohydrates were released from the plant tissue into the liquid fraction of the milling slurry (Figure 12). Notably, the separation of the fraction after milling with distances 1.5 and 2.0 mm did not lead into a two-phase system with a pellet and a supernatant. Instead an interface occurred where “algae-particles” interacted with the liquid face and enhanced the liquid fraction artificially with particles from the solid material.

Figure 13 displays the carbohydrates containing in their derived solid (Figure 13A) and liquid (Figure 13B) fractions. The liquid fractions consisted almost exclusively of glucose and mannitol. The reserve carbohydrates laminarin (i.e. glucose) and mannitol are stored in the lumina of the plant tissue of brown seaweed (Michel et al. 2010b). Milling most likely liberated these carbohydrates into the liquid fraction, with a harsher milling in greater solubilization. At refiner disc distances below 0.6 mm quantities of mannitol and glucose found the liquid equaled or exceeded those found in the solid fraction (Figure 13).

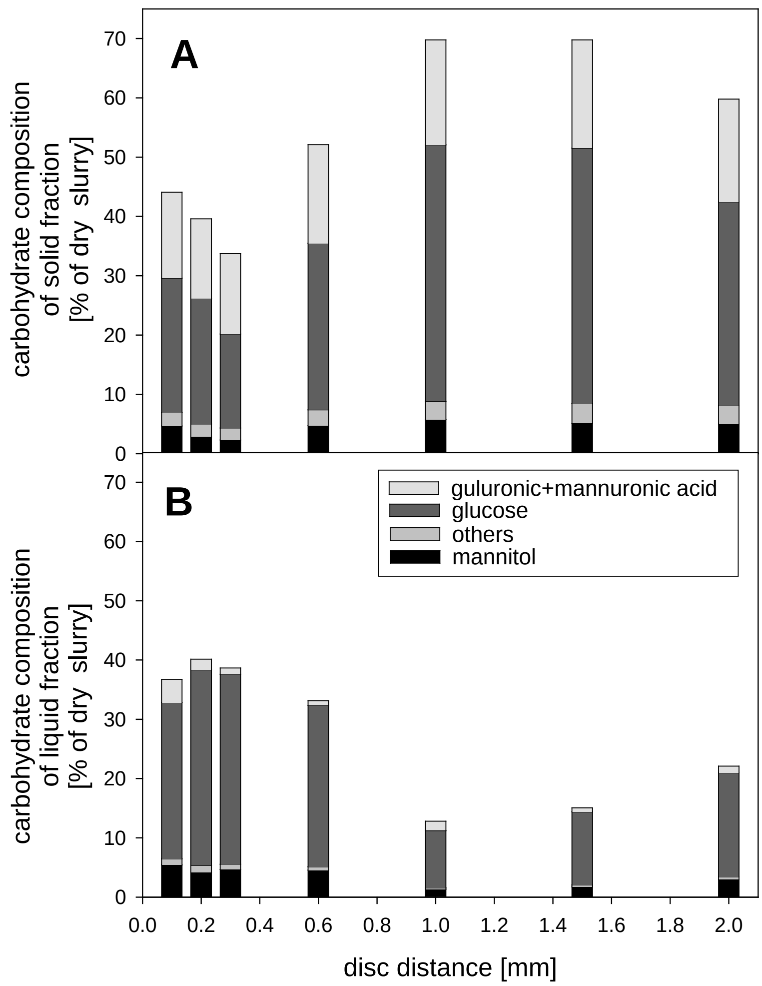

Figure 13: Carbohydrate compositions of milling slurry fractions [% w/w_{DM} of the total milling slurry] over increasing severities (i.e. decreasing disc distances from 2.0 mm to 0.1 mm), (**A**) solid fraction and (**B**) liquid fraction. All values are given as hydrated monomers determined by HPAEC analysis post sulfuric acid treatment (*PAPER III*); other carbohydrates: fucose, galactose, mannose, xylose and glucuronic acid.

Alginate (i.e. guluronic and mannuronic acids) and the other carbohydrates (i.e. fucoidan related carbohydrates) were increasingly present in the liquid fraction of the harsher millings, especially with disc distances below 0.3 mm (Figure 13B). However, levels of alginic acid of both liquid and solid fractions did not correspond to the values expected from the raw material, hereby underlining formerly gained experiences on the difficulty of detection of alginic acid monomers in general (see section 2.2.1. p. 13ff).

In conclusion, wet refiner milling with different, controlled rotating disc distances of 0.1-2 mm, was found to generate heterogeneous particles of decreasing sizes (100-0.1 mm^2) over increasing milling degree. Furthermore, viscosity measurements emphasized that milling with disc distances below the thickness of the algae increased the particle volume. Moreover, higher milling degree caused increased spontaneous carbohydrate solubilization of particularly glucose and mannitol in the milled seaweed slurries. Allover, smaller particles, increased particle volume and solubilization of laminarin indicated an enhanced availability to the seaweed glucan for the further investigated release of glucose.

3.2.2. Effect of milling on the enzymatic glucose release

Ravanal et al. (2016) applied different pretreatments on cut and dried (cutoff sieve up to 3.5 mm) brown algae *Macrocystis pyrifera* containing of 8.2 % w/w_{DM} glucose. The effect of the pretreatments was evaluated by the enzymatic release of glucose using a cellulase preparation for 4 hours and uronic acid using alginate lyases for 1 hour, respectively. No further treatment beside cutting and drying led to a glucose release of >10 % of the potential glucose but sulfuric acid pretreatment enabled the cellulase preparation to release 68 % w/w_{DM} of the potential glucose after 4 hours (Ravanal et al., 2016). Similarly, Kim et al. (2011b) found highest sugar release by cellulases preparation after hydrochloric acid pretreatment.

Throughout the literature particle size diminution, mostly in combination with drying, was applied to brown seaweed as prerequisite prior further chemical pretreatments. Milling has been applied previously on brown seaweeds such as *Saccharina latissima* after cutting the blades into smaller pieces of ~5 cm^2 (Adams et al., 2009, 2011), *Laminaria hyperborea* milled to pass a 7 mm sieve (Horn et al., 2000a/b), *Laminaria japonica* and *Sargassum fulvellum* to particles <0.5 cm (Kim et al., 2011b), and e.g. *Alaria crassifolia* to particles of <0.5 mm (Yanagisawa et al., 2011) employing different types of milling technologies from blending to ultra-centrifugal milling. However, all these investigations did not investigate the influence of the pretreatment on the subsequent enzymatic carbohydrate release exclusively by milling treatment.

The refiner milling pretreated slurries of the glucan rich *L. digitata* was enzymatically treated with a mixture of 2 % w/w_{DM} alginate lyase (Sigma-Aldrich) and the cellulase preparation Cellic®CTec2 (Novozymes) of 10 % v/w_{DM}. The reduction of the particle size after refiner milling did not improve the enzymatic decomposition of *L. digitata* biomass (Figure 14). Furthermore, no positive effect of substrate milling on glucose yields compared to the non-milled starting material was observed since all available glucose was enzymatically released after 24 hours (1440 min) both with and even without milling pretreatment (Figure 14) (*PAPER III*).

However, intense milling facilitated the release of free glucose monomers, resulting in detection of glucose monomers before the initiation of the enzymatic treatment of up to 6.4 % w/w_{DM} (timepoint zero for milling with 0.1 mm disc gap; Figure 14). In contrast, the glucose monomers in the non-milled sample and in the samples milled at higher disc gap were released only during the enzymatic treatment (Figure 14). Autumn harvested brown seaweed was reported to contain free glucose monomers (Østgaard et al., 1993). As a consequence, the milling induced liberation of free glucose monomers from the raw material thus affected the release rate (*PAPER III*).

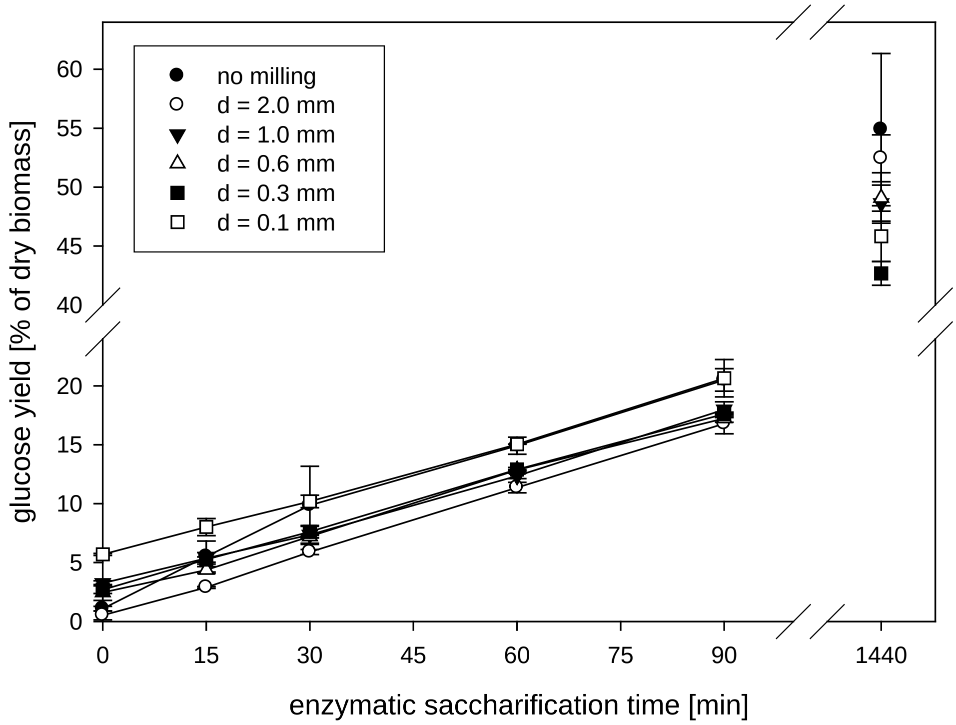

Figure 14: Glucose yields of refiner milled wet *L. digitata* slurries with different milling degrees (d = disc distance) and non-milled *L. digitata* over enzymatic saccharification time. Each data point represents the average value of independent duplicates hydrated monomer; vertical bars indicate the standard deviation (for statistical analysis see supplementary material in *PAPER III*).

During the combined treatment of alginate lyase and cellulase it is presumed that the alginate lyase activity catalyzes the cleavage of alginate. Consequently, the viscosity decreased and the cell wall matrix (Figure 2, p. 12) decomposed (Figure 15) which improved the access for the cellulases to attack the glucan of the brown seaweed tissue (*PAPER III*, *IV*). This perception of the alginate lyase action is in accordance with the described embedding matrix and an inner cell wall skeleton of brown seaweed where cellulose is part of the cell wall the laminarin stored in the sieve elements (Michel et al., 2010a/b; Deniaud-Bouët et al., 2014).

Figure 15: Pretreated (wet refiner milling at disc distance 2.0 mm) *Laminaria digitata*. Top: before enzymatic treatment. Bottom: after 90 min of enzymatic treatment with the cellulase preparation Cellic®CTec2 (Novozymes) and alginate lyase (Sigma Aldrich).

The catalysis of the enzyme cocktail apparently induced selective removal of alginate promoting enzymatic glucan saccharification. This is also evident from Figure 15 where the plant tissue of milled *L. digitata* at the lowest milling severity was attacked and solubilized directly by the enzymes at the substrate surface. Hence, a harsher milling for a further increase of the surface area was not required. Conclusively, the investigations concerning the effect of refiner milling showed that, compared to non-milled brown seaweed samples, no increased glucose yields after combined cellulase and alginate lyase treatment could be detected. Thus, *"Brown seaweed processing: Enzymatic saccharification of Laminaria digitata requires no pre-treatment"* (*PAPER III*).

3.3. Enzymatic decomposition of glucan rich *L. digitata* (*PAPER III* / *PAPER IV*)

Glucose from brown seaweed can be released using cellulase preparations. For example, Yanagisawa et al. (2011) treated brown seaweed *Alaria crassifolia* with a commercial cellulase preparation derived from *Trichoderma viride* for 120 hours and released 82.3 % of the potential glucose. A mixture of commercial Celluclast 1.5L and Viscozyme L (β-glucanase and endo-glucanase activity) performed best on *Laminaria japonica* releasing 72.4 % of sugars of the theoretical yield (Kim et al., 2011b). Similarly, glucose was released from *S. japonica* after 48 h treatment with cellulase together with cellobiase (Ge et al., 2011), or cellulase Celluclast 1.5L plus ß-glucosidase Novozym 188 (Lee et al., 2013), by Celluclast from *M. pyrifera* (Ravanal et al., 2016), or from *Sargassum* spp. using cellulases for 100 h (Borines et al., 2013).

Laminarinases (active only on β-1,3 glucan) for hydrolysis of *L. digitata* (Adams et al., 2011), or the industrial enzyme Termamyl 120 L plus isolated from *Bacillus* sp. JS-1 on *Alaria crassifolia* have also been investigated (Jang et al., 2011). In all the previous mentioned investigations, seaweed biomass was subjected to physical and/or chemical pretreated seaweed biomass prior the enzymatic treatment. None-the-less, none of the subsequent saccharifications of brown seaweeds were able to release all potential glucose.

PAPPER III indicated that alginate lyases are a beneficial tool for the biocatalytical processing of brown seaweed. Moreover, without any physical pretreatment all potentially available glucose was releasable by the commercial cellulase preparation Cellic®CTec2. Primarily, the saccharification by the enzymatic treatment aimed to release the glucose by cellulases. Therefore, the enzymatic treatment conditions with pH 5 and 40 °C were chosen, targeting conversion of the glucan rather than the alginate of the seaweed (*PAPPER III*).

Besides the evaluation of the milling effect on the enzymatic glucose release another aim of *PAPER III* was to develop an optimal enzymatic saccharification treatment. The investigations within *PAPER IV* continued the study, with particular focus on the usage of alginate lyases. Hence, for the study of *PAPER IV* two additional endolytic alginate lyases from two different microorganisms were selected for further investigations along with the purchased lyase (also investigated in *PAPER III*) to assist the overall aim to achieve maximal glucose release from the glucan rich brown seaweed *Laminaria digitata*.

3.3.1. Alginate lyases (*PAPER IV*)

Most of the alginate lyases are classified into two polysaccharide lyase (PL) families, PL-5 and -7 with preferred activity to depolymerize the MM-blocks or the GG-block of alginates. According to the enzyme nomenclature alginate lyases are classified within the EC number EC4.2.2.- where specifity towards G-blocks (poly-guluronate lyase) is announced in the classification as EC4.2.2.11 and specifity towards M-blocks (poly-mannuronate lyase) as EC4.2.2.3 (Zhu and Yin, 2015, Kim et al., 2011a). Although a lyase is classified as either M or G specific, it usually degrades alternating blocks as well and has in addition some residual activity towards the other homopolymer. Additionally, lyases with high activity on both homopolymers have been isolated from various sources (Wong et al., 2000).

Miyake et al. (2004) isolated three endotype alginate lyases (A1-I, A1-II [family PL-7], and A1-III [family PL-5]) and additionally a transformant of A1-II (A1-II’) from the bacterium strain *Sphingomonas* sp. A1.

Endotype alginate lyases catalyze alginate degradation via a β-elimination reaction, i.e. catalyzing bond cleavage within the alginate backbone chain (Figure 16). The reaction produces unsaturated oligoalginates at the reducing end, which are UV-visible due to the formation of double bonds (Wong et al., 2000). Furthermore, another type of lyase (A1-IV) exists in the bacterium (Miyake et al., 2003). Unsaturated monosaccharides, such as products from exolytic alginate lyase A1-IV, convert non-enzymatically to the stable 5-keto structure (Figure 16) and are not UV-visible (Wong et al., 2000; Miyake et al., 2003).

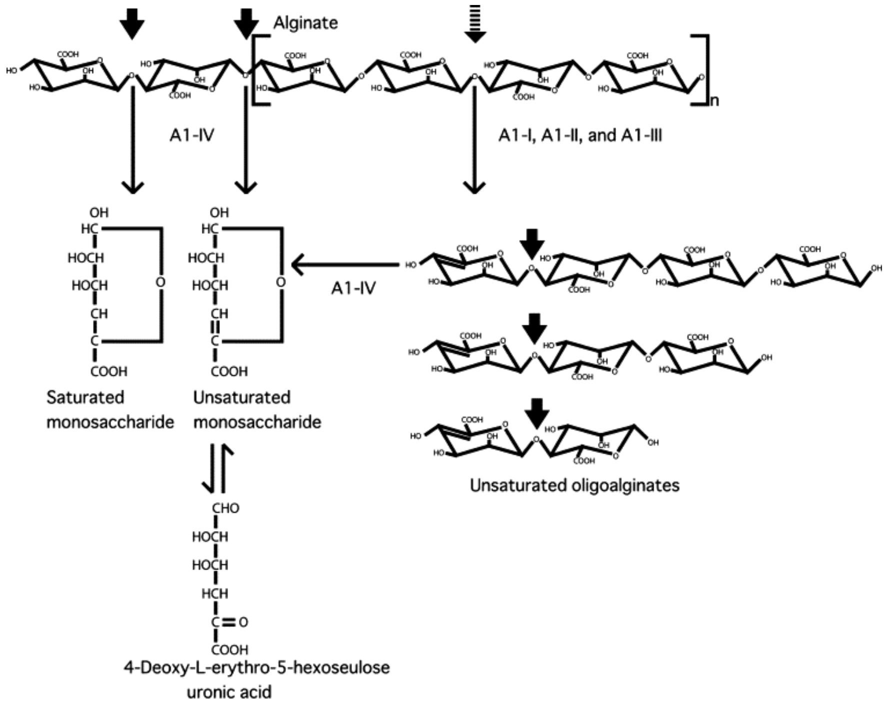

Figure 16: Alginate depolymerization process. In the displayed example, alginate is depolymerized to 4-deoxy-L-erythro-5-hexoseulose uronic acid through the consecutive reactions of four alginate lyases. The dotted arrow indicate the cleavage site by endolyases (A1-I, -II, and -III) and the thick arrows by the exolyase (A1-IV). The alginate depolymerization pathway is shown by the thin, elongated arrows (Miyake et al., 2003).

The endolytic alginate lyases were reported to have higher activity than the exolytic lyase (Miyake et al., 2003; 2004) and in particular A1-II’ was described having high activity on alginate (Ogura et al., 2008; Yamasaki et al., 2005). Recently, another alginate lyase was discovered from *Flavobacterium* sp. and particularly proposed as a pretreatment for production of biofuels (Huang et al., 2013).

Two bacterial alginate lyases (PL-7 family, EC 4.2.2.-) from *Sphingomonas* sp. (SALy) and *Flavobacterium* sp. (FALy), respectively, were selected for heterologous, monocomponent expression in *Escherichia coli*. Together with the purchased alginate lyase from Sigma Aldrich (SigmALy) the optimal pH and temperature were determined on commercially available sodium alginate using a statistical design. The enzyme activity was determined as the increase in absorbance due to the lyase induced β-elimination reaction (Figure 16, p. 37). The investigated reaction temperatures of 30-50 °C had no influence on the activity, suitable pH ranges were determined to be:

- SALy, pH 5.5-7.0 with optimum at pH 6.5
- FALy, pH 6.5-8.0 with optimum at pH 7.5, and
- SigmALy, pH 6.5-8.0 with optimum at pH 7.5 (*PAPER IV*).

Originally, optimal pH was 8.5 for FALy (optimal temperature: 40-45 °C) with 80 % relative activity at pH 8 or 9.5, respectively (Huang et al., 2013). 40 % remained at pH 7.5 (optimum in *PAPER IV*) and only 10 % at pH 6 while in house investigations revealed about 1/3 of its max activity at pH 6 and thermostability until 50 °C (*PAPER IV*). The purchased lyase SigmALy possessed about 50 % of the maximum activity at pH 6 but was not thermostable above 40 °C. However, the lower protein loading of FALy (30 % of that of SigmALy) indicated a general high activity of FALy (*PAPER IV*). In contrast to SigmALy and FALy, SALy was active at lower pH between pH 5.0-7.5 and thermostable until 50 °C (*PAPER IV*). Contradictory, Miyake et al. (2004) reported for the same enzyme highest activity at pH 7.5 and a temperature optimum of 40 °C including a fast decrease in activity above 45 °C.

The alginate degradation for the evaluation of the temperature and pH optima was measured as the increase of absorbance at 235 nm after 4 hours of reaction. However, in any case 80 % of the final absorbance was reached between 125 min and 165 min at the optimum pH range. This is in accordance with Ryu and Lee (2011), who reported similar reaction patterns (leveling off) for alginate degradation with endolytically active alginate lyases within the first 1-3 hours of reaction.

Figure 17 displays the activity of the lyases on different alginate substrates. There was no substrate specifity for the lyases SALy (Figure 17A) and FALy (Figure 17B) but SigmALy (Figure 17C) was strictly active on poly-guluronic acid (poly-(G)). The initial cleavage of SigmALy on poly-mannuronic acid (poly-(M)) was most likely due to impurity of the substrate with guluronic acid residues. The FALy preferred poly-(M) as substrate, but also exhibited activity on poly-(G) (Figure 17B). According to the initial rates of FALy on the alginate substrates the activity was almost double towards poly-(M) than poly-(G). The rates were for poly-(M): 0.111 ΔA_{235}/min and 0.102 ΔA_{235}/min; and for poly-(G): 0.058 ΔA_{235}/min) (*PAPER IV*). This was in contrast to Huang et al. (2013), who reported a preference of the enzyme FALy for poly-(G).

SALy had higher activity on poly-(G) than poly-(M) (Figure 17A), confirming earlier results (Yamasaki et al., 2005; Ogura et al., 2008). However, the specifity was not strongly pronounced since the degradation yield of poly-(M) was 63 % of the yield of SALy on poly-(G) (*PAPER IV*). This was higher than the earlier finding of only 20 % (Yamasaki et al., 2005). Differences in the specific activity between studies agree with another investigation where SALy was similarly active towards mannuronic and guluronic acid (Miyake et al., 2004). After 30 min of reaction, i.e. the initial reaction, SALy created per release of unsaturated M-unit (>5000 kDa) 1.5 unsaturated G-units while FALy 0.8 units of unsaturated G-blocks (Figure 17).

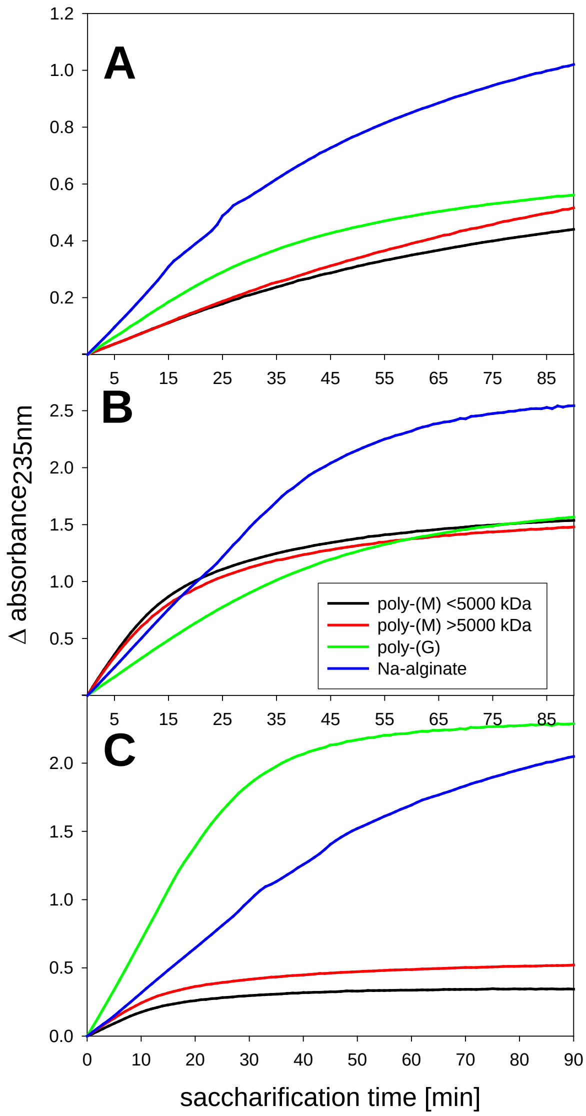

Figure 17: Substrate specifity of the alginate lyases (**A**) SALy, (**B**) FALy (**C**) and the purchased lyase SigmALy on poly-(M) < 5000 kDa, poly-(M) > 5000 kDa, poly-(G) and sodium alginate at pH 7 and 40 °C; activity recorded as Δ absorbance$_{\lambda=235\ nm}$ over 90 min of reaction. Enzyme dosages on the alginate substrates were 0.1 % w/w$_{DM}$ for SALy and SigmALy, respectively 0.03 % w/w$_{DM}$ for FALy.

Overall, taking into account that FALy had the lowest enzyme to substrate ratio (E/S) of 0.03 % w/w$_{DM}$ this enzyme had by far the highest decomposition ability towards alginates (figure 17). Whereas the enzyme dosages for SALy and SigmALy were set to 0.1 % w/w$_{DM}$ SALy performed with the lowest activity. Furthermore, the alginate constitution of *L. digitata* with M/G ratio of 3:1 (*PAPER I*) suggested higher degradation capability for lyases with poly-(M) preference. However, pH range of 5.5-7.0 emphasized SALy as an appropriate candidate to combine with fungi derived cellulase to release glucose from brown seaweeds. Conclusively, even though the investigations of *PAPER III* proved that SigmALy supports sufficient the glucose release from *L. digitata*, SALy and FALy were assumed to perform even better.

3.3.2. Alginate degradation versus glucose release (*PAPER IV*)

High viscosity, attributed to alginate, was pointed out to decrease the accessibility of glucan from *L. digitata* for enzymatic hydrolysis, to affect the enzymatic activity and thus reduce glucose recovery significantly (Hou et al., 2015). Therefore, the milling slurry with the highest viscosity was selected to assess the liquefaction of brown seaweed during the combined cellulase-lyase treatment of the glucan rich *L. digitata* (Figure 18). Furthermore, the induced viscosity reduction was compared to the alginate degradation by each of the three different alginate lyases with focus on each particular impact on the enzymatic saccharification of the glucan (the optimal glucose release is discussed in section 3.3.3.). The viscosity of the refiner milling slurry with disc distance 0.3 mm had a viscosity of 1050 cP, measured in water at substrate concentration of 7.5 % (Figure 11 in section 3.2.1, p. 30). In the presence of phosphate-citric buffer system at lower concentration of 5 % viscosity was 700 cP lower at around 450 cP (control, Figure 18).

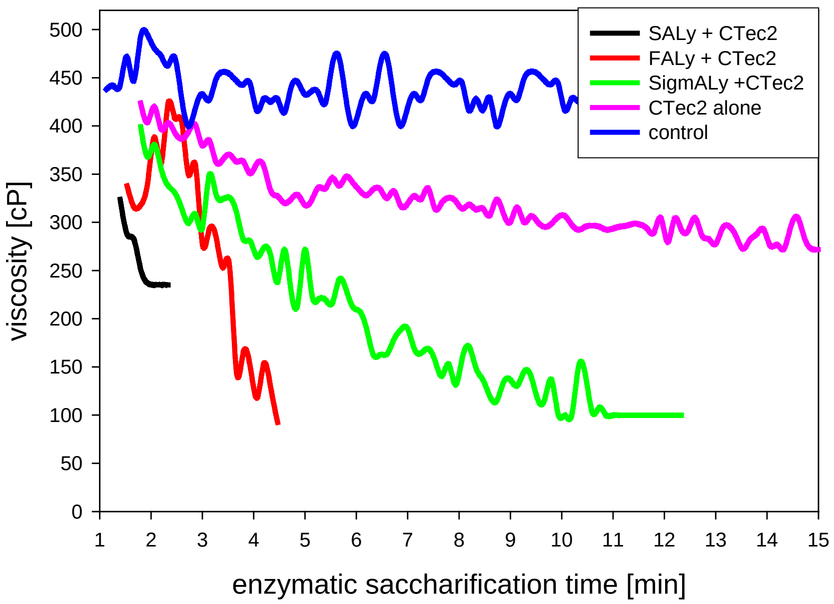

Figure 18: Evolution of received viscosities at shear rate of 60 rpm from the viscosimeter RVA over 15 min of enzymatic treatment at pH 6 and 40 °C with Cellic®CTec2 and alginate lyase (SALy, FALy and purchased lyase SigmALy); Cellic®CTec2 alone; and without any enzyme addition (control).

With application of alginate lyases the viscosity deduction occurred primarily in the first minutes of the reaction (Figure 18). This viscosity drop indicated the endo-type action of the ALys and was in agreement with previous data achieved on alginate lyases (Iwamoto et al., 2001; Inoue et al., 2014). The addition of the SALy to the cellulase preparation showed the fastest, and the addition of SigmALy the slowest viscosity reduction (Figure 18). However, the endolytical action of all alginate lyases decreased the viscosity quickly in the early phase of reaction (Figure 18). While the formation of unsaturated uronic acids (UA) still increased as the reaction proceeded (Figure 19B) but was not crucial for the measurement of the viscosity.

Regarding the enzyme catalyzed release of glucose, the data did not unequivocally reveal whether the initial viscosity decrease effected the initial glucose release rate. However, it was emphasized that the alginate lyases were moreso required to decompose the cell wall in order to guarantee access for the cellulase to the glucan (*PAPER IV*).

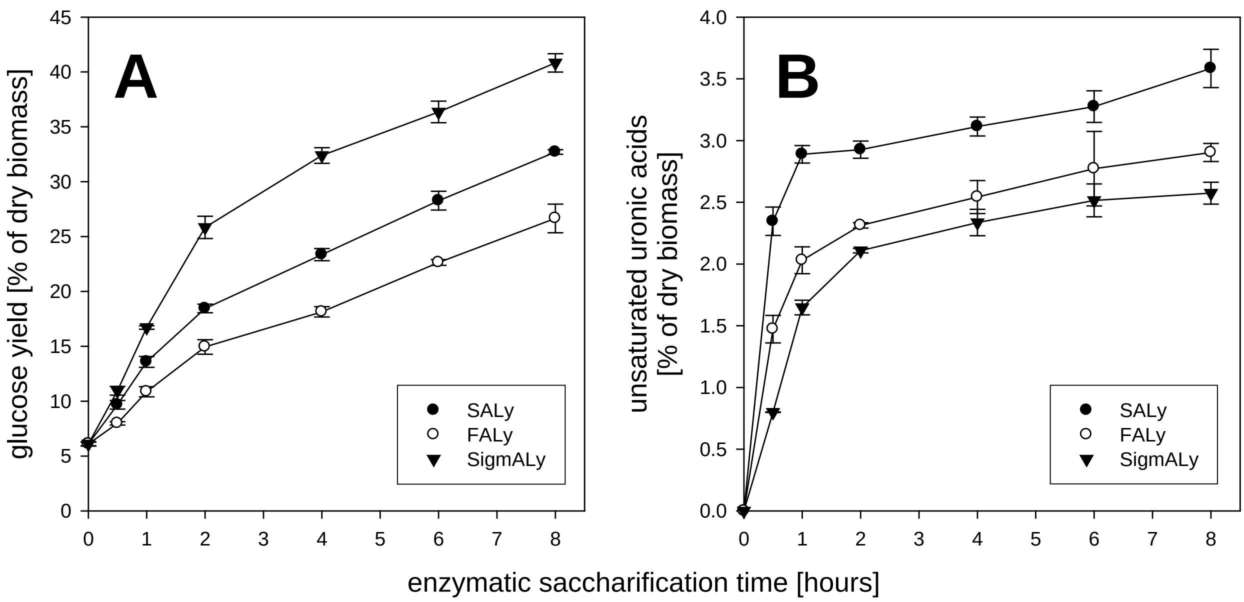

Figure 19: Yields over enzymatic saccharification time (0-8 h) of (**A**) glucose and (**B**) alginate degradation products after refiner milling of wet *L. digitata* (d = 0.3 mm). Enzymatic saccharification at pH 6 and 40 °C with Cellic®CTec2 concentration on substrate of 10 % v/w_{DM} and 1 % w/w_{DM} alginate lyase (SALy, FALy and SigmALy). Each data point represents the average value of independent duplicates; vertical bars indicate the standard deviation. All values are given as hydrated monomers.

The received milling slurry of *L. digitata* after refiner milling at disc distance 0.3 mm consisted of 46.6 % dry weight hydrated glucose. Subsequently, the seaweed was enzymatically treated with the cellulase preparation Cellic®CTec2 together with one of each characterized alginate lyase (Figure 19). A glucose yield of 40.8 % of dry weight milled seaweed supported by SigmALy corresponded to 87.6 % of the potential available glucose after 8 hours of treatment (Figure 19A). In comparison to SigmALy, SALy and FALy were expected to enhance the glucose release by the cellulase due to (a) the absence of substrate specifity (SALy and FALy), (b) the much higher activity (FALy) and (c) the more suitable pH working range for combined cellulase-lyase application at pH 6 (SALy). Surprisingly, for SALy and FALy the release of glucose was significantly lower (Figure 19A). After 8 hours FALy released 26.7 % w/w_{DM} and SALy 32.7 % w/w_{DM} of glucose from total seaweed by dry weight (Figure 19A). This corresponded to 65 %, respectively 80 % compared to what has been released by the cellulase preparation applied together with SigmALy (*PAPER IV*).

For saccharification of the alginate embedded in the *L. digitata* cell wall the first two hours of reaction were crucial. This was already described in section 3.3.1 for the different pure alginate substrates. Figure 19B displays the amount of unsaturated UA released by the different alginate lyases due to the degradation alginate in the seaweed. The cleavage mechanism of the lysases releases oligoalginates of undefined DP with an unsaturated acid at the reducing end (Figure 16, p. 37). The yield of unsaturated UA residues was of 2-3 % w/w_{DM} after 2 hours and only slightly higher (2.6 to 3.6 % w/w_{DM} unsaturated UA) after 8 hours (Figure 19B). Likewise, Thomas et al. (2013) reported an intermediate initial degradation to larger oligosaccharides with a degree of polymerization (DP) of 4 to 20. Over the following 12 h the lyases split the oligoalginates further to DP 2. Moreover, Figure 19B showed that the initial fast degradation of the alginate was achievable already within one hour by the use of SALy (2.9 % w/w_{DM} unsaturated UA). Potentially, this was a result of higher enzyme activity due to the more suitable pH conditions. The combined cellulase-lyase treatment was performed at pH 6 and the optimum for SALy was pH 6.5, whereas FALy and SigmALy had highest activity at pH 7.5 (*PAPER IV*).

The M/G ratio in the present *L. digitata* was 3:1 with a total amount of guluronic acid of 5.7 % w/w (*PAPER I*). Regardless of the mannuronic acid content, an efficient disruption of alginate requires a lyase with high activity on G-G linkages to break down the stiff “egg-boxes” (Figure 4 in section 2.2.1., p. 15) of the GG-blocks (Formo et al., 2014). The UV measurement of the amount of unsaturated UA (Figure 19) did not allow to distinguish between the types of cleavage. However, the relative ratio of G-G(M) cleavages to M-M(G) cleavages (G:M cleavage ratio) can be calculated based on the initial rates derived from the lyase activity on the pure poly-(M) and pure poly-(G) substrates. The G:M cleavage ratio for SALy was 1.5:1 and for FALy 0.6:1 whereas SigmALy was not active on mannuronic acid (Figure 17 in section 3.3.1., p. 39). Notably, alginates were reported to contain a smaller amount with much shorter DP of MG/GM-blocks than the homologous GG- and MM blocks (Aarstad et al., 2011 and section 2.2.1., p. 15). Assuming that the alginate of *L. digitata* consisted of only GG-blocks and MM-blocks, the released oligoalginates units from Figure 19 can be distinguished as unsaturated UA of G-units or M-units according to the G:M cleavage ratio (Figure 20). Hence, according to Figure 20 the degradation of mannuronic acid from *L. digitata* correlated negatively to the glucose release (*PAPER IV*).

With application of SigmALy approx. 37 % of all present guluronic acid (equal to 9.2 % $w/w_{DMalginate}$ or 2.1 % w/w_{DM}) underwent a ß-elimination (G-units, Figure 20C). Hence, the released unsaturated oligoalginates after 2 h were supposedly of DP 2-3. This was in accordance with the described mode of action for SigmALy, releasing mainly trimers (Huang et al., 2013). In the same study FALy was found to release oligomers of DP 5-7 within the first 20 h of reaction. Hence, the presence of longer oligomers could describe the lower yields of unsaturated uronic acids deriving from G-units generated by FALy (4 % $w/w_{DMalginate}$, Figure 20C) compared to the other two lyases (Figure 20). SALy was described to release tri- and tetrasaccharides (Yoon et al., 2000; Miyake et al., 2003, 2004). This corresponded with the release of unsaturated (G)-units from seaweed by SALy of 8.6 % $w/w_{DMalginate}$ (equal to 34 % of the present guluronic acid) after 60 min of reaction (Figure 20B). Accordingly, the degradation of the seaweed alginate by SALy occurred mainly during the first hour of reaction (Figure 19B) (*PAPER IV*).

After 1-2 hours of reaction potentially there were no more available guluronic acid bonds in the seaweed. In contrast, both poly-(M) active lyases (SALy and FALy) released unsaturated M-units of 4.3 % $w/_{DMalginate}$ (SALy), respectively 6.1 % $w/w_{DMalginate}$ (FALy) after 120 min (Figure 20C). While plenty of poly-(M) was theoretically still available as substrate, the degradation of the alginate leveled off (Figure 19B). Potentially, poly-(M) and poly-(G) interacted competitively with the lyases active on both substrates (*PAPER IV*). This was in accordance with Iwamato et al. (2001) indicating a strong reduced production of unsaturated mannuronates from poly-(M) in the presence of poly-(G), the higher the concentration of mannuronic acid the higher the reduction. The relatively high concentration of mannuronic acid in *L. digitata* (M/G ratio 3:1) supported this assumption.

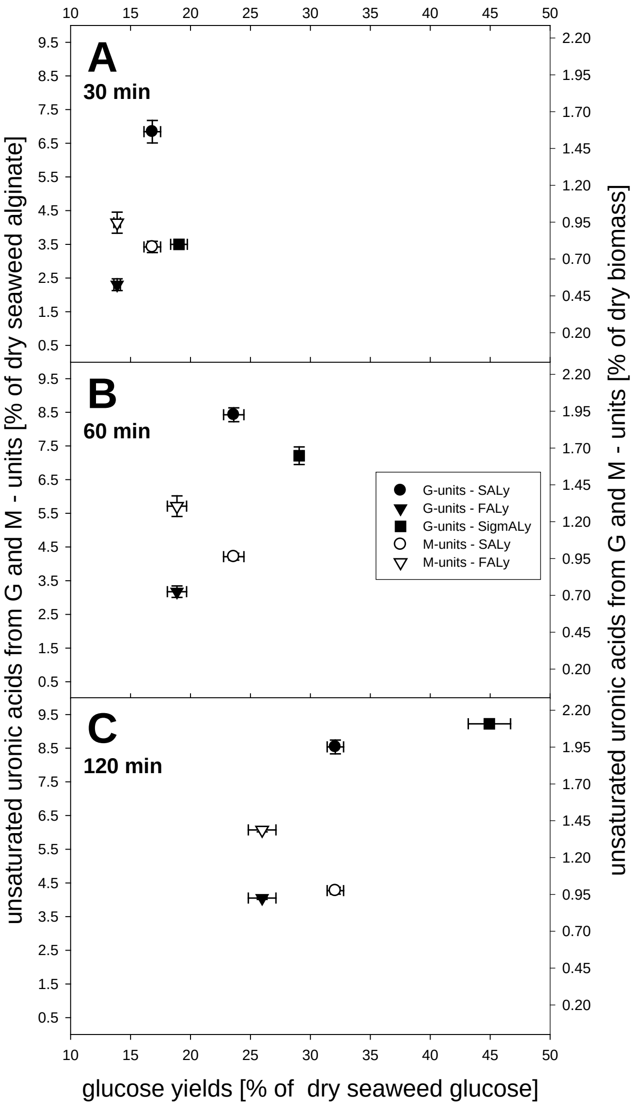

Figure 20: Yields of unsaturated uronic acids of *L. digitata* over glucose yields [% w/w$_{DM}$ potential glucose] deriving from cleavages of poly-guluronic acids (**G-units**) and poly-mannuronic acids (**M-units**) after combined cellulase-lyase treatments with SALy, FALy and SigmALy, (**A**) after 30 min, (**B**) after 60 min and (**C**) after 120 min calculated from the unsaturated UA of Figure 19.

Left y-axis: % w/w$_{DM}$ of potential seaweed alginate; right y-axis: % w/w$_{DM}$; for potential concentrations of glucose and alginate see Table 3 *L. digitata*, washed in in section 2.2.3., p. 20.

Each data point represents the average value of independent duplicates, bi-dimensional bars indicate the standard deviation. All values are given as hydrated monomers.

Conclusively, application of alginate lyase decreased the specific viscosity the initial minutes and alginate degradation occurred primarily within the first 1-2 hours of reaction. In particular, the guluronic acid blocks were degraded while only a minor portion of the mannuronic acid present in the seaweed was epimerized to unsaturated uronic acids. Moreover, a degradation of poly-(M) led into a decreased release rate of glucose from *L. digitata* by the cellulase preparation. Figure 20 demonstrated the more unsaturated M-units were released the lower were the correspondent glucose yields (*PAPER IV*).

3.3.3. Optimal glucose release (*PAPER III* / *PAPER IV*)

The experiments presented in Figure 19 and Figure 20 were performed at a total reaction volume of 13 mL. For the investigation of the lyase induced viscosity reduction (Figure 18) the analytical instrument required an up-scale to 30 mL reaction volume. Time-extended enzymatic treatment with Cellic®CTec2 and alginate lyase for 24 hours of this up-scaled experiment was sufficient to release all potential glucose (46.6 % w/w_{DM}, Figure 13A+B in section 3.2.2., p. 32) from the glucan rich *L. digitata* slurry (d = 0.3 mm) regardless the applied lyase (*PAPER IV*). Furthermore, in this experiment the cellulase preparation Cellic®CTec2 in combination with poly-(G) specific lyase SigmALy released 92 % (42.9 % w/w_{DM}) of the potential glucose after 4 h of treatment (Figure 21). After 8 h a total glucose yield of 50.6 % w/w_{DM} dry milling slurry was achieved. For the combined Cellic®CTec2-SALy treatment 14 h were sufficient to release a similar amount of glucose (48.0 % w/w_{DM}). After 14 h of treatment the enzyme mixture of Cellic®CTec2 and FALy had released 30.9 % but final glucose yield after 24 h increased to 46.0 % w/w_{DM} (Figure 21).

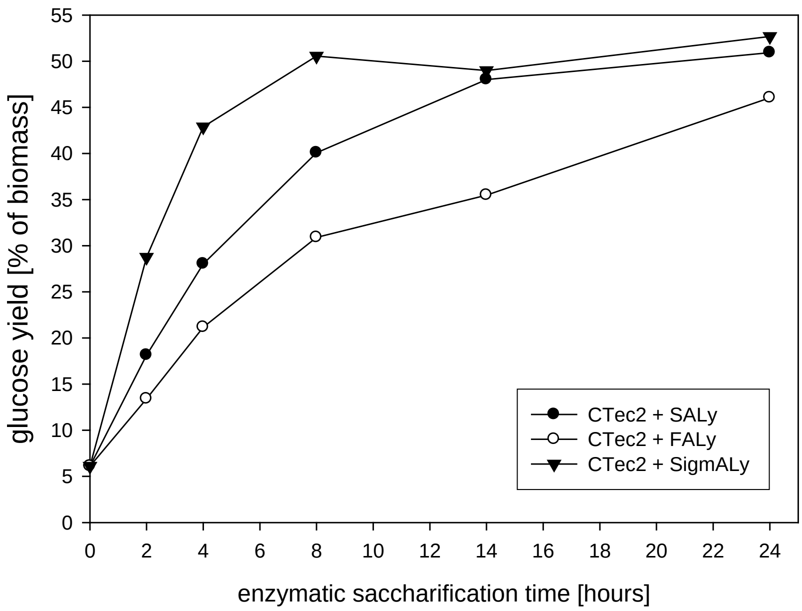

Figure 21: Glucose yields [% w/w_{DM}] over time of enzymatic saccharification (0-24 h) of refiner milled *L. digitata* (d = 0.3 mm). Enzymatic saccharification at pH 6 and 40 °C with enzyme concentration on substrate: 10 % v/w_{DM} Cellic®CTec2 and 1 % w/w_{DM} alginate lyase (SALy, FALy and purchased lyase SigmALy). Each data point represents a single experiment. All values are given as hydrated monomers.

Notably, glucose yields of the treatments including the lyases SigmALy and FALy extended the glucose potential. For example, the final glucose yield after 24 h for an application including the SigmALy was 52.7 % w/w_{DM}. Therefore, the yield was of 6.6 % w/w_{DM} higher than the determined potential glucose of the milling slurry after HPAEC analysis post H_2SO_4 treatment of 46.6 % w/w$_{DM}$ (Figure 13A+B in section 3.2.2., p. 32). The potential glucose in the biomass before milling was 56.6 % w/w$_{DM}$ (*L. digitata*, washed; Table 1 H_2SO_4 method A in section 2.2.1., p. 14). Possible reasons for the deviations could be explained by (a) the use of different glucose determination methods (glucose yields in Figure 21 determined by enzyme glucose assay; potential glucose yields in Figure 13 determined by HPAEC-PAD post H_2SO_4 treatment), (b) degradation of glucose by H_2SO_4 treatment (analysis of the raw material after cellulase hydrolysis revealed higher glucose yields than by H_2SO_4 hydrolysis, Table 1, p. 14), (c) challenges in regard to HPAEC-PAD analysis of brown seaweeds in general (described in section 2.2.1.), (d) heterogeneity in the investigated sample due to differences between the seaweed individuals (see Figure 9 in section 2.3.1, p. 24), and (e) heterogeneity due to losses of solubilized glucan during the refiner milling (see section 3.2.1).

Hou et al. (2015) applied the purchased lyase SigmALy at lower lyase concentration of 0.125 % w/w$_{DM}$ but also at lower substrate loading (2 % S/V). The maximum glucose recovery of the dried and milled material after 24 hours of enzymatic treatment was 80 % w/w$_{DM}$ (≈ 5 % after 8 h) (Hou et al., 2015). A sugar recovery (glucose and mannitol) of over 90 % was reached with a substrate concentration of 15 % S/V but was reduced to 78 % with increased solid loading (25 % S/V) after 29 hours, with no change over treatment extension of up to 48 hours (Alvarado-Morales et al., 2015). Both investigations were conducted on dried material using the cellulase preparation Celluclast 1.5L and Cellobiase 188 at about pH 5. In another study, the Celluclast 1.5L was found to release less reducing sugars from *L. digitata* than another commercially available cellulase (Vanegas et al., 2015). The investigation of Alvarado-Morales et al. (2015) and Hou et al. (2015) were conducted on the same glucan rich *L. digitata* using the Celluclast 1.5L. Hence, in *PAPER III* and *PAPER IV* the Cellic®CTec2 preparation most likely provided a higher decomposition capability compared to the cellulase preparation Celluclast 1.5L.

Furthermore, drying was shown to hinder glucose release, albeit from lignocellulosic material (Luo and Zhu, 2011; Luo et al., 2011). In regard to the pH, Celluclast retained an activity of 80 % at pH 6 (optimum was pH 5.2) when applied on brown seaweed *Macrocystis pyrifera*. In contrast, the activities of alginate lyases, including the endo-type lyase from Sigma-Aldrich, were lower than 10 % at pH 6 and about one third at pH 7 compared to pH 7.5 (Ravanal et al., 2016). With respect to temperature, glucose release could be enhanced by raising the temperature, for example at a temperature increase from 37 °C to 50 °C the yields were doubled (Ravanal et al., 2016). However, to allow suitable conditions for all alginate lyases temperatures in the experiments presented here were generally set to 40 °C. The purchased lyase from Sigma-Aldrich (SigmALy) exhibited significant activity losses for temperatures ≥50 °C. However, compared to the study of Ravanal et al. (2016), SigmALy retained 50 % of the maximum activity at pH 6 (*PAPER IV*).

The cellulase preparation Cellic®CTec2 enabled total glucose release of refiner milled wet *L. digitata* (disc distance 0.3 mm). Although the optimal temperature and pH for Cellic®CTec2 are 45–50 °C and pH 5.0–5.5 (Novozymes A/S, 2010), total glucose was released after 8 hours with the support of guluronic acid specific alginate lyase (SigmALy) using pH 6 and 40 °C (Figure 21). Further experiments concerning the optimal enzyme dosages were presented in *PAPER III*. Slurry having been subjected to the lowest milling intensity (d = 2.0 mm) was studied to investigate the effect of enzyme dosage of Cellic®CTec2 and alginate lyase (SigmALy) addition on the enzymatic glucose release from the seaweed at pH 5 and 40 °C (*PAPER III*).

Alginate lyase addition alone, without Cellic®CTec2, facilitated the release of glucose as glucose yields increased with time in the control experiments (Figure 22A, point 0.0). The effect of the alginate lyase must be a result of the alginate degradation initiating release of free glucose was corroborated by the findings that alginate lyase treatment alone on pure laminarin did not release more than 1-2 % glucose (*PAPER III*). The presence of free glucose monomers was discussed earlier (section 3.3.2 p. 33).

When varying the alginate lyase concentration at a fixed concentration of 10 % v/w_{DM} Cellic®CTec2, the glucose yields from the refiner-milled slurry of *L. digitata* increased over both hydrolysis time and enzyme concentration of alginate lyase (Figure 22B). Statistically, the alginate lyase dosage effect was significant at all hydrolysis times up to a concentration of 1 % (w/w_{DM}) lyase on the substrate (Figure 22B) (*PAPER III*).

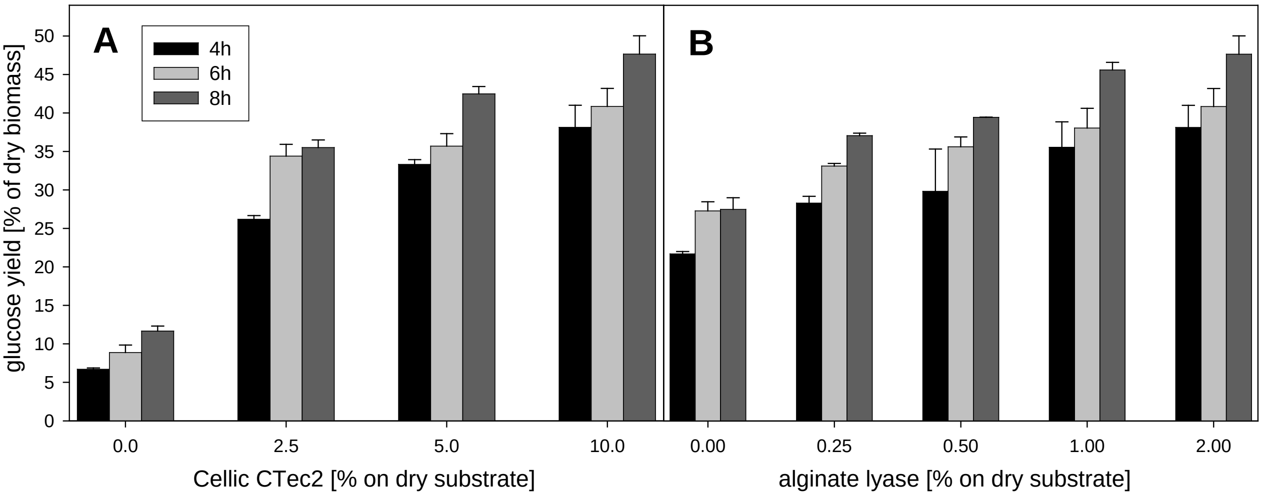

Figure 22: Glucose yields [% w/w_{DM}] for wet refiner milled *L. digitata* slurries (d = 2.0 mm). Enzymatic saccharification yields over (**A**) Cellic®CTec2 concentration at fixed alginate lyase SigmALy (2 % w/w_{DM}) and (**B**) over alginate lyase SigmALy concentration at fixed Cellic®CTec2 (10 % v/w_{DM}) at pH 5 and 40 °C.. Each data point represents the average value of independent duplicates; vertical bars indicate the standard deviation. All values are given as hydrated monomers (for statistical analysis see supplementary material in *PAPER III*).

Increased dosage of cellulase (Cellic®CTec2) with 2 % w/w_{DM} alginate lyase produced a steady increase in glucose yield after reactions of 4, 6, and 8 h, respectively (Figure 4A). Conclusively, a cellulase (Cellic®CTec2) concentration of 10 % v/w_{DM} and a reaction time of 8 hours were required to achieve release of the glucose present in the seaweed. The eight hours treatment with an enzyme mix of 1 % w/w_{DM} alginate lyase and the cellulase containing preparation Cellic®CTec2 10 % v/w_{DM} was sufficient to release 95 % of the available glucose. 75 % of the potential glucose could be released within four hours while the remaining 20 % were only released during the last two hours of enzymatic hydrolysis (Figure 22A). Further increase in cellulase dosage to 15 and 20 % v/w_{DM} did result in an increase of glucose yield after 8 hours (Figure 23).

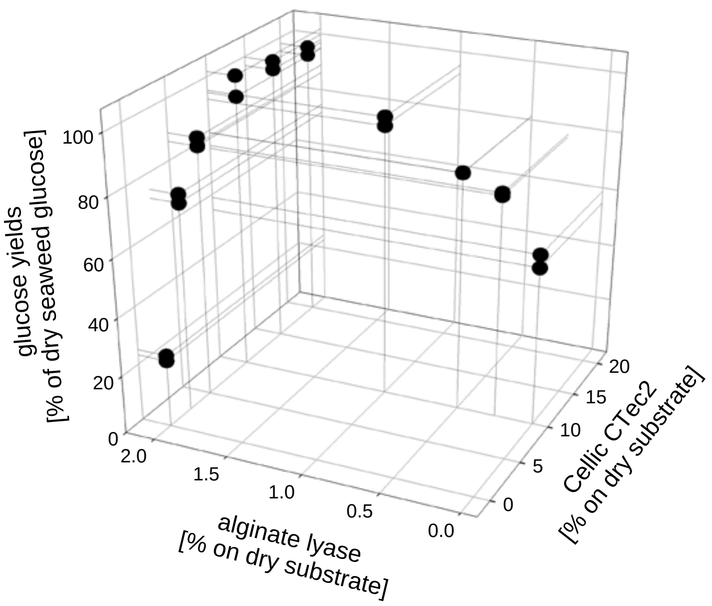

Figure 23: Glucose yields [% w/w_{DM} of potential seaweed glucose] for wet refiner milled *L. digitata* slurries (d = 2.0 mm). Enzymatic saccharification yields displayed over varying Cellic®CTec2 concentrations (0-20 % v/w_{DM}) and alginate lyase (SigmALy) concentrations (0-2 % w/w) after 8 hours of treatment at pH 5 and 40 °C. Each data point represents the average value of independent duplicates; all values are given as hydrated monomers (for statistical analysis see supplementary material in *PAPER III*).

Overall, the enzymatic glucose release from the glucan-rich *L. digitata* (harvested August 2012; Danish North Sea) through an enzyme mix of alginate lyase and Cellic®CTec2 was studied in-depth on two refiner milled slurries. These slurries were milled at refiner disc distances of 2.0 and 0.3 mm representing mild and severe milling conditions. Regardless of the milling intensity and the choice of pH 5 or pH 6 total glucose releases were achieved after 8 hours of enzymatic treatment. For this, an optimal enzyme dosage consisted of 10 % v/w_{DM} cellulase containing preparation Cellic®CTec2 and 1 % w/w_{DM} of the poly-(G) specific alginate lyase SigmALy (*PAPER III*, *IV*). In conclusion, *"Brown seaweed processing: enzymatic saccharification of Laminaria digitata requires no pre-treatment"* (*PAPER III*); while acknowledging the *"Impact of different microbial alginate lyases on combined cellulase-lyase saccharification of brown seaweed"* (*PAPER IV*).

3.3.4. Potential by-products: mannitol, protein and fucoidan (*PAPER IV*)

Applications of mannitol are extremely diverse, e.g. food (sweetener), addition in technical products such as varnish, paper or explosives and mannitol is also used in pharmaceuticals (Holdt and Kraan, 2011). Furthermore, investigations on yeast strains demonstrated the ability to convert mannitol to fructose by mannitol dehydrogenase for bioconversion into ethanol (Horn et al., 2000b; Ota et al., 2013). Protein levels in *L. digitata* from Danish waters can exceed 10 % w/w_{DM} (*PAPER II*). Algal protein contain all the essential amino acids interesting for food or feed application (Holdt and Kraan, 2011; Nielsen et al., 2016). Fucoidan are water soluble sulfated polysaccharides (Pereira et al., 2013). Their biological activities exhibit interesting applications for pharmaceutical and cosmetic products. However, classical extraction of fucoidans involves multi-step extended aqueous extractions usually with hot acid (Ale and Meyer, 2013).

L. digitata collected from the North Sea in August 2012 contained of 3.2 % total amino acids and 2.4 % fucose along with 2.4 % w/w_{DM} of other fucoidan related carbohydrates such glucuronic acid, mannose and xylose. The washed seaweed consisted of 8.0 % mannitol and in the milled slurry (refiner milling with d = 0.3 mm) 6.7 % w/w_{DM} mannitol was found (*PAPER I*, *III*). After enzymatic treatment of the milled seaweed for 24 hours (Figure 21) the liquefied fraction was separated from the insoluble residue (*PAPER IV*).

Treatment with the poly-(G) specific lyase SigmALy plus Cellic®CTec2 achieved the highest decomposition of the milled material and left behind an insoluble residue of 12.4 % w/w_{DM} after the enzymatic treatment (Table 5). After enzymatic treatment with the cellulase preparation Cellic®CTec2 and the alginate lyases FALy and SALy an insoluble residue of about 20 % w/w_{DM} of the refiner milled seaweed was observed (Table 5). The application of SigmALy or Cellic®CTec2 alone did not decompose the seaweed to this extent, indicated by an insoluble residue of 28.9 % w/w_{DM} or 51.8 % w/wDM (Table 5). Thus, the application of FALy and SALy (active on poly-(M)) did not only reduce the glucose release rate (Figure 20 in section 3.3.2., p. 43) but also the overall seaweed decomposition (*PAPER IV*). Furthermore, compared to a study that determined saccharification residues of about 26 % w/w_{DM} after enzymatic treatment (48 h with similar enzyme products) and following fermentation of dry milled *L. digitata* samples (Hou et al., 2015), the relatively low residues of 12 % w/w_{DM} after enzymatic saccharification of wet refining indicated wet refining as a gentle treatment has positive effects on the enzymatic yields.

Table 5: Amount of insoluble solid residues, nitrogen recovery from the residues, and yields of mannitol and fucose of the liquefied fraction after 24 h enzymatic treatment (Figure 21) of wet refiner milled *L. digitata* (d = 0.3 mm) with Cellic®CTec2 and alginate lyase (SALy, FALy and SigmALy), as well as treatments with SigmALy and Cellic®CTec2 alone.

treatment	solid residue[1] amount	solid residue[1] N-recovery[2]	solubilized carbohydrates[1] Mannitol[3]	solubilized carbohydrates[1] Fucose[3]
	[% w/w_{DM} of refiner slurry]		[% w/w_{DM} of refiner slurry]	
SALy + Cellic®CTec2	19.3	80.1	3.4	2.4
FALy + Cellic®CTec2	20.3	83.5	5.9	1.9
SigmALy + Cellic®CTec2	12.4	71.4	5.0	3.6
Cellic®CTec2 alone	51.8	68.4	3.9	0.7
SigmALy alone	28.9	78.5	2.3	2.1

[1] Separation of the solubilized carbohydrates from the insoluble residues by decanting after centrifugation at 14,000×g for 30 min

[2]after elemental analysis; raw material N=0.73 % (*PAPER I* and Table 3, p. 20)

[3]hydrated monomers after H_2SO_4 hydrolysis and HPAEC analysis

The received insoluble residue was analyzed for nitrogen contents. Compared to the raw material, 70-80 % by weight of nitrogen could be recovered indicating a high concentrations of proteins in the insoluble residue (Table 5) (*PAPER IV*). These data were in accordance with the recently published findings on the same glucan rich *L. digitata* by Hou et al. (2015). There, a residue rich in protein (after extensive saccharification and fermentation) having similar amino acid profile as the raw material remained insoluble (Hou et al., 2015). In addition to nitrogen, the insoluble residue also contained a mixture of carbohydrates. However, for the combined cellulase-lyase treatments this amount was equivalent to approx. 4 % w/w_{DM} indicating extensive enzymatic decomposition of the *L. digitata* milling slurry (*PAPER IV*).

The carbohydrate analysis by HPAEC-PAD showed that the liquefied fraction compromised monomeric mannitol and glucose. The measured mannitol levels (3.4 to 5.9 % w/w_{DM} of the original milled seaweed biomass; Table 5) made up about 51-88 % of the available original content of mannitol in the biomass (*PAPER IV*). Inhomogeneity in the seaweed individuals (*PAPER II*) and wash offs during the refiner milling process (*PAPER III*) could be potential reasons the high variations of the mannitol concentrations, among others mentioned earlier on p. 44f.

In order to complete mass balance of the carbohydrates, the enzymatically liquefied fraction was post-hydrolyzed with sulfuric acid. At this time, monomeric sugars of fucose, galactose, xylose, mannose, glucuronic acid, as well as alginic acid monomers appeared in HPAEC-PAD analysis along with glucose and mannitol. Beside the application of the cellulase preparation alone the yields of fucose were of the same magnitude as present in the original seaweed of 2.4 % of dry seaweed. Hence, this indicated that the fucoidan was dissolved but not hydrolyzed by the enzymatic treatment (*PAPER IV*).

In conclusion, the enzymatic treatment with alginate lyase and cellulases facilitated the release of glucose and mannitol from *L. digitata*. Furthermore, in the insoluble residues after enzymatic treatment contained of 70-80 % nitrogen of the original *L. digitata* raw material sample. Moreover, fucose containing molecules were solubilized and remained in the liquefied fraction after separation from the insoluble residue. The fucose concentration of these molecules corresponded closely to the same fucose amount present in the raw material. The highly valuable by-products of sulfated polysaccharides (fucoidan) and proteins can be potentially extracted after enzymatic treatment corroborating the *“Impact of different microbial alginate lyases on combined cellulase-lyase saccharification of brown seaweed”* (*PAPER IV*).

4. CONCLUSIONS AND FUTURE PERSPECTIVE

Similar to lignocellulosic material, acid hydrolysis with sulfuric acid (H_2SO_4) was the most appropriate treatment for subsequent quantitation of brown seaweed carbohydrates. After a 2-step treatment with 72 % H_2SO_4 for 1 hour at 30 °C followed by 4 % H_2SO_4 at 120 °C for 40 min HPAEC-PAD analysis enabled determination of the monomeric composition of neutral sugars, the sugar alcohol mannitol and uronic acids. Due to the lack of available pure standard mannuronic acid was quantified as galacturonic acid equivalents. However, the high heterogeneity in the type of monomeric compounds and the high amounts of β-bonds in the polysaccharides of brown seaweeds in combination with high ion load challenged the analysis and could cause elevated deviations. HPAEC-Borate is an accurate and highly reproducible method. However, it allowed only quantification of glucose, xylose and mannose monomers from the brown seaweed hydrolysates. With the HPAEC-PAD analysis for carbohydrates and additional determination of amino acids and lipids the matter of total organic compounds was determined. The matter of organic compounds was successfully cross-verified with the sum of C, H, N and O as total organic compounds through elemental analysis. Through the addition of ash content, a good closure on the compositional mass balance of brown seaweed was achieved, including a complete database of compositional compounds (*PAPER I*).

With the method for quantitative determination of the carbohydrate composition, developed within the studies of *PAPER I*, the potential of the brown seaweeds from Danish waters for glucose based biorefining was assessed in *PAPER II*. Therefore, the variation of the biochemical composition of four populations of *Saccharina latissima* and *Laminaria digitata* from three different locations was documented over one year of growth. Overall, the chemical composition of brown seaweed varied mainly in regard to the season but differed also with respect to year of sampling, species, location of growth and individuals of the population. Concentrations of ash and protein levels varied inversely to the carbohydrate levels, and total carbohydrate concentration were seasonally influenced, particularly by the storage carbohydrates glucose and mannitol. Generally, alginate was the most abundant carbohydrate at all sites from December to June/July with up to 36 % w/w_{DM}. In the summer the glucose levels were at least at the same magnitude. Alginate, or alginic acid, as the sum of its monomers mannuronic and guluronic acid was relatively independent of seasonal changes. However, M/G ratios differed strongly throughout the year from 1.3 to 3.6 but with no certain pattern regarding season, species or location (*PAPER II*).

Throughout the literature the total protein concentration is commonly determined by application of nitrogen-to-protein conversion factors after determination of total nitrogen content. Conversion of nitrogen to protein with factor 4, as suggested from *PAPER I*, emphasized high protein contents of up to 20 % w/w_{DM} in the seaweeds from February/March. However, total protein determination through amino acid analysis revealed concentrations of maximum 12.7 % w/w_{DM}. Based on the amino acid analysis the average N-to-protein conversion factor was 3.7, varying from 2.1 to 5.9, a smaller range compared to the variance in the total nitrogen. In this respect, application of a common nitrogen-to-protein factor was not satisfactorily. Amino acid analysis is instead recommended for the quantification of protein (*PAPER II*).

Nowadays, brown seaweed cultivation is practiced in nearshore (shallow) waters, with seedling production in hatcheries and sea growth phases usually occurring from winter to the following (late) spring (Sanderson et al., 2008; Nielsen et al., 2012; Marinho et al., 2015; Taelman et al., 2015). However, the highest concentration of glucose was found in August for wild growing *L. digitata* exposed to a windy and wavy site in the Danish North Sea. Moreover, biofouling organisms increasingly hampered growth of the *S. latissima* cultivation in the shallow and sheltered Limfjorden, Denmark. Glucose levels of 5 % w/w_{DM} in August indicated that nearshore cultivation might not achieve a favorable biochemical profile required for bioenergy purposes (*PAPER II*).

While biofouling for open sea cultivation was also reported (Handå et al., 2013), other studies underlined the general feasibility of offshore cultivation of brown seaweed (Buck and Buchholz, 2005; Taelman et al., 2015). Even in stormy conditions it was feasible to cultivate *S. latissima* in offshore wind farms in the North Sea (Buck and Buchholz, 2005). Likewise, *L. digitata* from the North Sea was exposed stronger to elaborated water motions. Furthermore, temperature was found to influence the chemical conditions and was more optimal at the North Sea compared to the Danish Kattegat (Bay of Aarhus) and the Limfjorden (*PAPER II*). Moreover, climate change is projected to increase the average sea surface temperature and so is predicted to cause a northward retreat of seaweed (Méléder et al., 2010; Raybaud et al., 2013). This might exclude locations such as the Limfjorden and also the Kattegat for cultivation of *L. digitata* and *S. latissima* in future, especially during the warmer summer months with potential increased temperatures of the surface water.

Generally, glucose levels of *L. digitata* appeared to be superior to *S. latissima*. Glucose levels in wild *L. digitata* from the Kattegat peaked in October with 37.0 % w/w_{DM} compared to 22.6 % in wild *S. latissima* from the same site. The higher glucose concentration was accompanied by lower ash content (18.5 % for *L. digitata* compared to 26.5 % w/w_{DM} of *S. latissima*). However, glucan concentrations of *L. digitata* specimens from the North Sea were >50 % w/w_{DM}, accompanied with mannitol concentrations of about 10 % and ash levels of 10-11 % w/w_{DM} for three sequential years (2012-2014). Harvested at the right time and place, *L. digitata* as the most suitable feedstock for application in glucose based biorefineries. Nevertheless, variations in the carbohydrate composition occurred also between seaweed individuals of the population, a fact to be considered for optimal cultivation strategies in regard to the cultivation density (*PAPER II*). Notwithstanding the superior offshore growing conditions, one must bear in mind that offshore cultivation is more energy, labor and capital intensive, would necessitate careful engineering of the cultivation structures and potentially includes detachment and therefore losses of the crop (Buck and Buchholz, 2005; Kerrison et al., 2015; Taelman et al., 2015).

Post washing *L. digitata* harvested from the Danish North Sea in August 2012 contained about 84 % w/w_{DM} organic matter dominated by glucan (51 % w/w_{DM}). Subsequently, this material was investigated for biocatalytical processing to achieve maximum glucose release. Refiner milling with disc distances between 0.1 and 2.0 mm generated differently sized particle fractions with surface areas from 0.1-100 mm^2. The tightest distances produced the smallest surface areas. Milling with disc distances below the thickness of the algae (≤1 mm) increased the particle volume of the milled seaweed slurries. Higher milling severity (lower rotating disc distance) also induced higher spontaneous carbohydrate solubilization from the material of particularly glucose and mannitol. Overall, smaller particles, increased particle volume and solubilization of glucan over milling intensity indicated an enhanced availability of the seaweed glucan. However, the particle size reduction did not improve the enzymatic glucose release. Milling was thus not required for enzymatic saccharification because all available glucose was released even from unmilled material during the combined use of purchased alginate lyase and the commercial cellulase preparation Cellic®CTec2. The alginate lyase (Sigma Aldrich) activity appeared to have catalyzed the cleavage of alginate from the substrate, which both decreases the viscosity of the substrate matrix and catalytically solubilizes the alginate to provide access to the glucan in the brown seaweed cell wall matrix. Nevertheless, in order to guarantee a homogenous processing particle size reduction is advisable (*PAPER III*).

Enzymatic hydrolysis of lignocellulosic feedstocks is inefficient without a preceding hydrothermal or other physicochemical biomass pretreatment to expose the cellulose (Alvira et al., 2010). However, such a harsh pretreatment was not required for enzymatic seaweed saccharification. Moreover, for mechanical pretreatment of chipped, debarked and screened wood chips had an energy consumption of 0.616 kWh/kg (Zhu et al., 2010). In contrast, for milling of *L. digitata* the energy consumption was estimated to be 0.38 kWh/kg biomass without a need of previous feedstock preparations (Alvarado-Morales et al., 2013). Additionally, while hydrothermal and most physicochemical biomass in case of lignocellulose pretreatment requires input of heat, this was not needed for pretreatment of seaweed biomass. Thus, lower energy consumption for necessary pretreatment favors seaweed as a superior feedstock over lignocellulosic material in terms of energy input.

The further investigations particularly focused on the support of alginate lyases on the overall goal of maximize glucose release from the glucan rich *L. digitata*. Therefore, two bacterial polysaccharide lyase (PL) of family 7 from *Sphingomonas* sp. (SALy) and *Flavobacterium* sp. (FALy) were selected after literature research for heterologous, monocomponent expression in *Escherichia coli*. Following, thermal stability, pH and temperature reaction optima, as well as the substrate specifity of the two expressed alginate endo-lyases (EC 4.2.2.- SALy, FALy) and the commercially available alginate lyase (SigmALy) were assed. The optimal pH range for SALy was pH 5.5-7.0 with optimum at pH 6. The optimum for FALy and SigmALy was pH 7.5. The investigated reaction temperatures of 30-50 °C had no influence on the activity. Above 50 °C the thermal stability of SALy and FALy was reduced. For SigmALy incubation >40 °C led to reduced activity. The FALy preferred poly-mannuronic acid as substrate, but also exhibited activity on poly-guluronic acid, whereas SALy had higher activity on poly-guluronic acid. SigmALy was only active on poly-guluronic acid. The alginate constitution of *L. digitata* with M/G ratio of 3:1 suggested higher degradation capability for lyases with activity on poly-mannuronic acid. Moreover, pH range of 5.5-7.0 emphasized SALy as an appropriate candidate to combine with fungi derived cellulase to release glucose release from brown seaweeds (*PAPER IV*).

Following, each of the three alginate lyases were subsequently combined and applied with the commercial, fungally derived cellulase preparation Cellic®CTec2 at pH 6 and 40 °C on the milling slurry of *L. digitata*, milled at disc distance of 0.3 mm. Viscosity of the slurry decreased in the initial minutes while alginate degradation occurred primarily within the first 1-2 hours of reaction. Expectedly, SALy and FALy showed higher activity on the alginate of the seaweed. Specifically, the guluronic acid was assumingly degraded to smaller oligomers by all lyases. However, after 2 h of reaction SALy and FALy released only 4-6 % $w/w_{DMalginate}$ unsaturated oligo-uronic acids from the mannuronic acid present in the seaweed. Moreover, with increasing mannuronic acid release lower glucose yields could be determined. This indicated that the degradation of mannuronic acid blocks inhibited the cellulase catalyzed glucose release from *L. digitata.* Only the strict action of SigmALy on guluronic acid enabled a 90 % glucose release within 8 hours by the cellulase preparation Cellic®CTec2. Nevertheless, combined alginate lyase and cellulase treatment for 24 hours released all potential glucose regardless of the applied lyase (*PAPER IV*).

The slurry having been subjected to the lowest milling intensity at disc distance 2.0 mm was treated with varying concentrations of SigmALy and Cellic®CTec2 at pH 5 and 40 °C. Treatment with a mixture of 1 % w/w_{DM} SigmALy and 10 % v/w_{DM} Cellic®CTec2 released 90 % of the available glucose during 8 hours. In detail, 75 % of the potential glucose could be achieved within the first four hours and two-thirds of the glucose with lower enzyme loading after 8 h. Simple application of only the cellulase preparation Cellic®CTec2 enabled the release of only half of the present glucose while the addition of lyase ensured accessibility for complete enzymatic glucan decomposition (*PAPER III*). Overall, the enzymatic glucose release by an enzyme mix of alginate lyase and Cellic®CTec2 was studied in more detail on two refiner milled slurries of the glucan rich *L. digitata* harvested in August 2012 from the Danish North Sea. Regardless of the milling intensity and the choice of pH 5 or pH 6, total glucose releases were achieved after 8 hours of enzymatic treatment. For this, an optimal enzyme dosage consisted of 10 % w/w_{DM} cellulase containing preparation Cellic®CTec2 combined with 1 % w/w_{DM} of the poly-guluronic acid specific alginate lyase SigmALy (*PAPER III*, *IV*).

The energy obtained from an energy production process compared to the energy required to produce that energy is the definition for the EROI. Hereby, an EROI ≥ 1 is considered as minimum for net energy gain and EROI ≥ 3 for a *'sustainable'* energy product (Hall et al., 2009). Aitken et al. (2014) calculated the EROI for ethanol fermentation by mannitol and glucose gained from brown algae to be <1. The calculation was based on ethanol production from *L. digitata* consisting of approx. 25 % w/w_{DM} glucose and 15 % mannitol (Adams et al., 2011). Although saccharification yields of 52.7 % w/w_{DM} glucose and 5.0 % w/w_{DM} mannitol *L. digitata* (*PAPER IV*) could potentially produce higher amounts of ethanol, *'sustainable'* production of ethanol from brown seaweed is rather illusory. However, life cycle assessment studies identified additional anaerobic digestion as a valuable option to overcome these limits (Alvarado-Morales et al., 2013). Nevertheless, the scenario including electricity from anaerobic digestion and the contribution of fertilizer as an energy credit to the production of ethanol achieved an EROI ≈ 2 (Aitken et al., 2014). According to definition of Hall et al. (2009) this EROI ≈ 2 does not meet *'sustainable'* production. Nevertheless, bioethanol production and anaerobic digestion including the extraction of the digestion residue for fertilizer from brown seaweed cultivations was proposed to reduce global warming and for bioremediation (Alvarado-Morales et al., 2013; Aitken et al., 2014).

To increase the energy potential of brown seaweeds, a more extensive exploitation of the biomass was promoted by early stage researches where developed bacterial systems demonstrated ethanol production from alginate (Takeda et al., 2011; Wargacki et al., 2012). The engineered *E. coli* bacterium was capable of simultaneously degrading alginate and fermenting it with glucose and mannitol to ethanol. The yield was 80 % of the maximum theoretical yield from the sugar composition in brown seaweed (Wargacki et al., 2012). The consideration of this ethanol yield in the life cycle assessment the EROI increased to 3.2 (Aitken et al., 2014). The total of glucose and mannitol content of *L. digitata* (August'12-14, North Sea) made up >70 % w/w_{DM} of the sugar composition and >60 % w/w_{DM} of the total biomass (*PAPER II*). Furthermore, glucose and mannitol was accessible with only minor pretreatment requirements (*PAPER III*). This indicates further energy saving potential. Moreover, after treatment with cellulase and endolytical alginate lyase the sulfated polysaccharides (fucoidan) remained dissolved in the soup of mannitol and glucose monomers. Proteins potentially remained in the solid saccharification residue after separation by centrifugation (*PAPER IV*). The extraction of these potential by-products can positively contribute to the life cycle of brown seaweed cultivation and biofuel production. Hence, production of ethanol, biogas for electricity and fertilizer from brown seaweed might meet an EROI = 3 (considered as *'sustainable'* production according to Hall et al. (2009)). Therefore, brown seaweed biorefining with state of the art technology can potentially meet *'sustainable'* if the biomass is harvested at the right time and place and appropriately pretreated.

Further investigations should be undertaken in order to extract fucoidan and proteins after the enzymatic treatment. Endo-attacking alginate lyases are proposed to play a key role in brown seaweed biorefining. Uncertainties remain however, about catalytical process especially regarding the catalysis of the mannuronic acid blocks and the inhibitory effect on the glucose release by cellulases. Beside this, for large scale brown seaweed biorefining the alginate lyase production is not yet industrialized. Furthermore, the addition of exolytic oligoalginate lyase to produce monosaccharide units of alginate was recently demonstrated (Ryu and Lee, 2011; Park et al., 2012) and should be verified as pretreatment for a subsequent improved anaerobic digestion or fermentation.

Services like greenhouse gas emission savings and bioremediation potential should further be considered and highlighted particularly with respect to limit the world temperature increase to 1.5°C as targeted from the COP21 (see introduction). Moreover, the total brown seaweed biorefining potential can include other products like seaweed for food and feed or extraction of the hydrocolloid alginate, iodine, lipids, vitamins or phenols or the use of the residue as fertilizer. Including as many by-products and services as possible could make brown seaweed an interesting candidate for application in biorefineries even in times when the glucose level becomes depleted. Here, too, *L. digitata* from the North Sea can be an interesting candidate for extraction of valuable products. In March *L. digitata* consisted of over 30 % w/w_{DM} of guluronic acid rich alginate and 12.5 % w/w_{DM} total amino acids. In summary, the results of this PhD study demonstrated that brown seaweed can be completely degraded enzymatically by cellulase and alginate lyase treatment after milling (*PAPER III* and *PAPER IV*). The work has also demonstrated, that biorefining of brown seaweed with current state of art technology is highly dependent on the cultivation, particularly growth site and season, of a suitable feedstock for achieving maximal glucan content and in turn allow maximum glucose release (*PAPER I* and *PAPER II*).

5. REFERENCES

Aarstad, O.A., Tøndervik, A., Sletta, H., Skjåk-Bræk, G. 2011. Alginate Sequencing: An Analysis of Block Distribution in Alginates Using Specific Alginate Degrading Enzymes. *Biomacromolecules*, **13**(1), 106-116.

Adams, J.M., Gallagher, J.A., Donnison, I.S. 2009. Fermentation study on Saccharina latissima for bioethanol production considering variable pre-treatments. *Journal of Applied Phycology*, **21**(5), 569-574.

Adams, J.M.M., Ross, A.B., Anastasakis, K., Hodgson, E.M., Gallagher, J.A., Jones, J.M., Donnison, I.S. 2011. Seasonal variation in the chemical composition of the bioenergy feedstock Laminaria digitata for thermochemical conversion. *Bioresource Technology*, **102**(1), 226-234.

Aitken, D., Bulboa, C., Godoy-Faundez, A., Turrion-Gomez, J.L., Antizar-Ladislao, B. 2014. Life cycle assessment of macroalgae cultivation and processing for biofuel production. *Journal of Cleaner Production*, **75**, 45-56.

Ale, M.T., Meyer, A.S. 2013. Fucoidans from brown seaweeds: an update on structures, extraction techniques and use of enzymes as tools for structural elucidation. *RSC Advances*, **3**(22), 8131-8141.

Alvarado-Morales, M., Boldrin, A., Karakashev, D.B., Holdt, S.L., Angelidaki, I., Astrup, T. 2013. Life cycle assessment of biofuel production from brown seaweed in Nordic conditions. *Bioresource technology*, **129**(0), 92-99.

Alvarado-Morales, M., Gunnarsson, I.B., Fotidis, I.A., Vasilakou, E., Lyberatos, G., Angelidaki, I. 2015. Laminaria digitata as a potential carbon source for succinic acid and bioenergy production in a biorefinery perspective. *Algal Research*, **9**, 126-132.

Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J. 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. *Bioresource technology*, **101**(13), 4851-4861.

Angell, A.R., Mata, L., Nys, R., Paul, N.A. 2015. The protein content of seaweeds: a universal nitrogen-to-protein conversion factor of five. *Journal of Applied Phycology*, **28**(1), 511-524.

Aresta, M., Dibenedetto, A., Barberio, G. 2005. Utilization of macro-algae for enhanced CO2 fixation and biofuels production: Development of a computing software for an LCA study. *Fuel Processing Technology*, **86**(14–15), 1679-1693.

Arnous, A., Meyer, A.S. 2008. Comparison of methods for compositional characterization of grape (Vitis vinifera L.) and apple (Malus domestica) skins. *Food and Bioproducts Processing*, **86**(C2), 79-86.

Balat, M., Balat, H. 2009. Recent trends in global production and utilization of bio-ethanol fuel. *Applied Energy*, **86**(11), 2273-2282.

Bilan, M.I., Grachev, A.A., Shashkov, A.S., Kelly, M., Sanderson, C.J., Nifantiev, N.E., Usov, A.I. 2010. Further studies on the composition and structure of a fucoidan preparation from the brown alga Saccharina latissima. *Carbohydrate Research*, **345**(14), 2038-2047.

Black, W. 1950. The seasonal variation in weight and chemical composition of the common British laminariaceae. *J. Mar. Biol. Assoc. U.K.* , **29**, 45-72.

Bold, H.C., Wynne, M.J. 1985. *Introduction to the Algae: Structure and Reproduction. 2nd ed*. Prentice-Hall, Inc., Englewood Cliffs, NJ.

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

Buck, B., Buchholz, C. 2004. The offshore-ring: A new system design for the open ocean aquaculture of macroalgae. *Journal of Applied Phycology*, **16**(5), 355-368.

Buck, B.H., Buchholz, C.M. 2005. Response of offshore cultivated Laminaria saccharina to hydrodynamic forcing in the North Sea. *Aquaculture*, **250**(3–4), 674-691.

Carstensen, J., Conley, D.J., Andersen, J.H., Ærtebjerg, G. 2006. Coastal eutrophication and trend reversal: A Danish case study. *Limnology and Oceanography*, **51**(1part2), 398-408.

Conley, D.J., Kaas, H., Møhlenberg, F., Rasmussen, B., Windolf, J. Characteristics of Danish estuaries. *Estuaries*, **23**(6), 820-837.

Cumashi, A., Ushakova, N.A., Preobrazhenskaya, M.E., D'Incecco, A., Piccoli, A., Totani, L., Tinari, N., Morozevich, G.E., Berman, A.E., Bilan, M.I., Usov, A.I., Ustyuzhanina, N.E., Grachev, A.A., Sanderson, C.J., Kelly, M., Rabinovich, G.A., Iacobelli, S., Nifantiev, N.E. 2007. A comparative study of the anti-inflammatory, anticoagulant, antiangiogenic, and antiadhesive activities of nine different fucoidans from brown seaweeds. *Glycobiology*, **17**(5), 541-552.

Davis, T.A., Volesky, B., Mucci, A. 2003. A review of the biochemistry of heavy metal biosorption by brown algae. *Water Research*, **37**(18), 4311-4330.

Dean, T.A., Jacobsen, F.R. Growth of juvenile Macrocystis pyrifera (Laminariales) in relation to environmental factors. *Marine Biology*, **83**(3), 301-311.

Deniaud-Bouët, E., Kervarec, N., Michel, G., Tonon, T., Kloareg, B., Hervé, C. 2014. Chemical and enzymatic fractionation of cell walls from Fucales: insights into the structure of the extracellular matrix of brown algae. *Annals of Botany*, **114**(6), 1203-1216.

Fertah, M., Belfkira, A., Dahmane, E.m., Taourirte, M., Brouillette, F. 2014. Extraction and characterization of sodium alginate from Moroccan Laminaria digitata brown seaweed. *Arabian Journal of Chemistry*, published online.

Formo, K., Aarstad, O.A., Skjåk-Bræk, G., Strand, B.L. 2014. Lyase-catalyzed degradation of alginate in the gelled state: Effect of gelling ions and lyase specificity. *Carbohydrate Polymers*, **110**(0), 100-106.

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

González López, C.V., García, M.d.C.C., Fernández, F.G.A., Bustos, C.S., Chisti, Y., Sevilla, J.M.F. 2010. Protein measurements of microalgal and cyanobacterial biomass. *Bioresource technology*, **101**(19), 7587-7591.

Hall, C., Balogh, S., Murphy, D. 2009. What is the Minimum EROI that a Sustainable Society Must Have? *Energies*, **2**(1), 25.

Handå, A., Forbord, S., Wang, X., Broch, O.J., Dahle, S.W., Størseth, T.R., Reitan, K.I., Olsen, Y., Skjermo, J. 2013. Seasonal- and depth-dependent growth of cultivated kelp (Saccharina latissima) in close proximity to salmon (Salmo salar) aquaculture in Norway. *Aquaculture*, **414–415**, 191-201.

Holdt, S.L., Kraan, S. 2011. Bioactive compounds in seaweed: functional food applications and legislation. *Journal of Applied Phycology*, **23**(3), 543-597.

Horn, S.J., Aasen, I.M., Østgaard, K. 2000a. Ethanol production from seaweed extract. *Journal of Industrial Microbiology & Biotechnology*, **25**(5), 249-254.

Horn, J.S., Aasen, M.I., Østgaard, K. 2000b. Production of ethanol from mannitol by Zymobacter palmae. *Journal of Industrial Microbiology and Biotechnology*, **24**(1), 51-57.

Hou, X., Hansen, J.H., Bjerre, A.-B. 2015. Integrated bioethanol and protein production from brown seaweed Laminaria digitata. *Bioresource technology*, **197**, 310-317.

Huang, L., Zhou, J., Li, X., Peng, Q., Lu, H., Du, Y. 2013. Characterization of a new alginate lyase from newly isolated Flavobacterium sp. S20. *Journal of Industrial Microbiology & Biotechnology*, **40**(1), 113-122.

Indergaard, M., Minsaas, J. 1991. Animal and Human Nutrition. 1 ed. in: *Seaweed Resources in Europe: Uses and Potential*, (Eds.) M.D. Guiry, G. Blunden, John Wiley & Sons. Chichester, UK, pp. 21-64.

Indergaard, M., Skjåk-Bræk, G., Jensen, A. 1990. Studies on the Influence of Nutrients on the Composition and Structure of Alginate in Laminaria saccharina (L.) Lamour. (Laminariales, Phaeophyceae). in: *Botanica Marina*, Vol. 33, pp. 277.

Inoue, A., Takadono, K., Nishiyama, R., Tajima, K., Kobayashi, T., Ojima, T. 2014. Characterization of an Alginate Lyase, FlAlyA, from Flavobacterium sp. Strain UMI-01 and Its Expression in Escherichia coli. *Marine Drugs*, **12**(8), 4693.

Iwamoto, Y., Araki, R., Iriyama, K.-i., Oda, T., Fukuda, H., Hayashida, S., Muramatsu, T. 2001. Purification and Characterization of Bifunctional Alginate Lyase from Alteromonas sp. Strain No. 272 and Its Action on Saturated Oligomeric Substrates. *Bioscience, Biotechnology, and Biochemistry*, **65**(1), 133-142.

Jang, J.-S., Cho, Y., Jeong, G.-T., Kim, S.-K. 2011. Optimization of saccharification and ethanol production by simultaneous saccharification and fermentation (SSF) from seaweed, Saccharina japonica. *Bioprocess and Biosystems Engineering*, **35**(1), 11-18.

Jiang, R., Ingle, K.N., Golberg, A. 2016. Macroalgae (seaweed) for liquid transportation biofuel production: what is next? *Algal Research*, **14**, 48-57.

John, R.P., Anisha, G.S., Nampoothiri, K.M., Pandey, A. 2011. Micro and macroalgal biomass: A renewable source for bioethanol. *Bioresource technology*, **102**(1), 186-193.

Jong, E.d., Higson, A., Walsh, P., Wellish, M. 2012. IEA Bioenergy - Task 42 Biorefinery: Bio-based Chemicals, Value Added Products from Biorefineries. (www.iea-bioenergy.task42-biorefineries.com; last accessed on 11.02.2016)

Kerrison, P.D., Stanley, M.S., Edwards, M.D., Black, K.D., Hughes, A.D. 2015. The cultivation of European kelp for bioenergy: Site and species selection. *Biomass and Bioenergy*, **80**, 229-242.

Kim, H., Lee, C.-G., Lee, E. 2011a. Alginate lyase: Structure, property, and application. *Biotechnology and Bioprocess Engineering*, **16**(5), 843-851.

Kim, N.-J., Li, H., Jung, K., Chang, H.N., Lee, P.C. 2011. Ethanol production from marine algal hydrolysates using Escherichia coli KO11. *Bioresource technology*, **102**(16), 7466-7469.

Kraan, S. 2013. Mass-cultivation of carbohydrate rich macroalgae, a possible solution for sustainable biofuel production. *Mitigation and Adaptation Strategies for Global Change*, **18**(1), 27-46.

Kumar, P., Barrett, D.M., Delwiche, M.J., Stroeve, P. 2009. Methods for Pretreatment of Lignocellulosic Biomass for Efficient Hydrolysis and Biofuel Production. *Industrial & Engineering Chemistry Research*, **48**(8), 3713-3729.

Lee, J.y., Li, P., Lee, J., Ryu, H.J., Oh, K.K. 2013. Ethanol production from Saccharina japonica using an optimized extremely low acid pretreatment followed by simultaneous saccharification and fermentation. *Bioresource technology*, **127**, 119-125.

Lourenco, S.O., Barbarino, E., De-Paula, J.C., Pereira, L.O.d.S., Lanfer Marquez, U.M. 2002. Amino acid composition, protein content and calculation of nitrogen-to-protein conversion factors for 19 tropical seaweeds. *Phycological Research*, **50**(3), 233-241.

Luo, X., Zhu, J.Y. 2011. Effects of drying-induced fiber hornification on enzymatic saccharification of lignocelluloses. *Enzyme and Microbial Technology*, **48**(1), 92-99.

Luo, X.L., Zhu, J.Y., Gleisner, R., Zhan, H.Y. 2011. Effects of wet-pressing-induced fiber hornification on enzymatic saccharification of lignocelluloses. *Cellulose*, **18**(4), 1055-1062.

Marinho, G., Holdt, S., Birkeland, M., Angelidaki, I. 2015. Commercial cultivation and bioremediation potential of sugar kelp, Saccharina latissima, in Danish waters. *Journal of Applied Phycology*, **27**(5), 1963-1973.

McHugh, D.J. 2003. A guide to the seaweed industry. FAO Fisheries. Technical Paper 441. Food and Agriculture Organisation of the United Nations, Rome.

Méléder, V., Populus, J., Guillaumont, B., Perrot, T., Mouquet, P. 2010. Predictive modelling of seabed habitats: case study of subtidal kelp forests on the coast of Brittany, France. *Marine Biology*, **157**(7), 1525-1541.

Michel, G., Tonon, T., Scornet, D., Cock, J.M., Kloareg, B. 2010a. The cell wall polysaccharide metabolism of the brown alga Ectocarpus siliculosus. Insights into the evolution of extracellular matrix polysaccharides in Eukaryotes. *New Phytologist*, **188**(1), 82-97.

Michel, G., Tonon, T., Scornet, D., Cock, J.M., Kloareg, B. 2010b. Central and storage carbon metabolism of the brown alga Ectocarpus siliculosus: insights into the origin and evolution of storage carbohydrates in Eukaryotes. *New Phytologist*, **188**(1), 67-81.

Middelboe, A.L., Sand-Jensen, K., Krause-Jensen, D. 1998. Patterns of macroalgal species diversity in Danish estuaries. *Journal of Phycology*, **34**(3), 457-466.

Mikkelsen, M.D., Harholt, J., Ulvskov, P., Johansen, I.E., Fangel, J.U., Doblin, M.S., Bacic, A., Willats, W.G.T. 2014. Evidence for land plant cell wall biosynthetic mechanisms in charophyte green algae. *Annals of Botany*, **114**(6), 1217-1236.

Miyake, O., Hashimoto, W., Murata, K. 2003. An exotype alginate lyase in Sphingomonas sp. A1: overexpression in Escherichia coli, purification, and characterization of alginate lyase IV (A1-IV). *Protein Expression and Purification*, **29**(1), 33-41.

Miyake, O., Ochiai, A., Hashimoto, W., Murata, K. 2004. Origin and Diversity of Alginate Lyases of Families PL-5 and -7 in Sphingomonas sp. Strain A1. *Journal of Bacteriology*, **186**(9), 2891-2896.

Morrissey, J., Kraan, S., Guiry, M.D., Institute, M.R.M.S., Board, I.S.F., Ireland, N.L.o. 2001. *A Guide to Commercially Important Seaweeds on the Irish Coast*. Bord Iascaigh Mhara/Irish Sea Fisheries Board.

Mueller, S., Llewellin, E.W., Mader, H.M. 2009. The rheology of suspensions of solid particles. *Proceedings of the Royal Society* **466**, 1201-1228.

Nielsen, M., Bruhn, A., Rasmussen, M., Olesen, B., Larsen, M., Møller, H. 2012. Cultivation of Ulva lactuca with manure for simultaneous bioremediation and biomass production. *Journal of Applied Phycology*, **24**(3), 449-458.

Nielsen, M.M., Manns, D., D'Este, M., Krause-Jensen, D., Rasmussen, M.B., Larsen, M.M., Alvarado-Morales, M., Angelidaki, I., Bruhn, A. 2016. Variation in biochemical composition of Saccharina latissima and Laminaria digitata along an estuarine salinity gradient in inner Danish waters. *Algal Research*, **13**, 235-245.

Novozymes, A.S. 2010 Application sheet: Novozymes Cellic®CTec2 and HTec2 - Enzymes for hydrolysis of lignocellulosic. Novozymes A/S, Bagsvaerd, Denmark. (http://bioenergy.novozymes.com/en/cellulosic-ethanol/CellicCTec3/Documents/AS_2010-01668-03.pdf; last accessed on 16.02.2016)

Obluchinskaya, E.D. 2008. Comparative chemical composition of the Barents Sea brown algae. *Applied Biochemistry and Microbiology*, **44**(3), 305-309.

Ogura, K., Yamasaki, M., Mikami, B., Hashimoto, W., Murata, K. 2008. Substrate recognition by family 7 alginate lyase from Sphingomonas sp. A1. *Journal of Molecular Biology*, **380**(2), 373-385.

Østgaard, K., Indergaard, M., Markussen, S., Knutsen, S.H., Jensen, A. 1993. Carbohydrate Degradation and Methane Production during Fermentation of *Laminaria saccharina* (Laminariales, Phaeophyceae). *Journal of Applied Phycology*, **5**(3), 333-342.

Ota, A., Kawai, S., Oda, H., Iohara, K., Murata, K. 2013. Production of ethanol from mannitol by the yeast strain Saccharomyces paradoxus NBRC 0259. *Journal of Bioscience and Bioengineering*, **116**(3), 327-332.

Park, H., Kam, N., Lee, E., Kim, H. 2012. Cloning and Characterization of a Novel Oligoalginate Lyase from a Newly Isolated Bacterium Sphingomonas sp. MJ-3. *Marine Biotechnology*, **14**(2), 189-202.

Pedersen, M., Meyer, A.S. 2009. Influence of substrate particle size and wet oxidation on physical surface structures and enzymatic hydrolysis of wheat straw. *Biotechnology Progress*, **25**(2), 399-408.

Percival, E., McDowell, R.H. 1967. *Chemistry and enzymology of marine algal polysaccharides*. Academic Press Inc. (London) Ltd.

Pereira, L., Gheda, S.F., Ribeiro-Claro, P.J.A. 2013. Analysis by Vibrational Spectroscopy of Seaweed Polysaccharides with Potential Use in Food, Pharmaceutical, and Cosmetic Industries. *International Journal of Carbohydrate Chemistry*, **2013**, 7.

Ravanal, M.C., Pezoa-Conte, R., von Schoultz, S., Hemming, J., Salazar, O., Anugwom, I., Jogunola, O., Mäki-Arvela, P., Willför, S., Mikkola, J.-P., Lienqueo, M.E. 2016. Comparison of different types of pretreatment and enzymatic saccharification of Macrocystis pyrifera for the production of biofuel. *Algal Research*, **13**, 141-147.

Raybaud, V., Beaugrand, G., Goberville, E., Delebecq, G., Destombe, C., Valero, M., Davoult, D., Morin, P., Gevaert, F. 2013. Decline in Kelp in West Europe and Climate. *Plos One*, **8**(6), e66044.

Rhein-Knudsen, N., Ale, M., Meyer, A. 2015. Seaweed Hydrocolloid Production: An Update on Enzyme Assisted Extraction and Modification Technologies. *Marine Drugs*, **13**(6), 3340.

Rioux, L.E., Turgeon, S.L., Beaulieu, M. 2007. Characterization of polysaccharides extracted from brown seaweeds. *Carbohydrate Polymers*, **69**(3), 530-537.

Rioux, L.-E., Turgeon, S.L., Beaulieu, M. 2010. Structural characterization of laminaran and galactofucan extracted from the brown seaweed Saccharina longicruris. *Phytochemistry*, **71**(13), 1586-1595.

Roesijadi, G., Jones, S.B., Snowden-Swan, L.J., Zhu, Y. 2010. Macroalgae as a Biomass Feedstock: A Preliminary Analysis, (Ed.) U.S.D.o.E. (DOE), Pacific Northwest National Laboratory. Richland, Washington, pp. 50.

Ross, A.B., Jones, J.M., Kubacki, M.L., Bridgeman, T. 2008. Classification of macroalgae as fuel and its thermochemical behaviour. *Bioresource Technology*, **99**(14), 6494-6504.

Rupérez, P. 2002. Mineral content of edible marine seaweeds. *Food Chemistry*, **79**(1), 23-26.

Ryu, M., Lee, E.Y. 2011. Saccharification of alginate by using exolytic oligoalginate lyase from marine bacterium Sphingomonas sp. MJ-3. *Journal of Industrial and Engineering Chemistry*, **17**(5–6), 853-858.

Sanderson, J.C., Cromey, C.J., Dring, M.J., Kelly, M.S. 2008. Distribution of nutrients for seaweed cultivation around salmon cages at farm sites in north–west Scotland. *Aquaculture*, **278**(1–4), 60-68.

Schiener, P., Black, K., Stanley, M., Green, D. 2015. The seasonal variation in the chemical composition of the kelp species Laminaria digitata, Laminaria hyperborea, Saccharina latissima and Alaria esculenta. *Journal of Applied Phycology*, **27**(1), 363-373.

Schultz-Jensen, N., Thygesen, A., Leipold, F., Thomsen, S.T., Roslander, C., Lilholt, H., Bjerre, A.B. 2013. Pretreatment of the macroalgae Chaetomorpha linum for the production of bioethanol – Comparison of five pretreatment technologies. *Bioresource technology*, **140**(0), 36-42.

Siddhanta, A.K., Prasad, K., Meena, R., Prasad, G., Mehta, G.K., Chhatbar, M.U., Oza, M.D., Kumar, S., Sanandiya, N.D. 2009. Profiling of cellulose content in Indian seaweed species. *Bioresource technology*, **100**(24), 6669-6673.

Silva, G.G.D., Couturier, M., Berrin, J.-G., Buléon, A., Rouau, X. 2012. Effects of grinding processes on enzymatic degradation of wheat straw. *Bioresource technology*, **103**(1), 192-200.

Singh, A., Nigam, P.S., Murphy, J.D. 2011. Renewable fuels from algae: An answer to debatable land based fuels. *Bioresource technology*, **102**(1), 10-16.

Slocombe, S.P., Ross, M., Thomas, N., McNeill, S., Stanley, M.S. 2013. A rapid and general method for measurement of protein in micro-algal biomass. *Bioresource technology*, **129**, 51-57.

Sluiter, A., Hames, B., Ruiz, R. Scarlata, C., Sluiter, J., Templeton, D., Crocker, D. 2011. *Determination of structural carbohydrates and lignin in biomass*. NREL Technical Report July 2011, NREL/TP-510-42618 (Version 07.08.2011).

Sutter, J.D., Berlinger, J., Ellis, R. 2015. *Final draft of climate deal formally accepted in Paris*. CNN. (http://edition.cnn.com/2015/12/12/world/global-climate-change-conference-vote/index.html.; last accessed on 18.12.2016)

Taelman, S.E., Champenois, J., Edwards, M.D., De Meester, S., Dewulf, J. 2015. Comparative environmental life cycle assessment of two seaweed cultivation systems in North West Europe with a focus on quantifying sea surface occupation. *Algal Research*, **11**, 173-183.

Takeda, H., Yoneyama, F., Kawai, S., Hashimoto, W., Murata, K. 2011. Bioethanol production from marine biomass alginate by metabolically engineered bacteria. *Energy & Environmental Science*, **4**(7), 2575-2581.

Thomas, F., Lundqvist, L.C.E., Jam, M., Jeudy, A., Barbeyron, T., Sandström, C., Michel, G., Czjzek, M. 2013. Comparative Characterization of Two Marine Alginate Lyases from Zobellia galactanivorans Reveals

Distinct Modes of Action and Exquisite Adaptation to Their Natural Substrate. *Journal of Biological Chemistry*, **288**(32), 23021-23037.

Torosantucci, A., Bromuro, C., Chiani, P., De Bernardis, F., Berti, F., Galli, C., Norelli, F., Bellucci, C., Polonelli, L., Costantino, P., Rappuoli, R., Cassone, A. 2005. A novel glyco-conjugate vaccine against fungal pathogens. *The Journal of Experimental Medicine*, **202**(5), 597-606.

Usov, A.I., Smirnova, G.P., Klochkova, N.G. 2001. Polysaccharides of algae: 55. Polysaccharide composition of several brown algae from Kamchatka. *Russian Journal of Bioorganic Chemistry*, **27**(6), 395-399.

Vanegas, C.H., Hernon, A., Bartlett, J. 2015. Enzymatic and organic acid pretreatment of seaweed: effect on reducing sugars production and on biogas inhibition. *International Journal of Ambient Energy*, **36**(1), 2-7.

Wang, J., Kim, Y.M., Rhee, H.S., Lee, M.W., Park, J.M. 2013. Bioethanol production from mannitol by a newly isolated bacterium, Enterobacter sp. JMP3. *Bioresource technology*, **135**, 199-206.

Wargacki, A.J., Leonard, E., Win, M.N., Regitsky, D.D., Santos, C.N.S., Kim, P.B., Cooper, S.R., Raisner, R.M., Herman, A., Sivitz, A.B., Lakshmanaswamy, A., Kashiyama, Y., Baker, D., Yoshikuni, Y. 2012. An Engineered Microbial Platform for Direct Biofuel Production from Brown Macroalgae. *Science*, **335**(6066), 308-313.

Wei, N., Quarterman, J., Jin, Y.-S. 2013. Marine macroalgae: an untapped resource for producing fuels and chemicals. *Trends in Biotechnology*, **31**(2), 70-77.

Wijesinghe, W.A.J.P., Jeon, Y.-J. 2012. Enzyme-assistant extraction (EAE) of bioactive components: A useful approach for recovery of industrially important metabolites from seaweeds: A review. *Fitoterapia*, **83**(1), 6-12.

Willför, S., Pranovich, A., Tamminen, T., Puls, J., Laine, C., Suurnäkki, A., Saake, B., Uotila, K., Simolin, H., Hemming, J., Holmbom, B. 2009. Carbohydrate analysis of plant materials with uronic acid-containing polysaccharides–A comparison between different hydrolysis and subsequent chromatographic analytical techniques. *Industrial Crops and Products*, **29**(2–3), 571-580.

Wong, T.Y., Preston, L.A., Schiller, N.L. 2000. Alginate lyase: review of major sources and enzyme characteristics, structure-function analysis, biological roles, and applications. *Annual review of microbiology*, **54**, 289-340.

Yamasaki, M., Ogura, K., Hashimoto, W., Mikami, B., Murata, K. 2005. A Structural Basis for Depolymerization of Alginate by Polysaccharide Lyase Family-7. *Journal of Molecular Biology*, **352**(1), 11-21.

Yanagisawa, M., Nakamura, K., Ariga, O., Nakasaki, K. 2011. Production of high concentrations of bioethanol from seaweeds that contain easily hydrolyzable polysaccharides. *Process Biochemistry*, **46**(11), 2111-2116.

Yeh, A.-I., Huang, Y.-C., Chen, S.H. 2010. Effect of particle size on the rate of enzymatic hydrolysis of cellulose. *Carbohydrate Polymers*, **79**(1), 192-199.

Yoon, H.-J., Hashimoto, W., Miyake, O., Okamoto, M., Mikami, B., Murata, K. 2000. Overexpression in Escherichia coli, Purification, and Characterization of Sphingomonas sp. A1 Alginate Lyases. *Protein Expression and Purification*, **19**(1), 84-90.

Zhu, B., Yin, H. 2015. Alginate lyase: Review of major sources and classification, properties, structure-function analysis and applications. *Bioengineered*, **6**(3), 125-131.

Zhu, W., Zhu, J.Y., Gleisner, R., Pan, X.J. 2010. On energy consumption for size-reduction and yields from subsequent enzymatic saccharification of pretreated lodgepole pine. *Bioresource technology*, **101**(8), 2782-2792.

Zimmerman, R.C., Kremer, J.N. 1986. In situ growth and chemical composition of the giant kelp, *Macrocystis pyrifera*: response to temporal changes in ambient nutrient availability. *Marine Ecoolgy. Progress Series,* **27**, 277–285.

PAPERS

PAPER I

Methodology for quantitative determination of the carbohydrate composition of brown seaweeds (Laminariaceae)

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PAPER

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Methodology for quantitative determination of the carbohydrate composition of brown seaweeds (Laminariaceae)

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The monosaccharide composition of four different samples of brown seaweeds *Laminaria digitata* and *Saccharina latissima* were compared by different high performance anion exchange chromatography (HPAEC) methods after different acid hydrolysis treatments or a cellulase treatment. A two-step treatment of 72% (w/w) H_2SO_4 + 4% (w/w) H_2SO_4 performed best, but cellulase treatment released more glucose than acid treatments. HPAEC with pulsed amperometric detection (PAD) allowed quantification of all present neutral sugars and the sugar alcohol mannitol. Furthermore, the use of guluronic, glucuronic, and galacturonic acid as standards enabled quantification of the uronic acids. A complete map of amino acids, fatty compounds, minerals, and ash was also achieved. *L. digitata* and *S. latissima* harvested in Denmark April (Baltic Sea, 2012) were dominated by alginic acid and ash (each ~30% by weight (w/w) of the dry matter) and 10% (w/w) protein. In contrast, the dominant compound of *L. digitata* harvested in August (North Sea, 2012) was glucose constituting 51% w/w of the dry matter, and with 16% w/w alginic acid. Washing prior to analysis mainly removed salts.

1 Introduction

Recently, carbohydrates from brown macroalgae (brown seaweeds) have received increased attention, also in Europe, as a new biomass resource for biofuels and manufacture of high-value carbohydrate products.[1,2] However, the proper assessment of the potential of this new resource for biorefinery purposes requires fast and reliable characterization of the biomass, notably with respect to the carbohydrate composition.

Several extraction and determination methods for particular compounds have been developed but no methods exist for total quantification of the carbohydrate contents and carbohydrate composition of brown seaweeds.

The composition of polysaccharides in (fibrous) terrestrial plant materials is usually determined by measuring the monosaccharide release after acid hydrolysis. The optimal type of acid hydrolysis treatment depends on the type of plant material, and no universal method exists. For pectinaceous plant materials, rich in uronic acids, treatment with hydrochloric acid (HCl) or trifluoroacetic acid (TFA) is usually favored,[3,4] whereas for lignocellulosic biomass acid hydrolysis with sulfuric acid (H_2SO_4) is generally the norm.[5–7] Analogously, different chromatography quantification techniques have subsequently been employed to assess the composition of the constituent monosaccharides.

Brown seaweeds (Phaeophyceae) are highly heterogeneous in their carbohydrate composition and the polysaccharides differ profoundly from those in terrestrial plants. Brown seaweed biomass is mainly composed of β-linked polysaccharides of neutral sugars and uronic acids but also harbor the sugar alcohol mannitol and proteins along with high ash contents. In the relatively cold Northern hemisphere, such as the European, North American, and Canadian waters, the carbohydrate composition varies throughout the year, with maximum ash, protein, and matrix polysaccharides (alginate, fucoidan) contents at the beginning of the spring, when the reserve compounds mannitol and laminarin are at a minimum. In the autumn the reverse is the case. Additionally, the carbohydrate structures and composition vary with the species, age of the algae population, and geographical location.[1,8,9]

Laminarin is the principal and unique carbohydrate reserve substance of brown seaweeds. This polysaccharide mainly consists of a backbone of (insoluble) β-1,3-bonded glucopyranoses of which some carry β-1,6-branched glucose residues. A typical laminarin chain is presumed to be made up of approximately 25 units that may be terminated with the other reserve substrate D-mannitol (M-chains) or glucose (G-chains), which are found in different ratios at the reducing end.[9–11] Mannitol, the alcohol form of mannose, is the first product of photosynthesis in brown macroalgae.[8,9] The amounts of laminarin and

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mannitol found in the most studied brown seaweed species *Laminaria digitata* and *Saccharina latissima*, both belonging to the Laminariaceae family, differ widely due to large seasonal variations. Hence, levels ranging from 0–33% by weight of the total dry matter (w/w) for laminarin and 2–20% w/w for mannitol have been reported depending on the harvest month.[1,12]

Alginic acid, or alginate, consists of 1,4-glycosidically linked α-L-guluronic acid (G) and β-D-mannuronic acid (M) in varying proportions forming linear chains with M/G ratio ranges of 1.2 to 2.1 and higher. Hence, alginic acid (alginate) does not designate one particular monosaccharide or one type of homopolysaccharide. The linear chains are made up of different blocks of guluronic and mannuronic acids, which are C-5 epimers.[9] The blocks are referred to as MM blocks or GG blocks, but less crystalline MG blocks may also occur. Alginate is the salt of alginic acid and is soluble with monovalent ions, *e.g.* K^+, Na^+, and insoluble with di-/polyvalent ions (except Mg^{2+}). In the presence of Ca^{2+} the GG blocks form ionic complexes to generate a stacked structure known as the "egg-box model", responsible for hard gel formation.[9,13,14]

Fucoidans constitute another unique type of brown seaweed polysaccharide. Primarily, fucoidans from the Laminariaceae are composed of a backbone of α-1,3-linked-L-fucopyranose residues with sulfate substitutions at C-4 and occasionally at the C-2 position in addition to 2-*O*-α-L-fucopyranosyl, other glycosyl such as galactose, and/or acetate substitutions.[15,16] However, the chemical structures and abundance of the sulfated fucans making up fucoidan in brown seaweeds vary significantly.[15] Alginate and fucoidan as matrix substances can be found at any time in the seaweeds of Laminariaceae, but their relative amounts vary with the season, for alginate the levels vary from 17 to 45%, for fucoidan between 3 and 10% (w/w).[12,17,18] However, exact determination is difficult due to high heterogeneity and the data also vary with the extraction method. Cellulose in brown seaweed has received less attention but has been mentioned in the literature as a structural monosaccharide present in minor amounts.[9,19] Besides polysaccharides, minerals and proteins constitute a significant proportion of the dry weight of brown seaweeds, mineral levels ranging from 15 to 39% w/w, and protein levels from 3 to 16% w/w. On the contrary, lipids always make up only a smaller fraction (below 2% w/w) in brown seaweeds.[19,20] The significant differences in the bond types and the types of monomeric carbohydrate building blocks dominating in terrestrial plants and brown seaweeds, respectively, call for attention to both the acid hydrolysis and the quantitative chromatography methodology used for compositional carbohydrate analysis of brown seaweeds.

The primary objective of this study was to examine the influence of different biomass material hydrolysis treatments and compare different high performance chromatography carbohydrate determination methods (borate *vs.* alkaline (NaOH) elution) in order to identify an optimal strategy for determination of all structural carbohydrate monomers from one hydrolysate of brown seaweed. Another objective was to assess the options for using cellulases for direct enzymatic glucose release from the structural laminarin in the brown seaweed. Different samples of *L. digitata* and *S. latissima* were used as raw materials for the study (Table 1).

2 Experimental

2.1 Materials

L. digitata and *S. latissima* were harvested in April 2012 from the Danish Baltic Sea and freeze-dried. Another harvest of *L. digitata* was obtained from the Danish North Sea coast late August 2012. One part of this latter material was washed successively four times with water to remove residual sand and salt. Another fraction remained untreated. Both the washed and the unwashed material were oven-dried at 40 °C until equilibrium moisture (Table 1). As a benchmark for the acid hydrolysis and carbohydrate analyses, hydrothermally pretreated barley straw fibers were used; the straw had been subjected to a triple heating treatment at 16% w/w dry matter (DM): 60 °C, 15 min; liquids removed; 180 °C, 10 min; and finally 195 °C, 3 min.[21] The pretreated barley straw was frozen, then defrosted and oven-dried at 40 °C until equilibrium moisture before use. Before analysis the dried seaweed materials and the pretreated straw material were ground by vibrating disc milling to pass a 100 μm sieve.

Chemicals. Boric acid, disodium tetraborate ($Na_2B_4O_7$), perchloric acid ($HClO_4$), sulfamic acid, sulphuric acid (H_2SO_4), trifluoroacetic acid (TFA), *m*-hydroxybiphenyl, dimethyl sulfoxide (DMSO), KOH, NaOH, all buffer salts, D-(+)fucose, L-rhamnose, L-(+)arabinose, D-(+)galactose, D-(+)xylose, D-(+)mannose, D-(+)galacturonic acid, and D-(+)glucuronic acid were from Sigma-Aldrich (Steinheim, Germany). Sodium acetate (NaOAc), D-mannitol, and 5-hydroxy-methyl furfural (5-HMF) were from Fluka/Sigma-Aldrich (Steinheim, Germany). Guluronic acid was purchased from Chemos GmbH (Regenstauf, Germany) and D-(+)glucose was from Merck (Darmstadt, Germany).

2.2 Methods

Hydrolysis methods

Sulfuric acid hydrolysis. A modified 2-step sulfuric acid hydrolysis of the NREL method[7] was applied exposing the

Table 1 Overview of origin and preparation of the received brown seaweed samples and barley straw used in the present study

Sample	Origin/preparation
L. digitata	April 2012 at Grenaa/Fornaes, Danish Baltic Sea coast (unwashed; freeze dried)
S. latissima	April 2012 at Grenaa/Fornaes, Danish Baltic Sea coast (unwashed; freeze dried)
L. digitata	End of August 2012 at Hanstholm, Danish North Sea coast (unwashed; oven dried)
L. digitata	End of August 2012 at Hanstholm, Danish North Sea coast (tap water washed to remove sand and salt; oven dried)
Barley straw	2006 at Funen, Denmark (hot water extracted by Rosgaard *et al.* 2007; fibers separated from liquid; oven dried)

ground material (100 mg dry material per mL) to 72% w/w H_2SO_4 at 30 °C for exactly 1 h; the reaction mixture was then diluted for the 2nd step to 4% w/w H_2SO_4 and the hydrolysis continued for 40 min at 120 °C in an autoclave (method A).[6] A milder 2nd step adapted from Moxley and Zhang[22] was performed using a 2% w/w solution of H_2SO_4 reacting for 30 min at 120 °C (method B). After hydrolysis, the hydrolysates were calibrated and filtered through a filter crucible (pore size 4; Schott, Germany).

Perchloric acid hydrolysis. A 2-step hydrolysis treatment was performed by adding 0.02 mL 70% w/w $HClO_4$ per 1 mg of dry sample and allowing the hydrolysis to proceed for 10 min at room temperature. The hydrolysate was then diluted with 0.2 mL water and the second hydrolysis step was then done at 120 °C for 60 min. After cooling, each sample was adjusted to neutral pH with 2 M KOH. Precipitated $KClO_4$ was separated by centrifugation. The supernatants were collected.[23] The remaining precipitate was re-dissolved in hot water and then passed through a filter crucible (pore size 4).

Trifluoroacetic acid (TFA) hydrolysis. Samples were weighed into screw-cap vials and 2 M TFA was added (10 mg dry material per mL). Each vial was tightly sealed and heated at 121 °C for 2 h. Hydrolysates were lyophilized at −20 °C under N_2. Prior to chromatographic analysis the lyophilized samples were re-dissolved in deionized water, calibrated and filtered through a filter crucible (pore size 4; Schott, Germany).[3] The acid-insoluble content, as well as the moisture content of all samples, were determined gravimetrically as the residue remaining after drying the filter crucibles at 103 °C overnight.

Enzymatic hydrolysis. The enzymatic treatment of the samples was conducted at 2% (w/w) substrate concentration in 0.1 M phosphate citrate buffer pH 5.1 at 50 °C and treated with 20% Cellic®CTec2 (enzyme/substrate level in % by weight). Cellic®CTec2 is a commercially available cellulase preparation derived from *Trichoderma reesei* containing at least the two main cellobiohydrolases EC 3.2.1.91 (Cel6A and Cel7A), five different *endo*-1,4-β-glucanases EC 3.2.1.4 (Cel7B, Cel5A, Cel12A, Cel61A, and Cel45A), β-glucosidase EC 3.2.1.21, β-xylosidase EC 3.2.1.37, and particular proprietary hydrolysis-boosting proteins (Novozymes A/S, Bagsværd, Denmark). The activity in filter paper units (FPU) of the enzyme preparation was 155 FPU mL^{-1}. During the enzymatic hydrolysis samples were taken out at 2, 4, 6 and 24 h. The reaction was stopped by mixing the sample with 5 M NaOH.

Carbohydrate analysis. Monomeric sugars, 5-hydroxy-methylfurfural (5-HMF), sugar alcohol mannitol and uronic acids in the hydrolysates were separated by a Dionex ICS-3000 HPAEC-PAD on a Dionex CarboPac PA20 column using the three eluents: A deionized water, B 200 mM NaOH and C 1 M NaOAc in 200 mM NaOH, all CO_2 free and dosed in % volume/volume (v/v). Prior to analysis, the samples were filtered through a 0.2 μm syringe tip filter and diluted appropriately in 200 mM NaOH. Chromatographic elution was carried out at a flow rate of 0.4 mL min^{-1} using B at 1% in A for 25 min for separation of neutral sugars and sugar alcohol. Subsequently, separation of uronic acids was performed by a linear gradient from 3 to 50% B plus 3 to 20% C in A for 20 min and completed with a linear gradient of C to 40% in 60% B and A within 5 min. The separated carbohydrates were detected using pulsed amperometric detection (PAD) with a gold working electrode. To increase the sensitivity of the detector after column addition of 200 mM NaOH was applied at a flow rate of 0.2 mL min^{-1} for the first 25 min and with a linear gradient down to 20 mM NaOH for the following 25 min.

The contents of glucose, xylose and mannose in the hydrolysates were also analyzed by borate-anion-exchange-chromatography with post column derivatization and UV detection at 560 nm (HPAEC-Borate) as described in detail by Sinner *et al.*[24] and Willfoer *et al.*[5] For identification and quantification of the carbohydrates the Dionex software Chromeleon 6.80 was used.

Total uronic acids (UAs) in the hydrolysates were detected spectrophotometrically at 525 nm based on the method described by Filisetti-Cozzi and Carpita.[25] Prior to the color reaction samples were filtered through a 0.2 μm syringe filter and diluted appropriately in deionized water. Then 4 M sulfamate (prepared after Filisetti-Cozzi and Carpita[25]) was added to the sample in proportion 1 : 10. The H_2SO_4 concentration was adjusted to 80% w/w by mixing the sample with H_2SO_4 (analytical grade) containing 120 mM $Na_2B_4O_7$. After adding the color reagent *m*-hydroxydiphenyl (prepared after van den Hoogen *et al.*[26]) the absorbance, 525 nm, was monitored for 20 min and the maximum was reported. Background absorbance was determined individually and subtracted before the UA content was determined as galacturonic acid (GalA) equivalents from the corresponding GalA reference curve. For estimation of the recovery factor (RF) GalA was treated according to the relevant sulfuric acid hydrolysis procedure and GalA was then quantified colorimetrically as described above.

Proximate, ultimate and metal analysis. C, H, N and S contents were measured by elemental analysis (vario EL cube, Elementar Hanau/Germany). The relative percentage of each was determined and the oxygen content was estimated as the difference and corrected for ash content. The ash contents were obtained and determined gravimetrically after low temperature oxidation (550 °C) of the samples in a furnace. For metal analysis the samples were digested with concentrated (65%) HNO_3 in a Milestone MLS Stat 1200 lab microwave and analyzed by inductively coupled plasma spectrometry (ICP) with mass spectrometric detection (Thermo Scientific iCAP 6300).

Analysis of amino acids and fatty compounds. Amino acid analyses (AAA) were performed according to Barkholt and Jensen.[27] Extraction of fatty compounds was carried out with the solvent petrol in an ASE apparatus (Accelerated Solvent Extractor, Dionex Corp.) in two cycles at 70 °C and 100 bar.[28]

FTIR spectroscopy. Residues from the 2-step sulfuric acid hydrolysis (method A) were measured on a Bruker Vector 33 FTIR-spectrometer. The spectra were recorded between 3750 and 583 cm^{-1} on a DTGS detector using attenuated total reflection; resolution 4 cm^{-1}; 60 scans; analysis software OPUS 6.5 (Bruker, Germany).[29]

2.3 Statistics

One-way analyses of variances (one-way ANOVA): 95% confidence intervals were compared as Tukey–Kramer intervals calculated from pooled standard deviations (Minitab Statistical Software, Addison-Wesley, Reading, MA).

3 Results and discussion

3.1 Monomeric carbohydrate yields from the decomposition techniques

Different plant polysaccharide acid hydrolysis methods for obtaining monomeric carbohydrates were investigated. Primarily, the employment of trifluoroacetic acid (TFA) hydrolysis (121 °C, 2 h) was inefficient on the brown seaweed samples (only April samples tested) and left behind a significant amount of residue making up approx. 30% by weight of the dry raw material weight (data not shown). In comparison, the amount of unhydrolysed residue on the same samples constituted ~5–10% w/w after the perchloric or the sulfuric acid hydrolysis treatments (Table 2). The amounts of hydrolysis residues obtained after perchloric acid hydrolysis on the seaweed were generally a little higher than those obtained for both sulfuric acid hydrolysis methods (Table 2). For the barley straw, the residue after perchloric acid was 41.6% w/w as opposed to that of ~30% w/w (also known as Klason Lignin) obtained after the sulfuric acid hydrolyses. Significantly lower monomeric carbohydrate yields, glucose, fucose and uronic acids, were obtained with the perchloric acid as compared to the strong acid hydrolysis, especially for the April harvested samples (Table 2). Determination of the fucose levels was less affected by the type of acid treatment, but as expected, the fucose levels tended to be higher in the samples harvested in the spring than in August (*L. digitata* Apr'12 *vs.* Aug'12, Table 2). The levels for mannitol were in the same range of 4 to 10% w/w for all brown seaweed samples after acid treatment, but the values tended, as expected, to be higher in the samples harvested in August (Table 2). Ostgaard *et al.*[30] measured mannitol directly in the supernatant of thawed *S. latissima* and found mannitol contents of 4% for spring and 16% for autumn respectively. Adams *et al.*[1] used a 5 mM sulfuric acid hydrolysis on ground *L. digitata* and also observed a seasonal variation of the mannitol ranging from a minimum of 5% w/w in the beginning of the year to a peak in June before the mannitol levels determined remained constant between 15 and 20% w/w.

Perchloric acid hydrolysis was demonstrated to give high glucose yields when applied on the highly polymerized substrate carboxy-methyl-cellulose.[23] Glucose levels determined for *L. digitata* and *S. latissima* from the April harvest, were significantly lower after $HClO_4$ treatment than after sulfuric acid hydrolysis, *e.g.* for *S. latissima* only 0.9% w/w compared to 4.6 and 6.8% w/w, respectively were recovered (HPAEC-PAD data, Table 2). A similar trend was observed for the glucose determined after acid hydrolysis on the pretreated straw (Table 2). Sulfuric acid hydrolysis performed by Ostgaard *et al.*[30] on *Laminaria saccharina* (now classified as *Saccharina latissima*) gave glucose concentrations, accounted for as laminarin, that were below 1% w/w for seaweed samples harvested in the spring, but 20% w/w for samples harvested in the autumn.

All acid hydrolysates were checked for 5-HMF as a degradation product of hexoses.[6] 5-HMF was not detected in any of the mildly treated sulfuric acid samples, *i.e.* with method B (except for the pretreated straw; 2 mg 5-HMF per g biomass). However, in the stronger sulfuric acid hydrolysates (method A) as well as after the $HClO_4$ treatment, 5-HMF was present in the samples having high glucose content, but only in minor amounts of <5 mg per g biomass (data not shown). Low contents of degradation products and hydrolysis residues indicated appropriate acid hydrolysis conditions for the decomposition of brown seaweed carbohydrates into monomers. Residues of the sulfuric acid hydrolysis (method A) were analyzed by FTIR, and this analysis indicated the presence of a variety of reaction products from the different polymers (data not shown). Elemental analysis revealed N contents below 3% by weight, very low contents of sulfur and 40–50% of C based on dry residues. Potentially, hydrolysis residues consist of condensed proteins, inorganic compounds and insoluble polysaccharides from incomplete hydrolysis, in particular alginic acid. Overall, the amounts of residue correlated with the ash content for all seaweed samples, but the amounts of residue were below 10% by weight of dry algae for all hydrolysis methods (Table 2).

Sulfuric acid hydrolysis with post-hydrolysis at 4% H_2SO_4 (method A) is widely used for lignocellulosic biomass analysis, and the method resembles the protocol recommended by the US National Renewable Laboratory (NREL) for acid hydrolysis of lignocellulosic feedstocks[7] – except that in NREL's protocol the second step includes autoclave heating for 60 min, not 40 min. Surprisingly, the highest monosaccharide levels of brown seaweed were generally achieved with H_2SO_4 hydrolysis (method A), notably with regard to the detection of uronic acids (UA), presumed to be mainly derived from alginate, as the uronic acid yields were significantly above those obtained with the other hydrolysis methods (Table 2). This finding was in accord with what was reported early by Percival and McDowell,[9] namely, that polysaccharides containing high levels of uronic acids like alginic acid, need drastic hydrolysis conditions to achieve a satisfactory decomposition into their carbohydrate monomers. The data obtained for uronic acids (Table 2) reflected the expected amount of alginic acid. Hence, the reported values for alginic acid content in *L. digitata* range from 17 to 44% by weight correlating with the seasonal variation – the highest levels are generally found in samples harvested winter/early-spring, whereas the lowest levels are found in samples harvested late summer/early autumn.[1,31] Uronic acids are discussed further in Section 3.2.

Additionally, the available glucans were enzymatically cleaved using the commercial enzyme preparation Cellic®CTec2 (Novozymes, Denmark). For the *L. digitata* samples harvested in August, high levels of hydrated glucose of 64 to 77% by weight were released by the enzymatic treatment within 6 h, and no further increase was noted. The HPAEC-PAD results for enzymatic glucose liberation from the April *L. digitata* harvest stayed constant at 10.7% already after 2 h of hydrolysis, whereas for the pretreated straw, the glucose yield increased over the whole duration of 24 h during the enzymatic treatment without releasing all potential monomeric glucose (Table 2). Adams *et al.*[1] used laminarinases, active only on β-1,3 glucan, to estimate the concentration of laminarin dependence on the season

Table 2 Monomeric carbohydrate yields (±SD) after different hydrolysis treatments and HPAEC-Borate or HPAEC-PAD analysis for brown seaweeds and barley straw. Hydrolysis residues were determined gravimetrically after acid treatment; post-hydrolysis with sulfuric acid at 4% concentration labelled as method A and 2% as method B. ANOVA analysis through the acidic hydrolysis treatments to determine significant differences per yield within each individual compound of sample. Different roman superscript letters indicate significant differences ($\alpha \leq 0.05$) column-wise per group[a]

		Mannitol [% dry material]	Fucose [% dry material]	Glucose [% dry material]		Others[1] [%dry material]		Uronic acids[3] [%dry material]	Residue [% dry material]
Samples	Hydrolysis treatment	PAD	PAD	Borate	PAD	Borate[2]	PAD	PAD	Gravimetric
	$HClO_4$	$4.2^{a} \pm <0.1$	$2.9^{a} \pm 0.1$	$1.1^{a} \pm <0.1$	$1.3^{a} \pm 0.1$	$0.7^{a} \pm 0.2$	$1.0^{a} \pm 0.2$	$7.6^{a} \pm 0.9$	$8.4^{a} \pm 1.3$
	H_2SO_4 (method A)	$4.1^{a} \pm 0.4$	$4.1^{b} \pm 0.4$	$7.9^{b} \pm 0.2$	$7.8^{b} \pm 0.2$	$1.2^{b} \pm 0.1$	$2.2^{b} \pm 0.3$	$32.5^{b} \pm 3.5$	$5.0^{a} \pm 1.6$
	H_2SO_4 (method B)	$3.7^{a} \pm 0.1$	$4.0^{b} \pm 0.1$	$7.4^{c} \pm 0.2$	$6.4^{c} \pm 0.2$	$1.2^{b} \pm 0.1$	$1.8^{b} \pm 0.1$	$26.0^{c} \pm 1.1$	$6.1^{a} \pm 2.1$
	enzym. Glc release[4]	5.0 ± 0.1	n.d.	8.7 ± 0.1	10.7 ± 0.4	$0.2 \pm <0.1$	$0.2 \pm <0.1$	n.d.	n.d.
S. latissima (Apr'12)	$HClO_4$	$6.1^{a} \pm 0.3$	$1.7^{a} \pm 0.1$	$0.8^{a} \pm 0.1$	$0.9^{a} \pm 0.1$	$0.5^{a} \pm 0.1$	$0.7^{a} \pm 0.1$	$7.2^{a} \pm 0.9$	$10.0^{a} \pm 0.4$
	H_2SO_4 (method A)	$6.5^{a} \pm 1.1$	$2.9^{b} \pm 0.5$	$6.5^{b} \pm <0.1$	$6.8^{b} \pm 1.2$	$0.7^{b} \pm 0.1$	$1.8^{b} \pm 0.4$	$31.8^{b} \pm 5.4$	$5.0^{b} \pm 0.1$
	H_2SO_4 (method B)	$5.1^{a} \pm 0.3$	$2.4^{ab} \pm 0.1$	$5.9^{c} \pm 0.4$	$4.6^{c} \pm 0.2$	$0.7^{b} \pm 0.1$	$1.2^{c} \pm 0.2$	$21.8^{c} \pm 0.9$	$8.4^{b} \pm 0.7$
	enzym. Glc release[4]	9.0 ± 2.1	n.d.	8.5 ± 0.1	13.1 ± 3.4	$0.2 \pm <0.1$	0.2 ± 0.1	n.d.	n.d.
L. digitata (Aug'12; washed)	$HClO_4$	$6.8^{a} \pm 0.1$	$2.0^{a} \pm 0.1$	$44.9^{a} \pm 2.3$	$53.3^{ab} \pm 1.7$	$0.6^{a} \pm 0.1$	$1.0^{a} \pm 0.1$	$19.3^{ab} \pm 0.5$	$7.4^{a} \pm 0.7$
	H_2SO_4 (method A)	$8.0^{a} \pm 0.3$	$2.4^{a} \pm 0.1$	$56.6^{b} \pm 1.2$	$57.1^{b} \pm 3.9$	$0.6^{a} \pm 0.1$	$1.3^{a} \pm 0.7$	$24.4^{b} \pm 0.7$	$2.7^{b} \pm 0.3$
	H_2SO_4 (method B)	$6.6^{a} \pm 0.7$	$2.1^{a} \pm 0.2$	$55.0^{b} \pm 0.2$	$43.9^{a} \pm 4.9$	$0.6^{a} \pm 0.1$	$0.9^{a} \pm 0.2$	$18.7^{a} \pm 2.6$	$3.5^{b} \pm 0.4$
	enzym. Glc release[4]	$8.1 \pm <0.1$	n.d.	63.7 ± 5.2	68.2 ± 0.3	0.2 ± 0.1	$0.1 \pm <0.1$	n.d.	n.d.
L. digitata (Aug'12)	$HClO_4$	$8.7^{a} \pm 0.2$	$1.6^{a} \pm 0.1$	$49.4^{a} \pm 4.4$	$53.7^{a} \pm 1.7$	$0.6^{a} \pm 0.1$	$0.7^{a} \pm 0.1$	$14.2^{a} \pm 0.8$	$6.7^{a} \pm 0.5$
	H_2SO_4 (method A)	$10.4^{a} \pm 1.8$	$2.1^{a} \pm 0.4$	$57.5^{b} \pm 0.8$	$56.5^{a} \pm 9.2$	$0.5^{a} \pm 0.1$	$1.3^{b} \pm 0.3$	$17.2^{a} \pm 2.5$	$1.8^{b} \pm 0.4$
	H_2SO_4 (method B)	$8.8^{a} \pm 0.5$	$1.9^{a} \pm 0.1$	$55.3^{ab} \pm 0.1$	$43.6^{a} \pm 2.8$	$0.6^{a} \pm 0.2$	$0.8^{a} \pm 0.1$	$13.9^{a} \pm 1.0$	$1.8^{b} \pm 0.6$
	enzym. Glc release[4]	$11.7 \pm <0.1$	n.d.	72.5 ± 0.4	77.0 ± 0.7	$0.3 \pm <0.1$	0.1 ± 0.1	n.d.	n.d.
Barley straw (pretreated)	$HClO_4$			$15.1^{a} \pm 7.5$	$14.0^{a} \pm 2.7$	$2.6^{a} \pm$	$3.8^{a} \pm 0.9$	n.d.	$41.6^{a} \pm 0.8$
	H_2SO_4 (method A)			$61.6^{b} \pm 0.8$	$57.7^{b} \pm 1.1$	$0.64.0^{b} \pm 0.1$	$4.5^{a} \pm 0.1$	n.d.	$30.0^{b} \pm 0.1$
	H_2SO_4 (method B)			$55.3^{b} \pm 0.8$	$43.6^{c} \pm 1.2$	$3.9^{b} \pm 0.2$	$3.7^{a} \pm 0.3$	n.d.	$29.3^{b} \pm 0.3$
	enzym. Glc release[4]			38.1 ± 7.1	39.3 ± 7.3	2.1 ± 0.4	1.9 ± 0.4	n.d.	n.d.

[a] All carbohydrate values are given from hydrated monomers; n.d. = not detected. [1]Mannose, rhamnose, arabinose, galactose and xylose; [2]only mannose and xylose; [3]uronic acids (UA) determined as galacturonic acid equivalents (GalA eq.); [4]after enzymatic hydrolysis for 6h.

for *L. digitata*. However, the data obtained by the use of a high dosage of the Cellic®CTec2 showed that the enzymatically released glucose levels were consistently higher than those obtained by any of the sulfuric acid hydrolysis methods or the $HClO_4$ method. The cellulase treatment thus catalyzed the decomposition of the glucose containing polysaccharides in the seaweed, and also efficiently catalyzed mannitol liberation (Table 2). No alginate degradation took place during cellulase treatment (the levels of uronic acids were nil), and cellulase treatment also released lower yields of other monomeric carbohydrates than the chemical hydrolysis methods (Table 2).

HPAEC-borate has been established as an optimal analytical method for analysis of lignocellulosic carbohydrates.[5,24] For separation of common compounds in acid hydrolysates of brown seaweed, glucose, xylose and mannose, this chromatography method produced highly reproducible results (Table 2). However, it was only possible to detect all carbohydrates especially sugar alcohols and uronic acids by HPAEC-PAD (Table 2).

3.2 Uronic acids

Uronic acids (UA) of brown seaweed can be separated and electrochemically quantified by HPAEC-PAD (Table 3). Small amounts of glucuronic acid, below 2% w/w in each sample, were determined in all the brown seaweed samples (Table 3). The detection of glucuronic acid was in agreement with what was reported in an early study by Knutson and Jeanes.[32]

Furthermore, guluronic acid was identified and quantified, but galacturonic acid was not found in any of the seaweed samples. Mannuronic acid (M) in its monomeric form is only available commercially as the lactone of mannuronic acid. Hence, mannuronic acid was quantified as galacturonic acid equivalents, but was found to be the dominant uronic acid in the brown seaweed samples (Table 3).

According to the literature M/G ratios depend on seaweed species but also vary within the different species. For *L. digitata* and *S. latissima* M/G ratios from 1.1 to 2.1 and up to 3.1 have been reported.[9,32] The M/G ratio for the *L. digitata* seaweed harvested in April 2012 from the Danish Baltic Sea was 2.0, for *S. latissima* it was 2.4, but ratios were higher (2.8–3.0) for the samples harvested from the North Sea in late summer 2012 (Table 3). Quantification of mannuronic acid (ManA) as galacturonic acid (GalA) equivalents and summation of the values with guluronic acid (GulA) as alginic acid led to estimated levels of about 32–33% w/w alginate in the seaweed samples harvested early spring *versus* ~20% w/w alginate in the samples harvested late summer (Table 3). The different fractions of alginic acid MM, GG, GM and MG blocks depolymerize at different rates in response to acid treatment,[9] and GulA has a relatively high acid lability.[32] Nevertheless, despite the uncertainties regarding the application of GalA as a standard for ManA and monomer recovery, the total amounts of the individually quantified uronic acids (Table 3) reflected those reported previously in the literature. Moreover, the response factor of ManA for HPAEC analysis can tentatively be concluded to be similar to the response of GalA and likely between that of glucuronic and guluronic acid. In this regard, the application of the present method also provides a reasonably reliable option for presenting all uronic acids directly as GalA equivalents probably because the response factor of GalA is close to that of the dominant uronic acid. Values were in the same range as the total of all individual monomers, but only when expressed as GalA equivalents (Table 3).

Filisetti-Cozzi and Carpita[25] recommend the measurement of total uronic acids as GalA equivalents by colorimetric analysis with the absorption of GalA being close to that of ManA after addition of 120 mM tetraborate to the reaction. However, Percival and McDowell[9] noted an influence of the M/G ratio on the absorbance. In this colorimetric method uronic acids react with concentrated sulfuric acid producing 5-formyl-2-furancarboxylic acid (5FF) which, in the absence of water, further reacts with 3-phenylphenol to produce a colored red-pink chromogen.[33] In the present work, yields quantified in galacturonic acid equivalents for total uronic acids only gave half of the amount of uronic acids as the HPAEC-PAD analysis on the same sulfuric acid hydrolysate (Table 3). The values were nevertheless in agreement with those reported previously for *S. latissima*,[30] where low contents of total uronic acids of 15% and 23% in the spring were noted by use of a similar method. Spectrophotometric determination of alginic acid after HCl treatment gave slightly higher quantities of 20 to 30%,[31] whereas Rioux *et al.*,[34] by use of the 3-phenylphenol method, reported total uronic acids mostly being below 10% w/w for different brown seaweeds.

Table 3 From left to right: yields (±SD) of individual determined monomeric uronic acids (UA) and ratio of mannuronic acid to guluronic acid after pre-treatment with 72% H_2SO_4, 4% post-hydrolysis and subsequent HPAEC-PAD analysis; determined as total UA displayed as equivalents (eq.) after HPAEC-PAD or colorimetric analysis out of the same hydrolysates; and corrected with recovery factor for colorimetric measurement[a]

	UA monomers by HPAEC[1]					Total UA by HPAEC[1] as equivalents			Total UA by UV	
Sample	GulA [%]	GluA [%]	ManA [%][2]	Total [%]	M/G [3][–]	GalAeq [%]	GluAeq [%]	GulAeq [%]	GalAeq [%]	GalAeq RF[4] [%]
L. digitata (Apr'12)	10.4 ± 1.1	1.7 ± 0.2	20.6 ± 2.2	32.7 ± 3.5	1.99 ± 0.04	32.5 ± 3.5	20.3 ± 2.2	38.8 ± 4.2	17.2 ± 1.4	28.0 ± 2.3
S. latissima (Apr'12)	9.0 ± 1.6	1.4 ± 0.2	21.4 ± 3.6	31.8 ± 5.4	2.41 ± 0.04	31.8 ± 5.4	19.9 ± 3.4	38.0 ± 6.5	15.3 ± 3.6	24.9 ± 5.9
L. digitata (Aug'12; washed)	5.7 ± <0.1	1.0 ± <0.1	17.2 ± 0.6	23.9 ± 0.8	3.00 ± 0.09	24.4 ± 0.7	15.2 ± 0.4	29.1 ± 0.8	10.3 ± 6.5	16.7 ± 10.6
L. digitata (Aug'12)	4.5 ± 0.7	0.7 ± 0.1	12.2 ± 1.8	17.4 ± 2.6	2.81 ± 0.06	17.2 ± 2.5	10.8 ± 1.5	20.6 ± 2.9	8.7 ± 2.9	14.2 ± 4.8

[a] Gu1A = guluronic acid; G1uA = glucuronic acid; ManA = mannuronic acid; GalA = galacturonic acid; eq. = equivalent. [1]All values are given from hydrated monomers; [2]given as GalA equivalents; [3]ratio of ManA (M) to GulA (G); [4]recovery factor (RF) 61.4 ± 5.9 [%].

HPAEC-PAD measurement is principally superior to the chromogenic measurement of total uronic acids, since the HPAEC assesses the actual individual monomer(s) and not the reactivity of a degradation product. Potentially, the gap between the methods may be due to the formation of further degradation products during the recurrent exposure of the hydrolysate to strong acid during preparation of the colorimetric measurement. An assessment of the recovery factor for galacturonic acid was performed along the sample chronology. For the first two step sulfuric acid hydrolysis (method A), a recovery of 57.0 ± 3.0% of galacturonic acid was achieved by HPAEC-PAD analysis. The overall recovery including the preparation for UV-measurement with 80% sulfuric acid was 61.4 ± 5.9% of the 5FF–chromogen by colorimetric analysis. This factor was applied and found to be more in agreement with the results of the HPAEC measurements (Table 3). However, application of the 57% as recovery factor for galacturonic acid to the HPAEC results produced a too high recovery in relation to the overall mass balances. An independent second determination for the recovery of galacturonic acid after 2-step sulfuric acid hydrolysis gave a recovery of only ~42% which further challenges the applicability of recovery factors for determination of uronic acid based polysaccharides[5,6]. Hence, determination of recovery factors by exposing monomers, particularly uronic acids, to the same acid hydrolysis conditions as the sample containing the hetero-polymeric polysaccharides appears error-prone due to different degradation behaviors.

3.3 Amino acids, fats, minerals and ash

Generally, brown seaweed contains significantly more protein than lignocellulosic biomass, but variations in the amounts and the amino acid composition are significant. *L. digitata* and *S. latissima* from April contained about 9% and 10% by weight of amino acids, respectively (Table 4), whereas *L. digitata* from August only contained about 3% w/w and the pretreated straw only of 0.4% w/w (Tables 4 and 7 in the Appendix). The protein content is known to range from 3–21% by weight for *L. digitata* and *S. latissima*,[12,20] the difference in the levels being due to the source and harvest season but also affected by the application of different nitrogen-to-protein factors, the most commonly used being 6.25. Lourenco *et al.*[35] collected seaweed (although not *L. digitata* or *S. latissima*) along the Brazilian coast line and found 75–99% of N related to protein with a factor of 5.38 ± 0.5, amino acid residues divided by nitrogen, for brown seaweed. By dividing the total amino acids by nitrogen content *L. digitata* revealed an N-to-protein ratio of 3.4 for the April harvest and 4.4 for the August harvest, and the ratio for *S. latissima* was found to be 3.8 (Table 4). This indicates that application of nitrogen-to-protein factors should be used carefully in order to avoid a potential risk of overestimation. Oppositely, the degradation of proteins during acid hydrolysis, considered to be 5–10% of most of amino acids, could also be taken into account.[27]

Fatty compounds were quantified gravimetrically with maximum amounts of 1% by weight after extraction with petrol and the levels were in accordance to the literature.[12] Ash content and mineral composition differed highly from terrestrial plants and varied with the harvest time (Tables 5 and 8 in Appendix). In general, the brown seaweeds have higher ash contents than other seaweed types.[36] A significantly low content of approx. 3% ash and 0.4% w/w minerals was found for the straw sample compared to the brown algae. Seaweeds from April contained more than 6% by weight of minerals and had an ash content of over 30% w/w (Table 5). In contrast, when carbohydrate contents of glucose and mannitol were high, *L. digitata* contained only 11.9% w/w of ash (Table 5), a level similar to that reported by Adams *et al.*[1] By applying washing as pretreatment the ash content was lowered to 7.9% and the mineral content to 2% w/w (Table 5). The lower level of minerals after washing was primarily due to the removal of sodium and potassium as salts by the washing. Together with sodium and potassium, calcium, phosphorus, and sulfur are the major minerals in brown seaweed.

For *L. digitata* Ruperez[36] found an ash content of 37% and total cations of 17% by weight. Ross *et al.*[37] noted ash contents of 11% to 38% w/w along with 6 to 15% minerals and up to 11 mol g^{-1} of halogens for different brown seaweeds (*L. digitata*: 25.8% ash and 11.3% minerals). Adams *et al.*[1] studied the seasonal variation of *L. digitata* and found total metal content in samples harvested in April of 13.7% and about 7% for samples collected in August and September. Seaweed ash is known to contain carbonates and sulfates.[36] The contents of carbonates and sulfates may partly explain the discrepancy between the total of ICP tracked minerals and determination of the ash content, not considering the amount of halogens like iodine and chlorine. The high discrepancy in mineral contents to the literature derived mainly from the concentration of Na, where analyzed *L. digitata* gave low contents of maximum 10 000 ppm.

Table 4 Total of amino acids (AA) after amino acid analysis (±SD), nitrogen (N) content determined by elemental analysis (±SD) and N-to-protein factor (AA divided by N) for brown seaweed samples and the overall average. (For complete amino acid analysis see Table 7 in Appendix)

Sample	AA [% dry material]	N factor	N-to-protein
L. digitata (Apr'12)	9.3 ± 0.4	2.7 ± <0.1	3.44 ± 0.13
S. latissima (Apr'12)	10.1 ± 0.1	2.6 ± <0.1	3.83 ± 0.04
L. digitata (Aug'12; washed)	3.2 ± 0.4	0.7 ± <0.1	4.34 ± 0.61
Average	6.4	1.7	4.02

Table 5 Total of minerals after ICP-MS (±SD) and ash content after incineration (±SD) for brown seaweeds and barley straw. (For complete mineral analysis see Table 8 in Appendix)

Sample	Minerals [%]	Ash [%]
L. digitata (Apr'12)	6.2 ± 0.1	31.0 ± 0.1
S. latissima (Apr'12)	6.4 ± 0.1	34.6 ± 0.2
L. digitata (Aug'12; washed)	2.0 ± <0.1	7.9 ± <0.1
L. digitata (Aug'12)	2.9 ± <0.1	11.9 ± 0.1
Barley straw (pretreated)	0.4 ± 0.1	2.8 ± 0.2

Table 6 Mass balance of analyzed brown seaweeds and barley straw. From left to right: yields (±SD) of elemental CHNO;[2] individual determined organic compounds and added up to its total organic matter (TOM) after treatment with 72% H_2SO_4, 4% post-hydrolysis and subsequent HPAEC-PAD analysis for carbohydrates;[1] amino acid hydrolysis for proteins and extraction for fats; ash contents (±SD) after incineration and gravimetric determination; and the overall amount of total of individual determined organic compounds plus ash content. Levels of each compound were compared (ANOVA) to determine significant differences per yield within each individual compound of sample. Significant differences are denoted by superscript letters a and b for differences between the species; f and g for seasonal variation; n and o for effect of washing; and r to v for overall differences[a]

	CHNO[2]	Protein	Fats	UA[1,5]	Glucose[1]	Mannitol[1]	Fucose[1]	Others[1,6]	TOM[3]	Ash	Total[4]
Sample	EA [%]	AAA [%]	ASE [%]	HPAEC [%]	HPAEC [%]	HPAEC [%]	HPAEC [%]	HPAEC [%]	Calc. [%]	Incin. [%]	Calc. [%]
L. digitata (Apr'12)	$67.3^{a,f,r} \pm 0.2$	$9.3^{a,f,r} \pm 0.4$	$0.7^{a,f,r} \pm 0.1$	$29.7^{a,f,r} \pm 3.5$	$7.0^{a,f,r} \pm 0.2$	$4.1^{a,f,r} \pm 0.4$	$3.6^{a,f,r} \pm 0.4$	$1.9^{a,f,r} \pm 0.3$	$56.4^{a,f,r} \pm 5.3$	$31.0^{a,f,r} \pm 0.1$	$87.4^{a,f,r} \pm 5.1$
S. latissima (Apr'12)	$64.0^{b,s} \pm 0.3$	$10.1^{a,s} \pm 0.1$	$0.5^{a,s} \pm 0.1$	$28.9^{a,r} \pm 4.9$	$6.1^{a,r} \pm 1.1$	$6.5^{b,rs} \pm 1.1$	$2.6^{a,s} \pm 0.5$	$1.6^{a,r} \pm 0.4$	$56.3^{a,r} \pm 8.2$	$34.6^{b,s} \pm 0.2$	$90.9^{a,r} \pm 8.4$
L. digitata (Aug'12; washed)	$91.3^{n,t} \pm 0.2$	$3.2^{n,t} \pm 0.4$	$1.0^{n,t} \pm 0.1$	$21.8^{n,rs} \pm 0.7$	$51.4^{n,s} \pm 3.5$	$8.0^{n,st} \pm 0.3$	$2.1^{n,s} \pm 0.1$	$1.2^{n,r} \pm 0.6$	$88.7^{n,s} \pm 5.7$	$7.9^{n,t} \pm <0.1$	$96.6^{n,r} \pm 5.7$
L. digitata (Aug'12)	$87.3^{g,o,u} \pm 0.1$	$3.1^{g,n,t} \pm 0.2$	$1.0^{f,n,t} \pm 0.1$	$15.8^{g,n,s} \pm 2.4$	$50.9^{g,n,s} \pm 7.4$	$10.4^{g,n,t} \pm 1.8$	$1.7^{g,n,s} \pm 0.4$	$1.2^{g,n,r} \pm 0.3$	$84.1^{g,n,s} \pm 12.6$	$11.9^{g,n,s} \pm 0.1$	$96.0^{f,n,r} \pm 12.7$
Barley straw (pretreated)	$97.0^{v} \pm 0.3$	$0.4^{u} \pm <0.1$	$2.1^{u} \pm 0.1$	n.d.	$51.9^{s} \pm 1.0$	n.d.	n.d.	$4.1^{s} \pm 0.1$	$88.5^{7\ s} \pm 1.3$	$2.8^{v} \pm 0.2$	$91.3^{r} \pm 1.5$

[a] EA = elemental analysis; AAA = amino acid analysis; ASE = accelerated solvent extraction; HPAEC = HPAEC-PAD; incin. = incineration; calc. = calculated; n.d. = not detected. [1]All values are given as dehydrated monomers (conversion factors for dehydration on polymerization: UA = 0.91; glc, gal, man = 0.90; fuc, rha = 0.89; xyl, ara = 0.88); [2]CHNO as total of carbon, hydrogen, nitrogen and oxygen determined by elemental analysis; [3]TOM (total organic matter) as total of individual determinations of amino acids, fats and carbohydrates; [4]total of all detected compounds; [5]total of GulA, GluA and ManA (ManA given as GalA equivalents); [6]total of arabinose, rhamnose, galactose, xylose and mannose; [7]including Klason lignin (30.0 ± 0.1%) determined after sulfuric acid hydrolysis.

3.4 Overall map of compounds

Additional determination of total amino acid and fats to carbohydrate analysis allowed quantification of total organic matter (TOM). For both April harvested *L. digitata* and *S. latissima* Table 6 accounts about 56% for TOM with only minor differences along protein and dehydrated monomeric carbohydrate composition. Hence, *L. digitata* from August consisted of about 84% TOM, about 30% more compounds of organic matter compared to April's *L. digitata*. This was primarily due to the extremely change in the glucose content to 51% which was dominant in this sample. In April the most dominant organic compounds were the uronic acids. The uronic acids constituted about 30%, mainly derived from the alginic acid, but also the level of proteins was higher in April. The difference of measurements of all neutral sugars, mannitol, proteins and fats as total organic matter to determination of C, H, N and O detected by elemental analysis (Table 6) was calculated as the theoretical amount of uronic acids. For the early spring harvested samples, the calculated averages were found to be slightly elevated as compared to those from August, 39.1% *vs.* 32.7% for *L. digitata* and 35.4% *vs.* 31.8% for *S. latissima*. In general, taking the standard deviations into account, all HPAEC-PAD measurements agreed satisfactorily with the theoretical calculations.

As stated above, washing mainly affected the ash content but also mannitol appeared to be washed out. Overall, the relative proportion of organic matter compounds increased from about 84 to 89 % even though the mannitol level decreased from 10.4 to 8% (Table 6).

By summing up the overall map of compounds, the recovery added up to about 90% for all samples by the addition of the ash content to the TOM (Table 6). The difference to a fulfilled composition (of 100%) can probably be found in the heterogeneous hydrolysis residues. For straw this difference was accounted for as lignin, but the nature of the remaining mass is uncertain for seaweed. On the other hand, inaccuracies due to application of four different methods – carbohydrate analysis, amino acid analysis, quantification of fatty compounds and incineration – including their losses should be kept in mind. In particular, the values for total organic matter (TOM) are below estimation of CHNO by elemental analysis. For seaweed samples from April only 56% of the TOM were estimated as compared to 67.3% to of C, H, N and O after elemental analysis, respectively 64% for *S. latissima*, whereas estimation for TOM of *L. digitata* from August was close to CHNO analysis. The values of individually determined TOM were only about 3% below the sum of elements of 87%, and 91%, respectively for the washed seaweed (Table 6).

However, taking standard deviations into account the total of individually determined organic matters of all samples agreed well with the sum of the elementals CHNO (Table 6) which does not specify the origin of the carbon. Adams *et al.*[1] found CHNO contents of *L. digitata* with less seasonal variation between 66 and 83% along with a maximum of 25% glucose determined as laminarin. Ostgaard *et al.*[30] similarly found less seasonal deviation for total organic matter. Like the results for April collected seaweed their compositions for spring harvested *S. latissima* were dominated by ash and alginate. In contrast, the dry matter composition of samples in autumn was almost equally distributed between ashes, laminarin, mannitol and alginate. However, not all organic matter could be identified. Rioux *et al.*[34] analyzed all compounds from brown seaweed. A sum-up of all extracted fractions of carbohydrate including proteins and lipids leads to a maximum yield of 2/3 of what was expected as carbohydrates by difference of ash, proteins and lipids. However, even if uncertainties probably derived from the carbohydrate analysis remain by adding the ash the balance was acceptable for all brown seaweed samples and the benchmark data for straw (Table 6).

4 Conclusions

HPAEC-PAD analysis after a 2-step treatment with first 72% sulfuric acid for 1 h at 30 °C and then 4% at 120 °C for 40 min turned out to be the best methodology for quantitative determination of the brown seaweed carbohydrate composition. The high heterogeneity in the type of monomeric compounds and the high amounts of β-bonds in the polysaccharides in the brown seaweed along with high ion load challenged the analysis and could cause elevated deviations compared to lignocellulosic material. In contrast to the underestimating colorimetric measurements of total uronic acids the HPAEC-PAD analysis of the total individually measured uronic acids reflected the expected values. Furthermore, additional measurements for amino acids and fats the matter of total organic compounds was determined and successfully cross-verified with the sum of C, H, N and O as total organic compounds received from elemental analysis. Thereby, a full map of brown seaweed compounds was achieved. In contrast to pulsed amperometric detection (HPAEC-PAD), HPAEC-borate is an accurate and highly reproducible method but only detects glucose, xylose and mannose monomers. HPAEC analysis of enzymatically decomposed seaweed with a commercial enzyme solution revealed higher glucose yields as compared to all acid treatments for all the seaweed samples. Nevertheless, decomposition was incomplete as almost only glucose and mannitol were released.

The brown seaweeds *Laminaria digitata* and *Saccharina latissima* collected in April in the Danish Baltic Sea showed only minor differences in their composition. *L. digitata* harvested in August in the Danish North Sea had a total of organic matter (TOM) of 84% dominated by glucose (51% w/w) and therefore predestinated for *e.g.* biofuels. In the samples harvested in April the content of alginic acid and ash dominated where changes in the M/G ratio from 2 in April to 2.8 in August also indicate different structures in the composition of alginic acid (although it cannot be ruled out that some of the differences were also caused by geographical differences). Total amino acid content of 3% in August is low compared to 10% present in April. In contrast, the N-to-protein factor was higher in August. Addition of the ash content to the TOM completes the mass balance. With the optimal 2-step sulfuric acid hydrolysis followed by HPAEC-PAD analysis a procedure for obtaining the full monomeric composition of neutral sugars, the sugar alcohol mannitol, and the uronic acids, where mannuronic acid was quantified as galacturonic acid equivalents, was achieved. Overall, a conclusive map of compounds for all brown seaweed samples was thus obtained.

5 Appendix

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Table 7 Amino acid (AA) composition after amino acid analysis (±SD) for brown seaweed and barley straw (additional information to Table 4)

	L. digitata (Apr'12)	*S. latissima* (Apr'12)	*L. digitata* (Aug'12; washed)	*L. digitata* (Aug'12)	Barley straw (pretreated)
Amino acid	AA/biomass [μg mg^{-1}]	AA/biomass [μg mg^{-1}]	AA/biomass [μg mg^{-1}]	AA/biomass [μg mg^{-1}]	AA/biomass [μg mg^{-1}]
Total	93.3 ± 3.7	101.0 ± 1.0	31.7 ± 4.5	31.3 ± 2.4	3.8 ± 0.1
Asp	12.6 ± 0.4	12.8 ± 0.3	3.7 ± 0.5	3.2 ± 0.1	0.4 ± <0.1
Thr	5.1 ± 0.2	5.2 ± 0.4	1.8 ± 0.3	1.3 ± 0.1	0.4 ± <0.1
Ser	4.5 ± 0.2	4.7 ± <0.1	1.6 ± 0.2	1.2 ± 0.1	0.3 ± <0.1
Glu	12.0 ± 0.3	15.2 ± 0.6	4.4 ± 0.6	3.5 ± 0.3	0.9 ± <0.1
Pro	4.3 ± 0.2	4.6 ± <0.1	1.6 ± 0.2	1.3 ± 0.1	0.6 ± <0.1
Gly	4.7 ± 0.2	5.1 ± <0.1	1.8 ± 0.2	1.4 ± 0.1	0.4 ± <0.1
Ala	10.8 ± 0.6	11.0 ± 0.2	2.6 ± 0.4	2.2 ± 0.2	0.5 ± <0.1
TPCys	2.4 ± 0.5	1.9 ± 0.1	0.5 ± <0.1	0.4 ± 0.1	<0.1
Val	5.0 ± 0.1	5.6 ± <0.1	1.9 ± 0.3	1.6 ± 0.1	<0.1
Met	1.9 ± 0.1	2.2 ± <0.1	0.8 ± 0.1	0.7 ± 0.1	0.1 ± <0.1
Ile	3.7 ± 0.1	4.1 ± 0.1	1.4 ± 0.2	1.1 ± 0.1	0.3 ± <0.1
Leu	6.2 ± 0.2	7.4 ± 0.1	2.5 ± 0.4	2.1 ± 0.1	0.6 ± <0.1
Tyr	3.4 ± 0.2	3.5 ± 0.1	1.1 ± 0.2	1.0 ± 0.1	0.2 ± <0.1
Phe	4.7 ± 0.1	5.5 ± 0.1	1.9 ± 0.3	1.6 ± 0.2	0.4 ± <0.1
His	2.7 ± 0.2	1.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.2 ± <0.1
Lys	5.2 ± 0.1	5.4 ± 0.1	1.7 ± 0.3	1.7 ± 0.1	0.1 ± <0.1
Arg	4.3 ± 0.2	4.8 ± 0.1	1.7 ± 0.3	1.5 ± 0.1	<0.1

Table 8 Mineral composition after ICP-MS (±SD) for brown seaweeds and barley straw. (additional information to Table 5)

Sample	Al [Ppm]	B [Ppm]	Ba [Ppm]	Ca [Ppm]	Cr [Ppm]	Cu [Ppm]	Fe [Ppm]	K [Ppm]	Mg [Ppm]	Mn [Ppm]	Na [Ppm]	P [Ppm]	Pb [Ppm]	S [Ppm]	Si [Ppm]	Zn [Ppm]	Total [Ppm]
L. digitata (Apr'12)	139.3 ± 0.4	121.9 ± 1.6	51.3 ± 1.9	1642 ± 33.9	6.0 ± 1.1	3.9 ± 0.1	194.4 ± 1.6	216 00 ± 594.0	7742 ± 140.7	37.3 ± 1.9	102 80 ± 226.3	3685 ± 47.4	0.8 ± 0.1	166 55 ± 148.5	53.5 ± 3.9	49.3 ± 0.7	622 61 ± 1205
S. latissima (Apr'12)	106.5 ± 1.2	142.4 ± 1.3	39.3 ± 1.0	1290 ± 6.4	5.9 ± 0.1	2.3 ± 0.3	133.9 ± 0.9	25 530 ± 834.4	7969 ± 30.4	10.4 ± 0.2	12 260 ± 424.3	4439 ± 5.7	1.5 ± 0.2	12 110 ± <0.1	51.1 ± 4.8	44.4 ± 0.1	64 135 ± 1311
L. digitata (Agu'12; washed)	33.1 ± 1.3	49.6 ± 0.4	13.0 ± 0.1	902.0 ± 16.7	0.4 ± <0.1	1.7 ± <0.1	95.6 ± 1.1	2255 ± 229.8	5367 ± 87.0	8.6 ± 0.3	4306 ± 59.4	529.9 ± 7.9	0.3 ± <0.1	6666 ± 74.2	52.1 ± 10.1	67.2 ± 4.0	20 346 ± 492
L. digitata (Agu'12)	27.9 ± 0.2	68.3 ± 0.9	9.1 ± 0.5	651.9 ± 8.6	0.6 ± 0.3	1.6 ± 0.3	81.5 ± 1.3	8778 ± 67.9	5147 ± 36.8	3.2 ± <0.1	7581 ± 82.0	488.9 ± 0.1	n.d. n.d.	6531 ± 12.0	38.5 ± 0.1	29.3 ± 0.2	29 437 ± 211
Barley straw (pretreated)	113.5 n.d.	4.1 n.d.	15.0 n.d.	105.7 n.d.	18.6 n.d.	16.0 n.d.	2326 n.d.	165.2 n.d.	40.1 n.d.	15.4 n.d.	22.5 n.d.	152.7 n.d.	0.8 n.d.	369.3 n.d.	176.1 n.d.	27.1 n.d.	3568 n.d.

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Notes and references

1 J. M. M. Adams, A. B. Ross, K. Anastasakis, E. M. Hodgson, J. A. Gallagher, J. M. Jones and I. S. Donnison, *Bioresour. Technol.*, 2011, **102**, 226–234.
2 D. Ashok, Y. Huang, S. Rezvani, D. McIlveen-Wright, M. Novaes and N. Hewitt, *Bioresour. Technol.*, 2013, **135**, 120–127.
3 A. Arnous and A. S. Meyer, *Food Bioprod. Process.*, 2008, **86**, 79–86.
4 I. Meseguer, M. Boix, M. C. M. Para and M. V. Aguilar, *J. Anal. Chem.*, 1999, **54**, 428–433.
5 S. Willfoer, A. Pranovich, T. Tamminen, J. Puls, C. Laine, A. Suurnakki, B. Saake, K. Uotila, H. Simolin, J. Hemming and B. Holmbom, *Ind. Crops Prod.*, 2009, **29**, 571–580.
6 J. Puls, in *Biotechnology in Agriculture; Bioconversion of Forest and Agricultural Plant Residues*, ed. J. N. Saddler, CAB International, Wallingford, UK, 1993, pp. 13 – 32.
7 A. Sluiter, B. Hames, R. Ruiz, C. Scarlata, J. Sluiter, D. Templeton and D. Crocker, *Determination of structural carbohydrates and lignin in biomass*, NREL Technical Report July 2011, NREL/TP-510-42618 (Version 0.7.08.2011):1, 2011.
8 W. Black, *J. Mar. Biol. Assoc.*, U.K., 1950, vol. 29, pp. 45–72.
9 E. Percival and R. H. McDowell, in *Chemistry and enzymology of marine algal polysaccharides*. Academic Press Inc. Ltd., London, 1967.
10 L. E. Rioux, S. L. Turgeon and M. Beaulieu, *Phytochemistry*, 2010, **71**, 1586–1595.
11 A. O. Chizhov, A. Dell, H. R. Morris, A. J. Reason, S. M. Haslam, R. A. McDowell, O. S. Chizhov and A. I. Usov, *Carbohydr. Res.*, 1998, **310**, 203–210.
12 S. L. Holdt and S. Kraan, *J. Appl. Phycol.*, 2011, **23**, 543–597.
13 M. Indegaard, G. Skjåk-Bræk and A. Jensen, *Bot. Mar.*, 1990, **33**, 277–288.
14 B. Kloareg and R. S. Quatrano, *Mar. Biol.*, 1988, **26**, 259–315.
15 M. T. Ale and A. S. Meyer, *RSC Adv.*, 2013, **3**, 8131–8141.
16 M. I. Bilan, A. A. Grachev, A. S. Shashkov, M. Kelly, C. J. Sanderson, N. E. Nifantiev and A. I. Usov, *Carbohydr. Res.*, 2010, **345**, 2038–2047.
17 E. D. Obluchinskaya, *Appl. Biochem. Microbiol.*, 2008, **44**, 305–309.
18 A. Jensen and A. Haug, *Geographical and seasonal variation in the chemical composition of Laminaria hyperborea and Laminaria digitata from the Norwegian coast*, Norwegian Institute of Seaweed Research, Report 14, Oslo, 1956, pp. 1–8.
19 M. Indegaard and J. Minsaas, in *Seaweed Resources in Europe: Uses and Potential*, ed. M. D. Guiry and G. Blunden, John Wiley & Sons, Chichester, UK, 1991, pp. 21–64.
20 J. Morrissey, S. Kraan and M. D. Guiry, *Guide to Commercially Important Seaweeds on the Irish Coast*, Bord Iascaigh Mhara/Irish Sea Fisheries Board, Ireland, 2001.

21 L. Rosgaard, S. Pedersen and A. S. Meyer, *Appl. Biochem. Biotechnol.*, 2007, **143**, 284–296.
22 G. Moxley and Y. H. P. Zhang, *Energy Fuels*, 2007, **21**(6), 3684–3688.
23 S. Horner, J. Puls, B. Saake, E. A. Klohr and H. Thielking, *Carbohydr. Polym.*, 1999, **40**, 1–7.
24 M. Sinner, M. H. Simatupang and H. H. Dietrichs, *Wood Sci. Technol.*, 1975, **9**, 307–322.
25 T. M. C. C. Filisetti-Cozzi and N. C. Carpita, *Anal. Biochem.*, 1991, **197**, 157–162.
26 B. M. v. d. Hoogen, P. R. v. Weeren, M. Lopes-Cardozo, L. M. G. v. Golde, A. Barneveld and C. H. A. v. d. Lest, *Anal. Biochem.*, 1998, **257**, 107–111.
27 V. Barkholt and A. L. Jensen, *Anal. Biochem.*, 1989, **177**, 318–322.
28 S. Willför, J. Hemming and A. Leppänen, *Analysis of extractives in different pulps – Method development, evaluation, and recommendations*, Laboratory of Wood and Paper Chemistry, Åbo Akademi, Finland, 2006.
29 O. Faix, D. S. Argyropoulos, D. Robert and V. Neirinck, *Holzforschung*, 1994, **48**, 387–394.
30 K. Ostgaard, M. Indegaard, S. Markussen, S. H. Knutsen and A. Jensen, *J. Appl. Phycol.*, 1993, **5**, 333–342.
31 A. I. Usov, G. P. Smirnova and N. G. Klochkova, *Russ. J. Bioorg. Chem.*, 2001, **27**, 395–399.
32 C. A. Knutson and A. Jeanes, *Anal. Biochem.*, 1968, **24**, 482–490.
33 A. Ibarz, A. Pagán, F. Tribaldo and J. Pagán, *Food Control*, 2006, **17**, 890–893.
34 L. E. Rioux, S. L. Turgeon and M. Beaulieu, *Carbohydr. Polym.*, 2007, **69**, 530–537.
35 S. O. Lourenco, E. Barbarino, J. C. De-Paula, L. O. d. S. Pereira and U. M. Lanfer Marquez, *Phycol. Res.*, 2002, **50**, 233–241.
36 P. Ruperez, *Food Chem.*, 2002, **79**, 23–26.
37 A. B. Ross, J. M. Jones, M. L. Kubacki and T. Bridgeman, *Bioresour. Technol.*, 2008, **99**, 6494–6504.

PAPER II

Compositional variations of brown seaweeds *Laminaria digitata* and *Saccharina latissima* in Danish waters

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Compositional variations of brown seaweeds *Laminaria digitata* and *Saccharina latissima* in Danish waters

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ABSTRACT

Carbohydrates and proteins of brown seaweeds (kelps) can meet requirements to substitute oil derived products and energy. Around Denmark *Laminaria digitata* and *Saccharina latissima* are especially common species. Glucose levels of *L. digitata* appeared to be superior *S. latissima* for bioenergy applications. Glucose levels in wild *L. digitata* from Kattegat peaked in October with 37.0 % by dry weight compared to 22.6 % in wild *S. latissima*, accompanied by lower ash contents (18.5 compared to 26.5 % w/w). However, wild *L. digitata* from the North Sea exceeded these values with >50 % glucose and a lower ash content of approx. 11 % w/w in August for three consecutive years (2012-2014). Cultivation of *S. latissima* for bioenergy applications in Limfjorden, Denmark was not possible, due to the occurrence of biofouling in the summer. Variation in the composition of the seaweeds was found for all compounds, mainly relating to season but there was also variation between species, locations, years, and even within populations. Total alginate content was less variable but M/G ratios differed highly between species and location from 1.33 to 3.64. Of the analyzed environmental variables, only temperature was found to correlate with the chemical

composition of the seaweeds. Application of a common N-to-protein factor cannot be recommended, since total nitrogen content fluctuated more between samples than the actual protein contents. Amino acid profiles were dominated by glutamic acid, aspartic acid and alanine and varied with season (especially for *L. digitata* from the North Sea) but also with the location.

Keywords: glucose, biochemical composition, *Laminaria digitata*, *Saccharina latissima*, carbohydrates, bioenergy, nitrogen-to-protein factor

INTRODUCTION

A growing portion of the total anthropogenic emission can be attributed to the production of goods that are then globally traded (IPCC 2014). Cultivation of biomass as a resource for the production of bioenergy and biomass derived chemicals has the potential to reduce global interdependencies and provide positive socio-economic and environmental effects (Venghaus and Selbmann 2014; Creutzig et al. 2014; Raman et al. 2015).

In the search for new biomass resources, there is growing interest in carbohydrates from macroalgae (Brown and Tustin 2010; Dave et al. 2013). Currently, neutral sugars, such as glucose, mannose, xylose, galactose, etc., are the most developed candidates for bioenergy production, due to intensive research into lignocellulosic biomass. Moreover, the most common fermenter *Saccharomyces cerevisiae* uses glucose as resource (Lee et al. 2011). Of these sugars, glucose is potentially available in sufficient quantities in brown seaweeds. In the Northern Hemisphere, brown seaweeds such as *S. latissima* and *L. digitata* have been described as being rich in carbohydrates (Adams et al. 2011; Manns et al. 2014; Schiener et al. 2015).

The structural polymer cellulose contributes up to 10 % w/w of the total glucose content of brown seaweed (Black 1950; Siddhanta et al. 2009). More significant amounts of glucose can be found in the principal and unique carbohydrate reserve substance, laminarin. Laminarin consists of β-1,3-linked glucose moieties with β-1,6-linked branches (Rioux et al. 2010). However, laminarin composition varies strongly with season by 0-33 % by weight of the total dry matter (w/w) (Adams et al. 2011; Holdt and Kraan 2011). In a recent study, we found that *L. digitata* harvested from the Danish North Sea in August 2012 contained about 51 % (w/w) glucans (Manns et al. 2014). The level of the second reserve substance, mannitol in brown seaweed varies widely due to seasonal variation from 5-26 % w/w (Adams et al. 2011; Schiener et al. 2015). Mannitol cannot be easily fermented, and only a few organisms can utilize it. Mannitol needs to be converted to fructose-6P before being further metabolized (Wei et al. 2013).

The major cell wall polysaccharide in brown seaweeds, alginate, consists of glycosidically linked guluronic acid (G) and mannuronic acid (M), which are present in varying proportions and form linear chains with M/G ratio ranges of 1.2 to 3.0 or higher for *S. latissima* and *L. digitata* (Percival and McDowell 1967; Kloareg and Quatrano 1988; Manns et al. 2014). The seasonal variation of the total alginate concentration in brown seaweed is less pronounced (Black 1950; Schiener et al. 2015). Nevertheless, recorded concentrations of alginate still vary from 17 to 45 % within the literature (Jensen and Haug 1956; Holdt and Kraan 2011). Fucoidans constitute another unique type of brown seaweed polysaccharide. Primarily, fucoidans from *S. latissima* and *L. digitata* are composed of a backbone of fucopyranose residues with sulfate substitutions and occasionally other glycosyl such as galactose, xylose, glucuronic acid and/or acetate substitutions (Bilan et al. 2010; Ale and Meyer 2013). Fucoidan makes up up to 10 % of the total dry matter of brown seaweed per weight (Obluchinskaya 2008; Holdt and Kraan 2011).

Currently, the two matrix carbohydrates from brown seaweed, fucoidans and alginate, are not utilized for bioconversion to biofuels. However, they are highly valuable in industries related to food, pharmaceuticals and cosmetics (Morrissey et al. 2001; Kraan 2013). Furthermore, new attempts to metabolize alginate into biofuels are under investigation (Wargacki et al. 2012). Seaweed carbohydrates have enormous potential as the basis for generating renewable bioenergy and bio-based products in the future. However, since glucan based carbohydrates are of primary interest at the moment, the choice of brown seaweed harvest time is crucial for optimal exploitation. Due to the seasonal variation laminarin, i.e. glucose, and mannitol have are at their peak concentrations in autumn (Black 1950; Adams et al. 2011).

Besides polysaccharides, the minerals and proteins that compose a significant proportion of the dry weight of brown seaweeds, are interesting for application as food for human and animals (Holdt and Kraan 2011). In contrast to carbohydrate concentrations, protein and mineral contents are highest in winter to early spring. Mineral levels range from 15 to 39 % w/w and the protein content is known to range from 3-21 % by weight for *L. digitata* and *S. latissima* (Indegaard and Minsaas 1991; Morrissey et al. 2001). The observed protein levels are dependent on seaweed species, location and harvest season, but are also artificially affected by the application of different N-to-protein factors, 6.25 being most commonly used for biomass in general (Lourenco et al. 2002; Angell et al. 2015).

Although variations and changes in composition of brown seaweed biomass have been known for long time in Europe (Black 1950), the topic of seasonal variation in macroalgae composition is currently of high interest due to increasing demands, particularly as a biofuel feedstock (Adams et al. 2011; Schiener et al. 2015). Also, in Denmark there is a need for thorough analysis of the parameters important for the growth of wild and cultivated *S. latissima* and *L. digitata*, in order to understand which areas could be suited for future cultivation practices. Danish marine waters are subjected to large temporal and spatial variations in environmental growth parameters, such as salinity, temperature, nutrients, exposure and light, among many others factors which may influence biomass yield and biochemical composition (Nielsen et al. 2016; Jørgensen and Richardson 1996; Conley et al. 2000).

The present study attempts to fill a gap in knowledge regarding the occurrence of variation in the chemical composition of brown seaweed compounds, with an emphasis on sugar compositions. Therefore, the compositions of four sources of brown seaweeds of *S. latissima* and *L. digitata* from three different Danish locations, including a cultivation site, were investigated and mapped for one year of growth.

MATERIALS AND METHODS

Materials

Seaweeds

This study was based on seasonal sampling of four brown seaweeds populations of *S. latissima* and *L. digitata* originating from three different geographic locations between October 2012 and November 2013:

- Wild *L. digitata* from Hanstholm, Danish North Sea (57° 7'9.86"N, 8°39'14.11"E) at a depth of 1-3 m, with a distance to shore of approx. 150 m and a salinity of approx. 30 ‰. The sandy seafloor was covered with small limestone boulders acting as a substrate for the seaweeds with strong exposure in terms of wind and waves in the area. In the summer months the North Sea is fully stratified, predominantly by a thermocline.

  Sampling: 2012-08-29, -11-28, 2013-01-18, -03-07, -04-02, -05-21, -07-01, -08-27, -11-28 and 2014-08-08.

- Wild *L. digitata* from the Bay of Aarhus, Danish Kattegat (56°10'8.74''N, 10°13'35.55''E) at a depth of 1-3 m, with distance to shore of 2-8 m and a salinity of approx. 22 ‰. Huge rocks build up the harbor's pier and act as substrate for the seaweeds. The shallow and calm water in the area may cause the build-up of a pycnocline during summer – therefore in summer months, salinity tends to drop below 19 ‰ above the pycnocline (Nielsen et al. 2014).

  Sampling: 2012-11-29, 2013-01-24, -02-20, -03-22, -04-25, -05-24, -06-28, -07-17, -08-20, -09-04 and 2013-10-24.
- Wild *S. latissima* from the same site (Bay of Aarhus; 56°10'8.74''N, 10°13'35.55''E)
- Cultivated *S. latissima* from lines deployed at depths of 1-3 m in September 2012 in Færker Vig, Limfjorden, Denmark (56°50'8.81''N, 9° 4'34.28''E). The total depth in this area was of 6-10 m, with a distance to shore of 100-200 m and an annual average salinity of approx. 27-28 ‰. The site is sandy/muddy with no suitable substrate for wild populations of kelps. The calm and protected area undergoes periods of reduced water movement which causes the build-up of a pycnocline with reduced salinity and elevated temperature.

  Sampling: 2013-02-06, -03-07, -04-03, -04-22, -05-07, -07-01 and 2013-08-27.

After collection, all seaweeds were frozen, transported in frozen conditions and remained at -20 °C until use.

Chemicals

Sulfuric acid (H_2SO_4), NaOH, D-(+)fucose, L-rhamnose, L-(+)arabinose, D-(+)galactose, D-(+)xylose, D-(+)mannose, D-(+)galacturonic acid, and D-(+)glucuronic acid were from Sigma-Aldrich (Steinheim, Germany). Sodium acetate (NaOAc), and D-mannitol were from Fluka/Sigma-Aldrich (Steinheim, Germany). Guluronic acid was purchased from Chemos GmbH (Regenstauf, Germany) and D-(+)glucose was from Merck (Darmstadt, Germany).

Biochemical analysis

Sample preparation. Before carbohydrate analysis, entire seaweed individuals were oven-dried at 40 °C until equilibrium moisture and weighed afterwards. Nitrogen and ash contents were determined from freeze dried material. The dried material was ground by vibrating disc milling to pass a 100 µm sieve. For studies on the variations of the four different populations from Kattegat, the North Sea and Limfjorden, three seaweed individuals were pooled proportionally to their weight into a single representative sample. For the study on the variation within the *L. digitata* population from the North Sea on analysis was performed on true biological seaweed individuals. Samples from November 2013 were separated into holdfast, stipe and blade, and analyzed individually.

Sulfuric acid hydrolysis. A 2-step sulfuric acid treatment was applied in triplicates according to Manns et al. (2014). For the study of the variation within the population, the samples were treated individually.

Carbohydrate analysis by HPAEC-PAD. Monomeric sugars, the sugar alcohol mannitol and uronic acids in the sulfuric acid hydrolysates were separated by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described in detail previously by Manns et al. (2014).

Determination of ash and nitrogen content. The ash contents were obtained and determined gravimetrically after low temperature oxidation (550 °C) of the samples in a furnace for 2 hours. Nitrogen (N) contents were analyzed using a Perkin Elmer 2400 Series II CHN Analyzer (PerkinElmer Inc.Waltham MA, USA). Seasonal variations in the total protein concentrations were calculated using an N-to-protein factor and the N contents determined by elemental analysis. An N-to-protein factor of 4 was chosen in accordance with Manns et al. (2014).

Amino acid analyses (AAA) were kindly performed by Annette Blicher, DTU System Biology, according to Barkholdt and Jensen (1989).

Calculation of N-to-protein factors, protein and non-protein related nitrogen (N). N-to-protein factors were calculated by dividing the total of amino acid residues by total N after elemental analysis. For protein related N, the specific amount of each amino acid residue was divided by its molar mass and multiplied with the molar mass of N present in the amino acid. Subtracting the protein related nitrogen from the total N gave the non-protein nitrogen.

Statistics

One-way analyses of variances (one-way ANOVA): 95 % confidence intervals were compared as Tukey–Kramer intervals calculated from pooled standard deviations (Minitab Statistical Software, Addison-Wesley, Reading, MA-United States).

Analytical data of the chemical compositions (carbohydrates mannitol, glucose, alginic acid monomers and other COH, as well as N and ash contents) were correlated against the corresponding environmental data in a partial least squares regression (PLS) using leave-one-out cross validation. Prior to analysis, both dependent and independent variables were preprocessed utilizing autoscaling and mean centering (PLS Toolbox 6.0.1., Eigenvector Research Inc., WA, USA). The location specific data of the physicochemical variables (salinity, oxygen, ammonia, nitrate and nitrite, total nitrogen, phosphate and total phosphorus) were retrieved from the nearest monitoring station of the Danish National Aquatic Monitoring and Assessment Program (DNAMAP). The data from a single date (±10 days of sampling date) represented the physicochemical conditions around the time of sampling.

RESULTS AND DISCUSSION

Variation in composition of carbohydrates

Seasonal and spatial variation

The seasonal variation of the composition of carbohydrates in brown seaweeds is well known, e.g. in the detailed documentation of Black (1950). At the beginning of spring (February/March), glucose and mannitol storages were exhausted at all sites. Subsequently, enhanced photosynthetic activity allowed the mannitol and laminarin (i.e. glucose) contents to rise again (Figure 1). Exceptionally, for the cultivation site at Limfjorden the carbohydrate levels, particularly mannitol, of *S. latissima* had already begun to decrease in May (Figure 1c). The low levels of carbohydrates were constantly accompanied by extraordinary high ash contents (discussed in Section 3.1.2). The data of the cultivated *S. latissima*, which is to our knowledge the first carbohydrate documentation for cultivated seaweed over a growth period, should be considered with care. First, after placement of the seedling lines in the cultivation site in September 2012, sampling of individuals was not possible until the following February. Second, from May onwards, growth was increasingly hampered by settling and growth of various biofouling organisms on the *S. latissima* fronds. In August, the biofouling caused massive losses and the last sample was obtained in August, which showed only small amounts of remaining carbohydrates (Figure 1c). Biofouling has been documented elsewhere at sheltered and shallow sites including Limfjorden (Buck and Buchholz 2004; Marinho et al. 2015b). Biofouling organisms have also been recorded in Norwegian waters on cultivated *S. latissima*, close to a salmon aquaculture and partly sheltered from the open ocean (Handå et al. 2013). Hence, cultivation of brown seaweed to generate glucose for bioenergy may not be possible at sheltered locations such as Limfjorden.

A high glucose content is essential for the optimal exploitation of brown seaweed in sugar based biorefineries, e.g. for the production of ethanol. However, glucose contents showed the most seasonal variation in all samples, followed by mannitol (Figure 1). Mannitol contents in the seaweeds always peaked before glucose contents, as mannitol synthesis occurs first in the metabolic process (Percival and McDowell 1967). For the wild brown seaweeds populations of Kattegat, the content of mannitol peaked in June for *S. latissima* at 18.7 % w/w (Figure 1a) and in May at 19.2 % w/w for *L. digitata* (Figure 1b). Glucose levels increased steeply to 20.2 % w/w in July for *S. latissima*, and 29.2 % w/w for *L. digitata*, respectively. After a decline (*S. latissima* 15 %, *L. digitata* 16 % w/w) in August, glucose contents more than doubled the following month to 37.0 % w/w, the peak glucose level for *L. digitata* at Kattegat (Figure 1b). The glucose content of *S. latissima* increased by approx. 3.5 % w/w/month from August to 22.6 % w/w in October (Figure 1a). In contrast, *L. digitata* collected from the North Sea demonstrated maximum concentrations of mannitol (19.9 % w/w) and glucose (54.0 % w/w) in July and August, respectively (Figure 1c). The outstandingly high glucose contents in August were also documented from samples from August 2012 and 2014 (Figure 2). The recorded glucose yield from the North Sea population exceeded levels of the site at Kattegat at the peak by 17 % when compared to *L. digitata* and 31 % when compared to *S. latissima* (compare Figure 1c to a/b). However, high glucose contents in samples of *S. latissima* and *L. digitata* have previously been discovered in Kattegat in August 2012, though only in more open water sites than Aarhus Bay (Nielsen et al., 2016).

The observation of higher glucose level for *L. digitata* growing in open-sea rather than in an inlet was contradictory to the results of Black (1950). However, Black (1950) only determined the glucose extracted from laminarin, whereas our measurement included the total glucose content. For mannitol, content levels in the open sea were similar to the levels in the inlet over the period of investigation (Black 1950). A shorter period between the maximum of the two reserve substances was observed by of Adams et al. (2011) for *L. digitata* from the Irish Sea, UK. There, the highest level of mannitol was recorded in June at 32.1 % w/w and glucose, derived from laminarin, at 24.6 % w/w in July. Further north, in Scotland, Schiener et al.

(2015) analyzed four brown seaweeds for the carbohydrates alginate, mannitol, cellulose and laminarin between August 2010 and October 2011. The cellulose content was between 10 and 15 % throughout. Total glucan, i.e. glucose from cellulose and laminarin, varied due to fluctuations in the laminarin content and peaked shortly after mannitol, later in the autumn. None of the four species investigated by Schiener et al. (2015) contained glucose concentrations higher than 25-30 % w/w, whereas mannitol levels (up to 26 % w/w) exceeded those of the samples presented in this study.

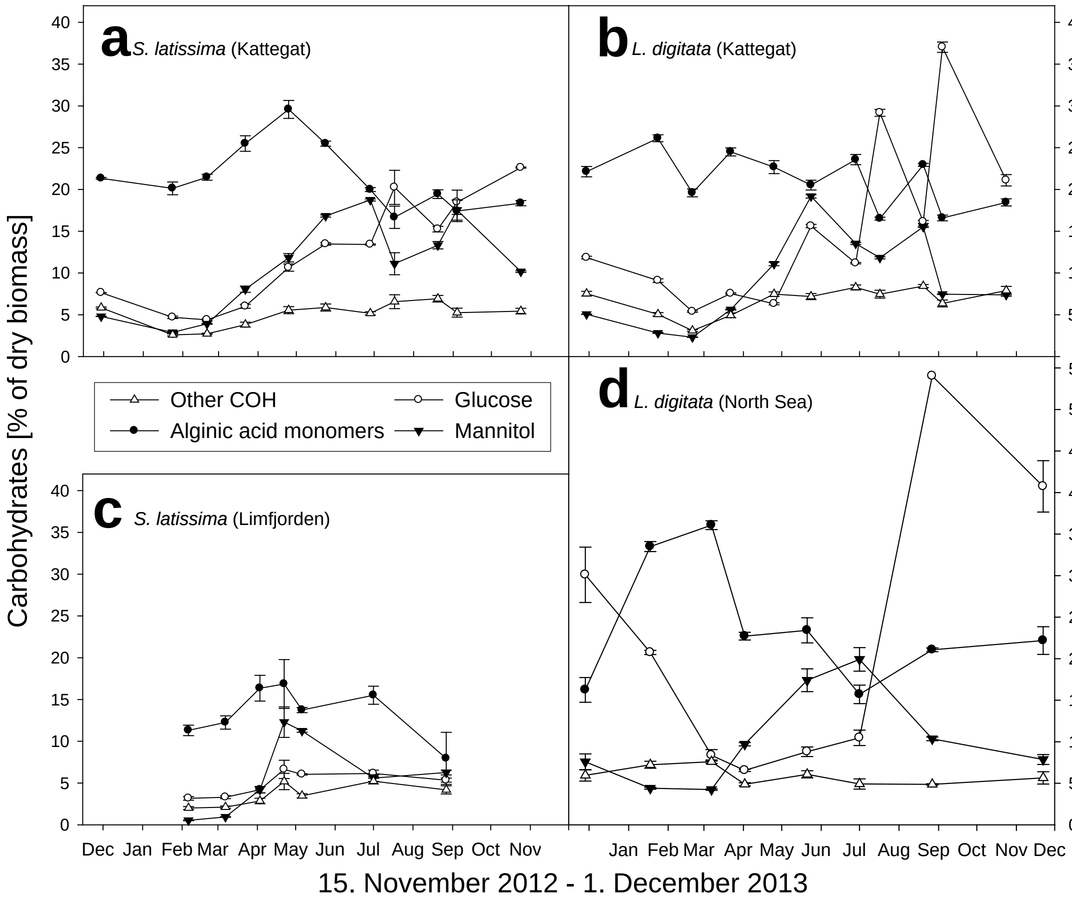

Figure 1: Seasonal variation from November 2012 to November 2013 of carbohydrate compositions of (**a**) *S. latissima* and (**b**) *L. digitata* from Danish Kattegat, (**c**) *S. latissima* from the cultivation in Limfjorden, Denmark and (**d**) *L. digitata* from the Danish North Sea. Each data point represents average values of independent triplicates; error bars indicate the standard deviation; population at given timepoint is represented of three randomly pooled seaweed individuals. All values are given as hydrated monomers; alginic acid: mannuronic acid and guluronic acid; other carbohydrates: fucose, galactose, arabinose, rhamnose, mannose, xylose and glucuronic acid.

Alginate or alginic acid consists of mannuronic (M) and guluronic acid (G) as blocks of MM, GG, MG and GM with different M/G ratios. High value compounds are currently predominately extracted from hydrocolloidal alginate, and preferably from seaweeds with high total alginate concentrations and proportionally high concentrations of guluronnate in the alginate. For Northern Europe in particular, *Laminaria hyperborea* with M/G ratios of >1 is exploited for high value compound extraction (McHugh 2003; Fertah et al. 2014). The total alginate content in our brown seaweeds was less fluctuant between seasons than the storage carbohydrates. In fact, there was no overall pattern of the total alginate concentration, because the patterns differed with species and site (Figure 1). Contrary to the storage carbohydrate levels, alginate levels were highest in spring, at up to 30 % and 36 % (w/w) for the species in Kattegat and North Sea, respectively (Figure1a, b, d). For more turbulent waters, higher contents of alginate have been recorded previously (McHugh 2003). The lowest levels of alginate, at about 16 % (w/w), were found for samples in July at all sites (Figure1). Seasonal variation in alginate concentrations agreed with previous reports, with alginate concentrations between 15 % and 35 % w/w (Schiener et al. 2015). However, a closer look revealed seasonal differences in the M/G ratio (Table 1). Generally, values varied from 1.3 to 3.6, but were mostly around 2.0 (Table 1). Therefore, the data were in agreement with the M/G ratios found and discussed previously (Manns et al. 2014). However, M/G ratios of the cultivated *S. latissima* (Limfjorden) differed without correlation to the season (1.3 to 1.8), being relatively rich in guluronic acid but with constantly low total alginate concentrations of <17 % w/w (Table 1 and Figure 1). In contrast, M/G ratios of *S. latissima* from Kattegat exhibited a strong correlation to the season. Starting with the highest value of 3.5 in November 2012, the M/G ratio decreased stepwise towards the summer of 2013. From May to September, the values were mainly around 1.8, except for June (2.3). In October 2013, the ratio increased to 2.2 again (Table 1; *S. latissima*, Danish Kattegat). In a qualitative study on *S. latissima* from the Norwegian coast, Indegaard et al. (1990) found a similar trend. Blades from wild seaweeds exhibited an M/G ratio of 1.8 in March and 1.4 in August, higher than the cultivated seaweed (Indegaard et al. 1990). In contrast to *S. latissima*, *L. digitata* of the North Sea had its lowest M/G ratios in November 2012 and May 2013 (2.0 ± 0.1). Higher ratios were found during the summer with 3.6 in July and 3.8 in August (Table 1). Nonetheless, the M/G ratio of *L. digitata* from Kattegat remained between 1.5 and 2.2 over the whole period of measurement. Overall, no general seasonal pattern for M/G ratios could be seen. The variation was apparently due to the specific location and the individual population, with no correlation to the total amount of alginate (data not shown). Most likely, factors in the process of epimerization of ß-D-mannuronic acid to α-L-guluronic acid were different between location and/or species (Indegaard et al. 1990). High concentrations of total alginate accompanied with high proportions of guluronic acid (i.e. low M/G ratio) were documented for *L. digitata* at both Kattegat and the North Sea. Between January and April *L. digitata* from Kattegat and the North Sea were particularly interesting for alginate extraction and application as hydrocolloids (Kraan 2013).

Table 1: Relation of mannuronic to guluronic acid (M/G ratios) of analyzed brown seaweeds from Danish Waters over the period of November 2012 to November 2013. Each data point represents average values of independent triplicates ± the standard deviation; population at given timepoint is represented of three pooled seaweed individuals; n.d. = not determined. Different roman superscript letters indicate significant differences ($\alpha < 0.05$) column-wise per season.

Month	*S. latissima* (Kattegat)	*L. digitata* (Kattegat)	*L. digitata* (North Sea)	*S. latissima* (Limfjorden)
Nov’12	$3.53^{a} \pm 0.02$	$2.08^{ab} \pm 0.08$	$1.91^{d} \pm 0.14$	n.d.
Jan’13	$3.12^{b} \pm 0.03$	$1.57^{d} \pm 0.01$	$2.57^{c} \pm 0.04$	n.d.
Feb’13	$3.04^{b} \pm 0.03$	$1.79^{c} \pm >0.00$	n.d.	$1.33^{d} \pm 0.03$
Mar’13	$2.48^{c} \pm 0.04$	$1.47^{d} \pm 0.05$	$2.81^{c} \pm 0.17$	$1.39^{c} \pm 0.01$
Apr’13	$2.52^{c} \pm 0.03$	$1.81^{c} \pm 0.05$	$1.89^{d} \pm 0.03$	$1.82^{a} \pm 0.03$
May’13	$1.80^{f} \pm 0.04$	$2.16^{a} \pm 0.11$	$2.06^{d} \pm 0.09$	$1.44^{c} \pm 0.02$
Jun’13	$2.34^{d} \pm 0.07$	$1.50^{d} \pm 0.03$	n.d.	n.d.
Jul’13	$1.82^{f} \pm 0.04$	$2.01^{b} \pm 0.03$	$3.64^{a} \pm 0.19$	$1.40^{c} \pm 0.01$
Aug’13	$1.76^{f} \pm 0.02$	$2.11^{ab} \pm 0.06$	$3.26^{b} \pm 0.08$	$1.64^{b} \pm 0.04$
Sep’13	$1.87^{f} \pm 0.01$	$2.09^{ab} \pm 0.08$	n.d.	n.d.
Oct’13	$2.21^{e} \pm 0.10$	$2.21^{a} \pm 0.05$	n.d.	n.d.
Nov’13	n.d.	n.d.	$2.50^{c} \pm 0.30$	n.d.

Other carbohydrates measured in the seaweeds, as presented in Figure 1, were generally a mix of mainly fucose but also mannose, xylose and glucuronic acid with occasional traces of galactose, arabinose and/or rhamnose (data not shown). Supposedly, these sugars compose sulfated fucans (Ale and Meyer 2013). Amounts of this matrix polysaccharide, also known as fucoidan, were generally below 9 % (w/w) and similar to those previously reported for *L. digitata* and *S. latissima* (Obluchinskaya 2008; Manns et al. 2014). Although the contribution of fucoidan related carbohydrates made up a negligible fraction of the total carbohydrates, fucoidans are of great economic interest (Holdt and Kraan 2011).

Total carbohydrates were subject to seasonal variation, especially the storage carbohydrates glucose and mannitol, with two peaks during the summer. Furthermore, the M/G ratio of alginate differed strongly with seasonal for the sites in Kattegat and the North Sea. Interestingly, erratic values occurred within the season, apparently contradictory to a general trend; see for example the drastic decline in glucose content for the Kattegat population of *L. digitata* in August (Figure 1b). Potentially, this could be a consequence of sudden nutrient impulses. The total nitrogen in the seaweed rose from 1.4 % w/w dry matter in July to 2.2 % in August. Following the assimilation of nutrients, glucose from laminarin was most likely remobilized and supported the growth of the seaweed. Consequently, this would have caused the decline of glucose in August for *L. digitata*, and, although less pronounced, for *S. latissima* (Figure 1b/a). The role of nutrients is further discussed with the impact of site-specific physicochemical conditions on the chemical profile.

Variation within the population of *L. digitata* (North Sea)

Variation of carbohydrate composition was not only influenced by season, species and location but also fluctated between the years and within a seaweed population (Figure 2). Seaweed individuals of *L. digitata* from the North Sea from August 2012 to 2014 and November 2012 and 2013 were analyzed individually. A significant difference between the individuals of a given population at a specific time and at the same time over the years was evident (Table S in the supplementary material). For example, in August 2013 the three individuals A, B and C contained 61.1, 54.6, 39.5 % w/w of glucose; 6.2, 8.4, 19.3 % w/w of mannitol and 22.3, 19.7, 30.1 % w/w of alginate. In August 2014 the differences between individuals were less severe but still significant (Table S in the supplementary material) and glucose levels varied from 46.9 to 55.4 % w/w, while alginate levels varied from 19.2 to 26.0 % w/w (Figure 2). Additionally, there was no correlation (ANOVA analysis, $\alpha>0.05$) between the individual carbohydrate concentrations, e.g. glucose concentration, and the weight, i.e. plant size, i.e. age approximately. Weight ranged from 9 g up to 239.7 g with median size of 54.8 g, but high glucose contents were found in both small individuals (e.g. Aug’13 #A, 36.7 g, 61.1 % w/w glucose) and large individuals (e.g. Aug’14 #B, 190.6 g, 55.4 % w/w glucose) as well as vice versa (e.g. Aug’13 #C and Aug’12 #A) (Figure 2). At higher growth densities, competition with respect to light and nutrition are known to influence the chemical composition of seaweeds (Kerrison et al. 2015). Furthermore, the three seaweed individuals from November 2013 were compartmentalized to plant parts, i.e. holdfast, stipe and blade (data not shown). There was variation in the relative composition and total of carbohydrates of the different fragments, in agreement with previous reports from Black (1950).

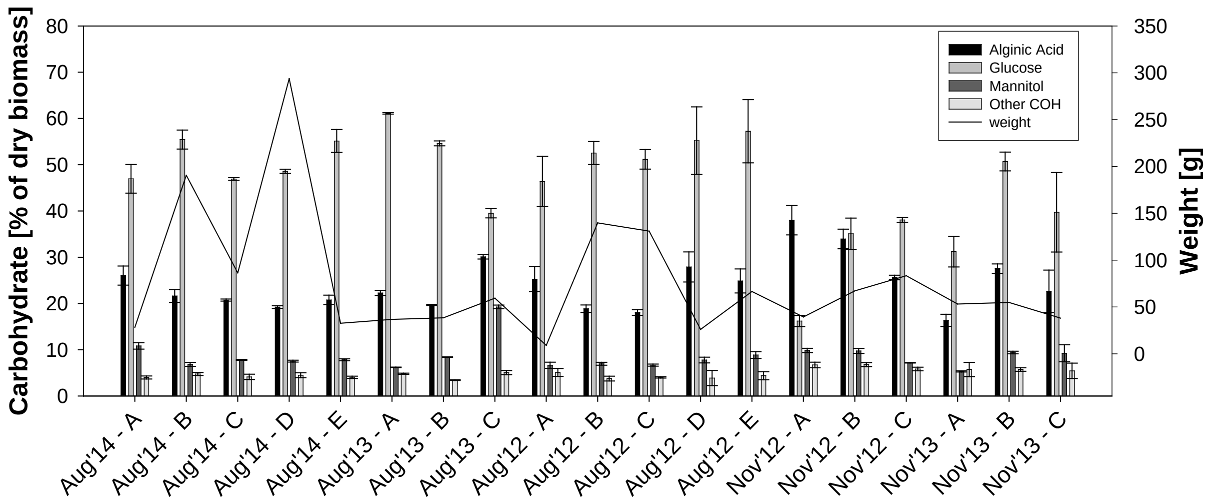

Figure 2: Variation of carbohydrate composition and weight (i.e. size) of seaweed individuals within the population of *Laminaria digitata* of the Danish North Sea at August 2014, ’13 and ’12, as well as at November 2012 and 2013. Each data point represents average values of independent triplicates; error bars indicate the standard deviation. All values are given as hydrated monomers; alginic acid: mannuronic acid and guluronic acid monomers; other carbohydrates: fucose, galactose, mannose, xylose and glucuronic acid. For ANOVA analysis through the different carbohydrate compounds to see Table S in the supplementary material.

Variation in composition of total carbohydrates, proteins and ash

In order to better understand the overall composition of the samples, the total amounts of carbohydrates, ash (as minerals) and protein were considered (Figure 3). The protein content was assessed from the total nitrogen content applying an N-to-protein-factor of 4 as proposed earlier (Manns et al. 2014). Carbohydrates were the dominant component in all seaweeds except the cultivated *S. latissima* from Limfjorden (Figure 3c). At the cultivation site, the carbohydrate content increased until May, before growth was severely hampered by massive biofouling. Samples from the North Sea had the highest amounts of carbohydrates ranging from 44 % w/w in April 2013 to 90 % w/w in August. In Kattegat both, *S. latissima* and *L. digitata*, had the lowest contents in February (30 % w/w) and remained around 60 % w/w from May to October (Figure 3). Concentrations of minerals (i.e. determined as ash) and protein were complementary to carbohydrate concentrations. Accordingly, the brown seaweeds were rich in protein and minerals during winter and spring (Figure 3). Ash contents of *L. digitata* from the North Sea were found to be 29.5 % in April and only 11.4 % w/w in August. For August 2012, correspondingly low levels of ash (11.9 % w/w) were reported for this site (Manns et al. 2014). Furthermore, in Wales, Adams et al. (2011) found an analogous ash profile (13.8 to 34.8 % w/w) for *L. digitata* from the Irish Sea. However, the ash content of *L. digitata* and *S. latissima* from Kattegat ranged between 20-30 % w/w (Figure 3a/b). The relative ash content of the cultivated seaweed remained high (33-40 % w/w) through the entire period of investigation (Figure3c). Marinho et al. (2015a) also described high ash contents of up to 40 % in young, cultivated *S. latissima* in Limfjorden, Denmark. However, ash concentrations were <30 % w/w between May and September (Marinho et al. 2015a). Generally, ash levels from 20 to 42 % of the dry matter present in the four analyzed seaweeds agree with recent findings of Schiener et al. (2015), who reported ash levels of 20 to 40 % of the dry matter of *L. digitata* and *S. latissima.* Seaweed minerals are used for fertilizers (Kraan 2013). The overall system productivity and sustainability of a biorefinery can be improved by integrating co-products, such as the production of fertilizers and the extraction of proteins (Kraan 2013).

The highest protein levels in the seaweeds from Kattegat and Limfjorden were achieved in February with about 20 % w/w (Figure 3a-c), while then highest protein level for *L. digitata* of the North Sea was in March with 15 % (Figure 3d). The lowest potential protein amounts were found in the months of July and August, constituting between 2.3 and 8.4 % of the dry weight depending on the site. Contents of 3-21 % by weight are well in agreement with protein levels previously summarized (Morrisey et al. 2001; Holdt and Kraan 2011).

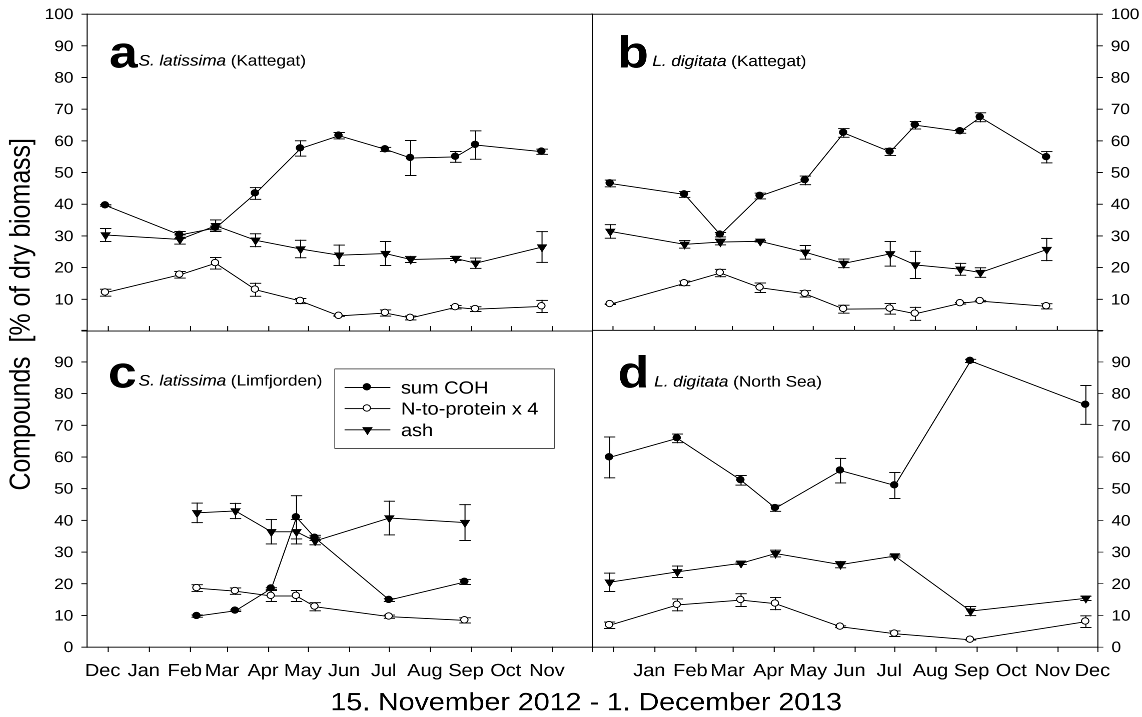

Figure 3: Seasonal variation from November 2012 to November 2013 of the main components minerals (determined as ash content, total protein (determined by N-to-protein factor of 4 after elemental analysis) and total carbohydrates (determined as the sum of hydrated monomers presented in Fig 1). (**a, b**) *S. latissima*, respectively *L. digitata* from the Danish Kattegat, (**c**) *S. latissima* from the cultivation in Limfjorden and (**d**) *L. digitata* from the Danish North Sea. Each data point represents average values of independent triplicates; error bars indicate the standard deviation.

Nitrogen to protein conversion factor

For the samples with the maximum and minimum protein concentrations (Figure 3) detailed amino acid analyses were performed. Samples from February and March had lower protein levels of 109 to 125 µg/mg dry biomass (Table 2) than calculated by total nitrogen from elemental analysis (Figure 3). For example, *S. latissima* from Kattegat was assumed to contain 21.4 % w/w from N-to-protein conversion, but was found to have a total of only

11.1 %, i.e. 111.1 µg amino acid residues per mg dry biomass after amino acid analysis (compare Figure 3a with first column of Table 2). For July samples of *S. latissima*, the N-to-protein factor of 4 underestimated the values found by amino acid analysis (compare populations of *S. latissima* from Figure 3 with Table 2). In contrast, a factor of four corresponded well to the total amino acid content of *L. digitata* samples from Kattegat in July and in the North Sea from August, respectively (55 µg/mg and 24 µg/mg in Table 2; compared to 54 µg/mg and 23 µg/mg in Figure 3b/d). In general, total amino acid concentrations varied between 23.9 and 127.0 µg/mg dry biomass for *L. digitata* populations and 48.1 to 141.6 µg/mg for *S. latissima* populations (Table 2). Similarly, Schiener et al. (2015) reported total protein contents of 49-82 µg/mg for *L. digitata* and 51-99 µg/mg dry weight for *S. latissima* over the season, using the Lowry method with bovine serum albumin (BSA) as the standard. Notably, the total amino acid concentration in *S. latissima* at the cultivation site at Limfjorden increased from February to July and was significantly higher than the other sites during summer (Table 2). However, the total nitrogen content decreased in the same period of time (February to July), for all sites documented (Figure 3). Hence, increased relative protein concentrations were most likely an artefact of the comparably very low carbohydrate concentrations. Nevertheless, total nitrogen levels of above 3 % w/w dry matter in May and >2 % in August (Figure 3) emphasizes the potential of cultivation of *S. latissima* as a bioremediation tool (Marinho et al. 2015b).

Table 2: Distribution of nitrogen, calculation of nitrogen-to-protein factors and mapping of amino acids, related to total biomass and relatively to the amino acid (AA) composition, of brown seaweeds from Danish waters containing the highest and the lowest level of nitrogen within the period of sampling (November 2012 to November 2013). ANOVA-analysis through the different samples (with pooled standard deviation of 10 %). Different roman superscript letters indicate significant differences ($\alpha < 0.05$) row-wise.

		S. latissima Kattegat Feb'13	*S. latissima* Kattegat Jul'13	*L. digitata* Kattegat Feb'13	*L. digitata* Kattegat Jul'13	*L. digitata* North Sea Mar'13	*L. digitata* North Sea Aug'13	*S. latissima* Limfjorden Feb'13	*S. latissima* Limfjorden Jul'13
$N_{biomass}$	[$\mu g/mg_{biomass}$]	53.4^{a}	10.3^{e}	45.8^{b}	13.6^{e}	37.1^{c}	5.8^{e}	46.5^{b}	24.0^{d}
$N_{non\text{-}protein}$	[$\mu g/mg_{biomass}$]	38.7^{a}	3.8^{d}	28.7^{b}	6.3^{d}	20.8^{c}	2.6^{d}	31.7^{b}	5.3^{d}
$N_{protein}$	[$\mu g/mg_{biomass}$]	14.8^{b}	6.4^{c}	17.0^{ab}	7.3^{c}	16.3^{ab}	3.23^{d}	14.8^{b}	18.7^{a}
total AA	[$\mu g/mg_{biomass}$]	111.1^{b}	48.1^{c}	127.0^{ab}	54.8^{c}	125.4^{ab}	23.9^{d}	109.0^{b}	141.6^{a}
$N_{biomass}$ to protein factor		2.1^{e}	4.7^{b}	2.8^{de}	4.0^{bc}	3.4^{cd}	4.1^{bc}	2.3^{e}	5.9^{a}
Asp	[$\mu g/mg_{biomass}$]	13.6^{ab}	6.4^{c}	15.7^{a}	8.0^{c}	11.9^{b}	3.5^{d}	12.8^{b}	16.2^{a}
	[% of total AA]	12.2^{abc}	13.4^{abc}	12.4^{abc}	14.6^{a}	9.5^{bc}	14.5^{a}	11.7^{abc}	11.4^{bc}
Thr	[$\mu g/mg_{biomass}$]	5.5^{ab}	2.9^{d}	6.3^{ab}	3.2^{d}	4.9^{c}	1.4^{e}	4.8^{c}	6.7^{a}
	[% of total AA]	4.9^{bc}	6.1^{a}	4.9^{bc}	5.9^{ab}	3.9^{c}	5.9^{ab}	4.4^{c}	4.7^{bc}
Ser	[$\mu g/mg_{biomass}$]	4.7^{c}	2.7^{d}	5.7^{b}	3.0^{d}	4.5^{c}	1.3^{e}	4.9^{bc}	7.0^{a}
	[% of total AA]	4.2^{c}	5.7^{a}	4.5^{bc}	5.4^{ab}	3.6^{c}	5.5^{ab}	4.5^{bc}	4.9^{ab}
Glu	[$\mu g/mg_{biomass}$]	17.5^{b}	6.1^{cd}	18.8^{b}	7.3^{c}	33.2^{a}	3.1^{d}	14.6^{b}	30.0^{a}
	[% of total AA]	15.7^{b}	12.7^{b}	14.8^{b}	13.4^{b}	26.5^{a}	12.9^{b}	13.4^{b}	21.2^{a}
Pro	[$\mu g/mg_{biomass}$]	5.0^{a}	2.4^{b}	5.7^{a}	2.8^{b}	5.8^{a}	1.2^{c}	5.3^{a}	6.2^{a}
	[% of total AA]	4.5^{a}	5.0^{a}	4.5^{a}	5.0^{a}	4.7^{a}	5.1^{a}	4.9^{a}	4.4^{a}
Gly	[$\mu g/mg_{biomass}$]	5.8^{bc}	2.9^{d}	6.7^{b}	3.0^{d}	5.0^{c}	1.4^{e}	6.8^{b}	11.0^{a}
	[% of total AA]	5.2^{bc}	6.1^{b}	5.2^{bc}	5.4^{b}	4.0^{c}	5.9^{b}	6.3^{b}	7.8^{a}
Ala	[$\mu g/mg_{biomass}$]	14.3^{b}	3.3^{de}	17.2^{a}	4.3^{d}	19.5^{a}	1.8^{e}	13.4^{b}	10.2^{c}
	[% of total AA]	12.9^{b}	6.8^{c}	13.5^{ab}	7.8^{c}	15.5^{a}	7.4^{c}	12.3^{b}	7.2^{a}
TPCys	[$\mu g/mg_{biomass}$]	1.7^{c}	1.3^{c}	2.5^{b}	1.7^{c}	2.9^{b}	0.6^{d}	-	3.8^{a}
	[% of total AA]	1.5^{d}	2.7^{ab}	1.9^{cd}	3.1^{a}	2.3^{bc}	2.3^{bc}	-	2.7^{ab}
Val	[$\mu g/mg_{biomass}$]	5.8^{ab}	2.6^{c}	6.5^{ab}	3.0^{c}	5.0^{b}	1.3^{d}	6.1^{ab}	7.1^{a}
	[% of total AA]	5.2^{ab}	5.4^{a}	5.1^{ab}	5.4^{a}	4.0^{b}	5.5^{a}	5.6^{a}	5.0^{ab}
Met	[$\mu g/mg_{biomass}$]	2.5^{a}	1.1^{c}	2.6^{a}	1.2^{c}	2.1^{b}	0.6^{d}	1.9^{b}	2.3^{ab}
	[% of total AA]	2.2^{ab}	2.3^{ab}	2.0^{abc}	2.3^{ab}	1.6^{c}	2.3^{a}	1.8^{bc}	1.6^{c}
Ile	[$\mu g/mg_{biomass}$]	4.4^{ab}	2.0^{c}	4.9^{a}	2.2^{c}	3.7^{b}	0.9^{d}	4.9^{a}	4.8^{a}
	[% of total AA]	3.9^{ab}	4.1^{ab}	3.9^{ab}	4.0^{ab}	3.0^{c}	3.9^{ab}	4.5^{a}	3.4^{bc}
Leu	[$\mu g/mg_{biomass}$]	8.2^{ab}	3.7^{c}	9.0^{a}	4.0^{c}	6.8^{b}	1.77^{d}	8.8^{a}	8.4^{ab}
	[% of total AA]	7.4^{ab}	7.7^{a}	7.1^{abc}	7.3^{ab}	5.4^{c}	7.4^{ab}	8.1^{a}	5.9^{bc}
Tyr	[$\mu g/mg_{biomass}$]	3.8^{bc}	1.9^{d}	4.4^{b}	1.9^{d}	3.3^{c}	0.8^{e}	3.9^{bc}	5.3^{a}
	[% of total AA]	3.4^{ab}	3.9^{a}	3.4^{ab}	3.5^{a}	2.6^{b}	3.5^{a}	3.6^{a}	3.7^{a}
Phe	[$\mu g/mg_{biomass}$]	5.7^{ab}	2.6^{c}	6.8^{a}	2.9^{c}	4.7^{b}	1.3^{d}	6.6^{a}	6.9^{a}
	[% of total AA]	5.1^{ab}	5.4^{ab}	4.9^{bc}	5.2^{ab}	3.7^{c}	5.5^{ab}	6.1^{a}	4.9^{bc}
His	[$\mu g/mg_{biomass}$]	1.9^{b}	1.0^{c}	1.9^{b}	1.0^{c}	1.9^{b}	0.6^{d}	1.7^{b}	2.4^{a}
	[% of total AA]	1.7^{bc}	2.0^{ab}	1.5^{c}	1.7^{bc}	1.5^{c}	2.3^{a}	1.6^{bc}	1.7^{bc}
Lys	[$\mu g/mg_{biomass}$]	5.8^{ab}	2.6^{c}	6.9^{a}	2.9^{c}	5.3^{b}	1.40^{d}	6.5^{a}	6.5^{a}
	[% of total AA]	5.2^{abc}	5.4^{ab}	5.5^{ab}	5.2^{abc}	4.2^{c}	5.9^{a}	6.0^{a}	4.6^{bc}
Arg	[$\mu g/mg_{biomass}$]	5.0^{b}	2.5^{c}	6.1^{ab}	2.8^{c}	5.1^{b}	1.2^{d}	5.9^{b}	6.9^{a}
	[% of total AA]	4.5^{ab}	5.2^{a}	4.8^{ab}	5.0^{ab}	4.0^{b}	5.1^{ab}	5.4^{a}	4.9^{ab}

The presence of non-protein nitrogenous substances such as pigments and dissolved inorganic nitrogen affects the N-to-protein conversion factor (Lourenco et al. 2002). Levels of inorganic nitrogen in seawater vary seasonally (Zimmerman and Kremer 1986; Carstensen et al. 2006). Hence, non-protein related nitrogen made up 20-30 µg/mg in *L. digitata* and 30-40 µg/mg in *S. latissima* of dry weight for the samples of early 2013, equal to 56 to 72 % of the total nitrogen. Later in the year, nitrogen contents decreased drastically. Hence, most of the nitrogen was attributed to the presence of amino acids, and non-protein N such as dissolved nitrates was <40 % of the total nitrogen content for *L. digitata* and >40 % of the total nitrogen content for *S. latissima* (Table 2). The conversion factors were low in winter, during times of high total algal nitrogen, and vice versa in summer (Table 2). Total algal nitrogen contents show more seasonal variation compared to the protein content (Zimmerman and Kremer 1986; Marinho et al. 2015a/b). A literature study on all available data for protein concentration of seaweeds revealed no correlation between internal N content and N-protein factor (Angell et al. 2015). Conclusively, the suggested protein concentrations in Figure 3 display the total nitrogen content rather than the real protein content, visualizing the problems related to the simple and often used N-to-protein conversion factor for protein estimation. The factor for conversion of N-to-protein depends on the applied method for the nitrogen determination. Generally, factors are higher for the Kjeldahl method than for total nitrogen measured by elemental analysis with a broad range from 2.7 to 5.4 (Gonzalez et al. 2010; Slocombe et al. 2013). Differences in N-to-protein factors for brown seaweed species were previously reported by Lourenco et al. (2002), with factors on average 5.4 ± 0.5 for four brown seaweeds collected during the austral winter (although not *L. digitata* or *S. latissima*). Overall, the average conversion factor of N-to-protein from Table 2 was calculated as 3.7, well in agreement with earlier reports for Danish seaweed (Manns et al. 2014; Nielsen et al. 2016). Also in agreement with these studies, the sampling site appeared to influence the protein content (Table 2). Therefore, the determination of the total protein content by application of global factor of 3.7 ± 1.3 risks enormous miscalculation due to the high standard deviation of ± 1.3. In the present study, the conversion factors for *L. digitata* were less variable, i.e. higher during winter (Kattegat: 2.8, The North Sea: 3.4) but lower in the summer (approx. 4 at both sites) compared to *S. latissima* populations varying between 2.1 to 5.9 (Table 2). The extreme variance of *S. latissima* protein factors was in agreement with a previous literature study of 459 brown seaweed samples (including 40 samples of *Laminaria/Saccharina*), which reported a variance of 2.1 to 6.25 with an average protein factor of 4.6 (Angell et al. 2015). In the study by Angell et al. red and green seaweed were included and a global N-protein factor of 5 was suggested. However, seasonal fluctuations of non-protein related nitrogen sources, such as nitrates and ammonia were not considered in the suggestion of a global conversion factor of 5 (Angell et al. 2015).

Table 2 presents the amino acid profiles of the analyzed brown seaweeds. Individual amino acid contents vary significantly between spring and summer (first rows of each amino acid in Table 2). But interestingly, the total individual amino acid levels are more dependent on the location than on the species of seaweed. ANOVA analysis revealed only minor differences between *L. digitata* and *S. latissima* from the same location i.e. Kattegat. However, the amino acid composition of both seaweeds differed significantly from the same seaweed at other locations. The amino acid compositions of Kattegat populations were quite similar at both sampling times, while the amino acid profile of *L. digitata* from the North Sea varied with the season. Glutamic acid made up 26.5 % of the total amino acids content of *L. digitata* from the North Sea in March, but only 12.9 % in August. In contrast, the concentration of aspartic acid increased from 9.5 to 14.5 % to become the most abundant amino acid in August (Table 2). *S. latissima* from Kattegat in the present study differed in profile and total protein content from a previous study of cultivated Kattegat seaweed (Marinho et al., 2015a). Overall, the most abundant amino acids were glutamic acid, aspartic acid and alanine, accounting for 30-

50 % of the amino acid content in all samples, which is in agreement with previous reports for most seaweed species (Holdt and Kraan 2011).

Impact of site-specific physicochemical conditions on the chemical profile

Several previous studies have shown the impacts of single variables such as nutrients, carbon dioxide/pH, salinity, water motions, temperature, light or salinity, among others mainly on the biomass growth and composition (Indegaard et al. 1990; Sanderson et al. 2008; Marinho et al. 2015a; Nielsen et al. 2015b). However, the correlation of a single variable with a single group of results disregards the possibility of interdependence of the multiple impacts of environmental variables and/or the biochemical composition. Hence, this study attempts to include all available input factors (environmental variables) and output data (biochemical composition) in a single statistical cross validation (PLS regression) in order to model the biochemical composition regarding the related specific condition.

Compositional analyses of the four populations of *S. latissima* (2 populations from 2 locations, n=18) and *L. digitata* (2 populations from 2 locations, n=19) from the three different locations over one season (2012/13) were correlated with the site-specific physicochemical conditions retrieved from the Danish National Aquatic Monitoring and Assessment Program (Figure 4). The PLS regression tool suggested two main influencing factors (loadings) to describe 71.0 % of the variance in the biochemical composition of the brown seaweeds (data not shown). The first loading was dominated by the seasonal variation of the reserve substances mannitol and glucose, and behaved contrary to nitrogen and ash content. This was in agreement with the findings above where mineral (ash) and protein levels were complementary to carbohydrate levels and total carbohydrate concentration were seasonally influenced by the storage carbohydrates laminarin, i.e. glucose, and mannitol, in particular (see section seasonal and spatial variation). Furthermore, not the alginate content but the group of other COH (supposed as fucoidan derived carbohydrates, see Figure 1 other COH) characterized loading 1. Alginate, though, was the main strong variable for loading 2. Fucoidan, nitrogen and glucose were acknowledged to influence loading 2 in addition. Generally, the finding of high statistical residuals indicated little or no correlation between the biochemical composition and the environmental parameters (data not shown).

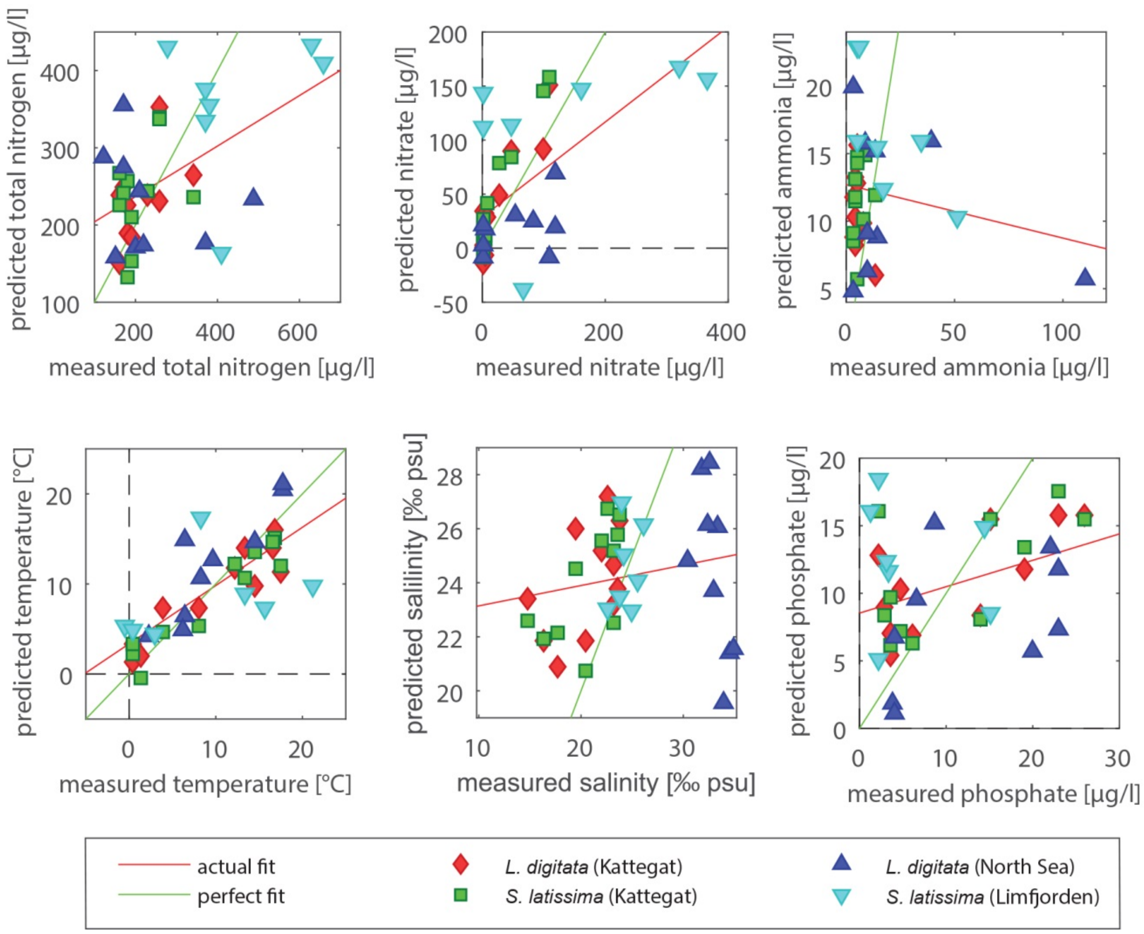

Figure 4: PLS regression of site-specific physicochemical variables total nitrogen, nitrate, ammonia, temperature, salinity and phosphate against the biochemical compositions (mannitol, glucose, alginic acid, other COH (see figure 1) as well as ash and nitrogen (see figure 3)) of *S. latissima* and *L. digitata* from the Danish Kattegat, *S. latissima* from the cultivation in Limfjorden, Denmark and *L. digitata* from the Danish North Sea. The closer perfect fit and actual fit the more significant the influence of the variable.

In accordance to high statistical residues, the statistical cross validation of the multiple environmental variables identified temperature ($R^2 = 0.616$) as a significant physicochemical parameter (Figure 4). The physicochemical conditions at the three different locations were very heterogeneous across the season. The heterogeneity of the studied sites was particularly apparent for salinity, with high salt content over 30 PSU in the North Sea (*L. digitata* at Hanstholm, dark blue upright triangles), around 25 PSU with little variance in Limfjorden at the cultivation site (light blue upside down triangles) and a seasonal variation from 15 to 25 PSU in Kattegat at the Bay of Aarhus (Figure 4). As salinity gradient of 16-31 PSU in Kattegat has been previously documented as a significant factor on the biochemical composition of *L. digitata* and *S. latissima* using one-way ANOVA analysis (Nielsen et al. 2016). However, in this study different salinities had no influence ($R^2 = 0.038$) on the biochemical composition of the seaweeds (Figure 4).

Nutrient levels in the seaweeds presented in Figure 4 differed with the seaweed growth location. At the Bay of Aarhus, Kattegat, yearly nutrient concentrations (>10 µg/L for ammonia) were steadily lower compared to higher and more fluctuating levels in Limfjorden (5-51 µg/L) as well as in the North Sea averaging 23 µg/L (Figure 4), in agreement with earlier reports (Conley et al. 2000; Carstensen et al. 2006). The optimal uptake requirements for bioavailable nitrogen (ammonia and nitrates) is ≥5 µM (5 µM = 85 µg/L ammonia or 5 µM = 310 µg/L nitrate) and ≥0.3 µM (28.5 µg/L) for phosphates (Kerrison et al. 2015). However, concentrations of phosphate never reached the optimum at any time or site and the sum of nitrate and ammonia only reached optimum levels for three measurements, once for *L. digitata* at Hanstholm (dark blue upright triangles) and twice for *S. latissima* at Limfjorden (light blue downright triangles) (Figure 4). The relationship between between nutrition requirements and uptake is not a simple because the algae assimilate and store nitrogen, it makes more sense to evaluate their nutritional status by looking at the tissue N concentrations

(Dalsgaard and Krause-Jensen 2006). A tissue concentration of <1.7 % N of dry seaweed is considered critical for optimal growth (Pedersen and Borum 1997). In contrast to the bioavailable nitrogen requirement, N concentrations of the seaweed exceeded the critical concentration of 1.7 % for most of the year. Only during summer months of May-July were there lower tissue concentrations for *S. latissima* (Kattegat), July (Kattegat) and May-August for *L. digitata* (North Sea) (Figure 3). In fact, the total nitrogen concentration of the seaweeds was a significant factor of the PLS regression in both loadings of the biochemical composition. Nevertheless, the nitrogen available in the sea water in forms of ammonia, nitrate and phosphate apparently did not influence the biochemical composition of the seaweed (R^2: nitrate = 0.377; ammonia = 0.030; phosphate = 0.134). Furthermore, no correlation was found between total sea water nitrogen concentration and biochemical composition of the seaweeds (R^2 = 0.257) (Figure 4). Therefore, tissue N might be a more reliable indicator of the physiological nutrient status of the seaweeds than the environmental concentrations. However, to verify this relationship it is necessary to distinguish between inorganic tissue N and cumulated organic tissue N (i.e. gained over the period investigation) from the organic tissue N present before the investigated period.

Temperature is known to influence growth rate, with the optimal temperature for brown seaweed in the range from 5 to 15 ºC (Kerrison et al. 2015) but no literature is currently available that describes a direct correlation with chemical composition. However, the temperature of sea water is directly linked to the amount of incoming light during the season and therefore the growth strategy of the perennial seaweed, linking the storage/exhaustion of nitrogen and buildup of the reserve carbohydrates mannitol and laminarin during periods of limited growth. Optimum temperature conditions were found at the North Sea in every month except March 2013 (2.3 ºC). In contrast, the temperature fluctuations were larger in Kattegat, where it was colder on average during winter and warmer throughout the summer months.

Moreover, the highest variance in temperature were seen at the cultivation site at Limfjorden with temperatures above 20 ºC in the summer, which potentially contributed to the restricted growth of the seaweed during the summer (Kerrison et al. 2015; Marinho et al. 2015b). Accordingly, the highest concentrations of glucose were found for the population of the North Sea, while for the seaweed at the cultivation site, glucose was present at the lowest concentrations (Figure 1).

The cross data validation identified temperature as the only physiochemical variable with significant influence on the chemical composition of the seaweeds. Further investigations should rule out if temperature is just a proxy for the incoming light. Light and other known influencing parameters, such as ultra-violet radiation, related depth, pH, carbon dioxide concentrations, water motion, water flow rate or population growth density were available and therefore could not be considered in this validation.

CONCLUSIONS

The chemical composition of brown seaweed varied with respect to location, season, species and environmental conditions. The cultivated seaweed from Limfjorden was very different from the three wild populations, but similar to other cultivated seaweeds from Denmark. Cultivation was feasible, but a favorable biochemical profile with high carbohydrate concentrations for bioenergy purposes was not achievable. The seasonal pattern of the composition depended on location and was more variable in populations of *L. digitata* than *S.latissima*. *L. digitata* accumulated more glucose than *S.latissima*, along with lower ash contents, making it preferable as a source for bioenergy. In particular, for the population in the North Sea, outstandingly high glucose levels (>50 % w/w) were documented for three sequential years (2012-2014) in August. Furthermore, in times when the glucose levels became depleted, *L. digitata* from the North Sea was an interesting candidate for the extraction of other valuable products, as it consisted of over 30 % total alginate, rich in guluronic acid and a concentration of amino acids of 12.5 % w/w of the dry seaweed. Variations within populations also have to be considered for optimal cultivation strategies. Analytical carbohydrate analysis of population individuals of *L. digitata* from the North Sea showed significant differences at the given months of sampling and between the different seasons. Generally, alginate was the most abundant carbohydrate polymer at all sites from December to June/July, before glucose levels rose to at least the same magnitude. Alginate, or alginic acid, as the sum of its monomers mannuronic and guluronic acid was relatively unaffected by seasonal changes. However, M/G ratios differed strongly throughout the year from 1.3 to 3.6, but with no certain pattern regarding season, species or location. Conversion of N-to-protein with a factor of four emphasized high protein contents of up to 20 % of dry weight in the beginning of the year. However, total protein determination through amino acid analysis did not confirm these findings. The average conversion factor was 3.7, with variance from 2.1 to 5.9, leading to less variance in the total protein content. Affected by season and location, the amino acid profile was dominated by aspartic and glutamic acid in the beginning of the year, while glutamic acid and alanine dominated during the summer. Many environmental parameters have been described throughout the literature as exerting an influence on brown seaweed growth conditions and composition. However, in this study temperature was the only variable found to influence the chemical composition of seaweeds with optimal conditions found at the site of Hanstholm, Danish North Sea. Furthermore, the promising brown seaweed *L. digitata* from the North Sea was exposed to stronger water motions than the other seaweed populations from the Danish inner waters, indicating correlations between water motion and chemical composition. Future work should include assessments of the local environmental conditions growing conditions such as wind exposure, light exposure, the availability and uptake of nutrients, or perhaps other factors like genetic differences and the demography of the population. Such work would help to develop strategies for cultivating and harvesting seaweed to ensure optimum chemical profile for developing bioenergy and bio-based products from macroalgae.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES

Adams, J.M.M., Ross, A.B., Anastasakis, K., Hodgson, E.M., Gallagher, J.A., Jones, J.M. & Donnison, I.S. 2011. Seasonal variation in the chemical composition of the bioenergy feedstock *Laminaria digitata* for thermochemical conversion. *Bioresour. Techn.* 102(1):226-234.

Ale, M.T. & Meyer, A.S. 2013. Fucoidans from brown seaweeds: an update on structures, extraction techniques and use of enzymes as tools for structural elucidation. *RSC Adv.* 3(22):8131-8141.

Angell, A.R., Mata, L., Nys, R. & Paul, N.A. 2016. The protein content of seaweeds: a universal nitrogen-to-protein conversion factor of five. *J. Appl. Phycol.* 28(1):511-524.

Barkholt, V. & Jensen, A.L. 1989. Amino acid analysis: Determination of cysteine plus half-cystine in proteins after hydrochloric acid hydrolysis with a disulfide compound as additive. *Anal Biochem.* 177(2):318-322.

Bilan, M.I., Grachev, A.A., Shashkov, A.S., Kelly, M., Sanderson, C.J., Nifantiev, N.E. & Usov, A.I. 2010. Further studies on the composition and structure of a fucoidan preparation from the brown alga *Saccharina latissima*. *Carbohydr. Res.* 345(14):2038-2047.

Black, W. 1950. The seasonal variation in weight and chemical composition of the common British laminariaceae. *J. Mar. Biol. Assoc. UK* 29:45-72.

Brown, A. & Tustin, J. 2010. *Algae – The Future for Bioenergy? Summary and conclusions from the IEA Bioenergy ExCo64 Workshop*. IEA Bioenergy: ExCo: 2010:02.

Buck, B.H. & Buchholz, C.M. 2004. The offshore-ring: A new system design for the open ocean aquaculture of macroalgae. *J. Appl. Phycol.* 16:355–368.

Carstensen, J., Conley, D.J., Andersen, J.H. & Ærtebjerg, G. 2006. Coastal eutrophication and trend reversal: A Danish case study. *Limnol. Oceanogra.* 51:1, part 2.

Conley, D.J., Kaas, H., Møhlenberg, F., Rasmussen, B. & Windolf, J. 2000. Characteristics of Danish Estuaries. *Estuaries* 23:820–837.

Creutzig, F., Goldschmidt, J.C., Lehmann, P., Schmid, E., von Blücher, F., Breyer, C., Fernandez, B., Jakob, M., Knopf, B., Lohrey, S., Susca, T. & Wiegandt, K. 2014. Catching two European birds with one renewable stone: Mitigating climate change and Eurozone crisis by an energy transition. *Ren. Sustain. Ener. Rev.* 38:1015-1028.

Dalsgaard, T. & Krause-Jensen, D. 2006. Monitoring nutrient release from fish farms with macroalgal and phytoplankton bioassays. *Aquaculture* 256:302-310.

Dave, A., Huang, Y., Rezvani, S., McIlveen-Wright, D., Novaes, M. & Hewitt, N. 2013. Techno-economic assessment of biofuel development by anaerobic digestion of European marine cold-water seaweeds. *Bioresour. Techn.* 135:120-127.

Fertah, M., Belfkira, A., Dahmane, Em., Taourirte, M. & Brouillette, F. 2014. Extraction and characterization of sodium alginate from Moroccan Laminaria digitata brown seaweed. *Arab. J. Chem.*

González López, C.V., García, M.d.C.C., Fernández, F.G.A., Bustos, C.S., Chisti, Y. & Sevilla, J.M.F. 2010. Protein measurements of microalgal and cyanobacterial biomass. *Bioresour. Techn.* 101(19):7587-7591.

Holdt, S.L. & Kraan, S. 2011. Bioactive compounds in seaweed: functional food applications and legislation. *J. Appl. Phycol.* 23(3):543-597.

IPCC. 2014. *Climate Change 2014: Mitigation of Climate Change*. Contribution of Working Group III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Edenhofer O, Pichs-Madruga R, Sokona Y, Farahani E, Kadner S, Seyboth K, Adler A, Baum I, Brunner S, Eickemeier P, Kriemann B, Savolainen S, Schlömer S, von Stechow C, Zwickel T, Minx JC (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Indergaard, M., Skjåk-Bræk, G. & Jensen, A. 1990. Studies on the Influence of Nutrients on the Composition and Structure of Alginate in *Laminaria saccharina* (L.) Lamour. (Laminariales, Phaeophyceae). *Bot. Mar.* 33:277-288

Indergaard, M. & Minsaas, J. 1991. Animal and Human Nutrition. 1 ed. *In* Guiry, M.D. Blunden, G. [Eds.] *Seaweed Resources in Europe: Uses and Potential*. John Wiley & Sons. Chichester, UK, pp. 21-64.

Jensen, A & Haug, A. 1956. Geographical and seasonal variation in the chemical composition of *Laminaria hyperborea* and *Laminaria digitata* from the Norwegian coast. Rep. Norw. Inst. Seaweed Res. No. 14. Laboratory of Biotechnology, Trondheim.

Jørgensen, B.B. & Richardson K [Eds.] 1996. *Eutrophication in Coastal Marine Ecosystems*. American Geophysical Union, Coastal and Estuarine Studies 52, Washington, D.C., 273 pp.

Kerrison, P.D., Stanley, M.S., Edwards, M.D., Black, K.D. & Hughes, A.D. 2015. The cultivation of European kelp for bioenergy: Site and species selection. *Biom. Bioener.* 80:229-242.

Kloareg, B & Quatrano, R.S. 1988. Structure of the cell-walls of marine-algae and ecophysiological functions of the matrix polysaccharides. *Oceanogr. Mar. Biol. Ann. Rev.* 26:259-315.

Kraan, S. 2013. Mass-cultivation of carbohydrate rich macroalgae, a possible solution for sustainable biofuel production. *Mitig. Adapt. Strateg. Glob. Change*. 18:27-46.

Lee, K.S., Hong, M.E., Jung, S.C., Ha, S.J., Yu, B.J., Koo, H.M., Park, S.M., Seo, J.H., Kweon, D.H., Park, J.C. & Jin, Y.S. 2011. Improved Galactose Fermentation of *Saccharomyces cerevisiae* Through Inverse Metabolic Engineering. *Biotechnol. and Bioeng.* 108(3):621-631.

Lourenco, S.O., Barbarino, E., De-Paula, J.C., Pereira, L.O.d.S. & Lanfer Marquez, U.M. 2002. Amino acid composition, protein content and calculation of nitrogen-to-protein conversion factors for 19 tropical seaweeds. *Phycol. Res.* 50(3):233-241.

Manns, D., Deutschle, A.L., Saake. B. & Meyer, A.S. 2014. Methodology for quantitative determination of the carbohydrate composition of brown seaweeds (Laminariaceae). *RSC Adv*. 4(49):25736-25746.

Marinho, G., Holdt, S. & Angelidaki, I. 2015a. Seasonal variations in the amino acid profile and protein nutritional value of *Saccharina latissima* cultivated in a commercial IMTA system. *J. Appl. Phycol.* 27(5):1991-2000.

Marinho, G., Holdt, S., Birkeland, M. & Angelidaki, I. 2015b. Commercial cultivation and bioremediation potential of sugar kelp, *Saccharina latissima*, in Danish waters *J. Appl. Phycol.* 27(5):1963-1973.

McHugh, D.J. 2003. *A guide to the seaweed industry*. FAO Fisheries Technical Paper 441. Food and Agriculture Organisation of the United Nations, Rome.

Morrissey, J., Kraan, S. & Guiry, M.D. 2001. *A Guide to Commercially Important Seaweeds on the Irish Coast*. Bord Iascaigh Mhara/Irish Sea Fisheries Board. Institute, M.R.M.S., Board, I.S.F., Ireland, N.L.o.

Nielsen, M.M., Krause-Jensen, D., Olesen, B., Thinggaard, R., Christensen, P. & Bruhn, A. 2014. Growth dynamics of *Saccharina latissima* (Laminariales, Phaeophyceae) in Aarhus Bay, Denmark, and along the species' distribution range. *Mar. Biol.* 161(9):2011-2022.

Nielsen, M.M., Manns, D., D'Este, M., Krause-Jensen, D., Rasmussen, M.B., Larsen, M.M., Alvarado-Morales, M., Angelidaki, I. & Bruhn, A. 2016. Variation in biochemical composition of *Saccharina latissima* and *Laminaria digitata* along an estuarine salinity gradient in inner Danish waters. *Algal Res.* 13: 235-245.

Obluchinskaya, E.D. 2008. Comparative chemical composition of the Barents Sea brown algae. *Appl. Biochem. Microbiol.* 44(3):305-309.

Pedersen, M.F. & Borum, J. 1997. Nutrient control of estuarine algae: growth strategy and the balance between nitrogen requirements and uptake. *Mar. Ecol. Prog. Ser.* 161:155 –163.

Percival, E. & McDowell, R.H. 1967. *Chemistry and enzymology of marine algal polysaccharides*. Academic Press Inc. (London) Ltd.

Raman, S., Mohr, A., Helliwell, R., Ribeiro, B., Shortall, O., Smith, R. & Millar, K. 2015. Integrating social and value dimensions into sustainability assessment of lignocellulosic biofuels. *Biom. Bioener.* 82:49-62.

Rioux, L.E., Turgeon, S.L. & Beaulieu, M. 2010. Structural characterization of laminaran and galactofucan extracted from the brown seaweed *Saccharina longicruris. Phytochem.* 71(13):1586-1595.

Sanderson, J.C., Cromey, C.J., Dring, M.J. & Kelly, M.S. 2008. Distribution of nutrients for seaweed cultivation around salmon cages at farm sites in north–west Scotland. *Aquaculture* 278:60-68.

Schiener, P., Black, K., Stanley, M. & Green, D. 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(1):363-373.

Siddhanta, A.K., Prasad, K., Meena, R., Prasad, G., Mehta, G.K., Chhatbar, M.U., Oza, M.D., Kumar, S. & Sanandiya, N.D. 2009. Profiling of cellulose content in Indian seaweed species. *Bioresour. Techn.* 100(24):6669-6673.

Slocombe, S.P., Ross, M., Thomas, N., McNeill, S. & Stanley, M.S. 2013. A rapid and general method for measurement of protein in micro-algal biomass. *Bioresour. Techn.* 129:51-57.

Venghaus, S. & Selbmann, K. 2014. Biofuel as social fuel: Introducing socio-environmental services as a means to reduce global inequity? *Ecol. Econom.* 97:84-92

Wei, N., Quarterman, J. & Jin, Y.-S. 2013. Marine macroalgae: an untapped resource for producing fuels and chemicals. *Trends Biotech.*, 31(2), 70-77.

Wargacki, A.J., Leonard, E., Win, M.N., Regitsky, D.D., Santos, C.N.S., Kim, P.B., Cooper, S.R., Raisner, R.M., Herman, A., Sivitz, A.B., Lakshmanaswamy, A., Kashiyama, Y., Baker, D. & Yoshikuni, Y. 2012. An Engineered Microbial Platform for Direct Biofuel Production from Brown Macroalgae. *Science* 335(6066):308-313.

Zimmerman, R.C. & Kremer, J.N. 1986. In situ growth and chemical composition of the giant kelp, *Macrocystis pyrifera*: response to temporal changes in ambient nutrient availability. *Mar*. *Ecol. Prog. Ser.* 27:277–285.

SUPPLEMENTARY MATERIAL

Table S: ANOVA-analysis through the different carbohydrate composition of *L. digitata* from August 2012, 2013 and 2014, as well as November 2012 and (see also figure 2). Different roman letters indicate significant differences between the individuals ($\alpha < 0.05$) row-wise per group; first letter within a population at the given date (August 2014, August, 2013, August 2012, November 2012, November 2013); second letter between the three years at the same month (August, November).

Sample	Alginic Acid	Glucose	Mannitol	Other COH
Aug'14 #A	a, b	b, cd	a, b	a, ab
Aug'14 #B	b, d	a, ab	c, fg	a, ab
Aug'14 #C	b, d	b, cd	b, de	a, ab
Aug'14 #D	b, d	b, cd	bc, ef	a, ab
Aug'14 #E	b, d	a, ab	b, de	a, ab
Aug'13 #A	b, cd	a, a	c, g	a, ab
Aug'13 #B	c, d	b, ab	b, cd	b, b
Aug'13 #C	a, a	c, d	a, a	a, a
Aug'12 #A	a, bc	a, cd	b, g	a, a
Aug'12 #B	b, d	a, bc	b, ef	a, ab
Aug'12 #C	b, d	a, bc	b, g	a, ab
Aug'12 #D	a, ab	a, ab	ab, de	a, ab
Aug'12 #E	a, bc	a, ab	a, c	a, ab
Nov'12 #A	a, a	b, cd	a, a	a, a
Nov'12 #B	a, a	a, b	a, a	a, a
Nov'12 #C	b, b	a, b	b, b	a, a
Nov'13 #A	b, c	b, b	b, b	a, a
Nov'13 #B	a, b	a, a	a, a	a, a
Nov'13 #C	a, b	ab, b	a, a	a, a

PAPER III

Brown seaweed processing: enzymatic saccharification of *Laminaria digitata* requires no pre-treatment

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Brown seaweed processing: enzymatic saccharification of *Laminaria digitata* requires no pre-treatment

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Abstract This study assesses the effect of different milling pre-treatments on enzymatic glucose release from the brown seaweed *Laminaria digitata* having high glucan (laminarin) content. Wet refiner milling, using rotating disc distances of 0.1–2 mm, generated populations of differently sized pieces of lamina having decreasing average surface area (100–0.1 mm^2) with increased milling severity. Higher milling severity (lower rotating disc distance) also induced higher spontaneous carbohydrate solubilization from the material. Due to the seaweed material consisting of flat blades, the milling did not increase the overall surface area of the seaweed material, and size diminution of the laminas by milling did not improve the enzymatic glucose release. Milling was thus not required for enzymatic saccharification because all available glucose was released even from unmilled material. Treatment with a mixture of alginate lyase and a cellulase preparation (Cellic®CTec2) on large-sized milled material released all available glucose within 8 h. Application of the cellulase preparation alone released only half of the available glucose. The alginate lyase catalysis apparently induced selective removal of alginate to improve the cellulase catalyzed degradation of laminarin and cellulose in the material.

Keywords Brown seaweed · Phaeophyceae · Milling · Enzymatic glucose release · Alginate lyase

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Introduction

Macroalgae, or seaweeds, have recently been prospected as a potential new biomass resource for bioenergy and chemicals production (Brown & Tustin, 2010; Roesijadi et al., 2010). In the Northern Hemisphere, mainly brown seaweeds of the type "kelp" (Phaeophyceae), including species such as *Saccharina latissima* and *Laminaria digitata*, have been studied to assess their glucose potential in relation to bioenergy production (Adams et al., 2011). It is well known that the biomass composition of brown seaweeds varies throughout the year and that also the carbohydrate composition differs with the algae species and the geographical location for growth (Adams et al., 2011; Black, 1950; Percival & McDowell, 1967). We recently found that *L. digitata* harvested from the Danish North Sea (off Hanstholm) in August 2012 contained about 84 % by weight of total organic matter dominated by glucose moieties constituting 51 % by weight of the dry matter (Manns et al., 2014). This high glucose content, which is accompanied by a content of 8 % by weight of mannitol and a low ash content (<10 %), indicates that *L. digitata* is particularly promising as a brown seaweed source for biorefining and bioenergy production when harvested at the right time and place (Manns et al., 2014).

The brown seaweed plant tissue is soft and in the case of kelp mainly made up of flat longitudinal blade structures (lamina) (John et al., 2011; Manns et al., 2014; Percival & McDowell, 1967; Roesijadi et al., 2010). Whereas enzymatic hydrolysis of lignocellulosic feedstocks is inefficient without a hydrothermal

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or other physicochemical pre-treatment to help increase enzymatic accessibility to the cellulose (Alvira et al., 2010; Kumar et al., 2009), such harsh pre-treatment may not be required for enzymatic seaweed saccharification since seaweed does not contain lignin. Pre-treatment of *Laminaria japonica* with very low sulfuric acid concentrations of <0.1 % followed by heat treatment at 170 °C for 15 min has been reported to enhance enzymatic glucan hydrolysis of the seaweed compared to just employing hot water pre-treatment (Lee et al., 2013). However, it has also been reported that some of the classical types of lignocellulose pre-treatments induce significant losses of convertible seaweed biomass (Schultz-Jensen et al., 2013). A comparison of five pre-treatment technologies for processing of the green seaweed *Chaetomorpha linum* for ethanol production showed that a ball milling pre-treatment producing particles of 2 mm was superior to classical lignocellulosic biomass benchmark pre-treatments (Schultz-Jensen et al., 2013). Mechanical grinding pre-treatment has also been shown to enhance ethanol yields on *S. latissima* biomass (Adams et al., 2009). The diminution of seaweed biomass to smaller particles by milling has been envisaged to increase the substrate surface area which in turn would enhance the enzymatic processing and fermentation to ethanol (Roesijadi et al., 2010; Wargacki et al., 2012). However, no systematic study assessing the influence of the degree of milling on brown seaweed particle size or the influence of substrate particle size on enzymatic saccharification response for brown seaweed is available.

Phylogenetically, the kelp type brown seaweeds (Phaeophyceae) belong to the Stramenopiles phylum that uses laminarin as storage polysaccharide. Recent genome annotation evidence has confirmed that pathways for sucrose, starch, and glycogen synthesis are absent in this type of seaweed (Michel et al., 2010). Laminarin is made up of a backbone of β-1,3-linked glucose moieties with β-1,6-linked branches (John et al., 2011; Rioux et al., 2010). In addition, brown seaweeds have been reported to contain some cellulose (Schiener et al., 2015; Siddhanta et al., 2009). The presence of laminarin and cellulose agrees with the experimental findings that enzymatic liberation of glucose from brown seaweeds is effectively accomplished by enzyme cocktails harboring β-1,3-glucanases and cellulases (Adams et al., 2009; Adams et al., 2011; Kim et al., 2011b; Yanagisawa et al., 2011). Another difference from terrestrial biomass is that in brown seaweeds, the main matrix polysaccharide is alginic acid or alginate as its salt. Alginate thus constitutes a key component of the brown seaweed cell walls but also appears to be present in the intercellular space matrix (Adams et al., 2011; Davis et al., 2003; Kloareg & Quatrano, 1988; Mabeau & Kloareg, 1987). Alginate consists of 1,4-glycosidically linked α-L-guluronic acid (G) and β-D-mannuronic acid (M), which are present in varying proportions in different brown seaweeds. The G and M moieties form linear chains with M/G ratio ranges of 1.2 to 3.0 and higher. Alginate lyase, encompassing EC 4.2.2.3 mannuronate lyase and EC 4.2.2.11 guluronate lyase, catalyzes alginate degradation via a β-elimination reaction and mainly acts via endo-attack, i.e., catalyzing bond cleavage within the alginate backbone chain (Wong et al., 2000). The potential of employing alginate lyases for pre-treatment or saccharification of macroalgae for biofuel production has been suggested in the literature (Kim et al., 2011a). However, an evaluation of the significance of alginate lyase in relation to enzymatic glucose release from brown seaweed is currently not available.

The objective of this study was to assess the significance of milling pretreatment and substrate particle size diminution on enzymatic saccharification of glucan-rich *L. digitata* biomass. Another aim was to develop an optimal enzymatic saccharification treatment to achieve maximal glucose release from the brown seaweed biomass.

Materials and methods

L. digitata was harvested from the Danish North Sea coast end of August 2012 (Manns et al., 2014). Prior to processing, the material was washed successively four times with water to remove residual sand and salt. After washing, the biomass was stored at −20 °C until use. The dry matter content was determined after thawing. By weight, the dry biomass consisted of 51 % glucose moieties (dehydrated monomers), 8 % mannitol, 23 % mannuronic and guluronic acid, and ~4.5 % of other carbohydrates (Manns et al., 2014).

Pure laminarin was from Sigma-Aldrich (Steinheim, Germany). D-(+)glucose was from Merck (Darmstadt, Germany).

Mechanical size reduction

Mechanical wet milling was performed in a Sprout-Bauer 12" lab refiner. Wet seaweed material was fed to the mill through a central screw feeding. The milling severity was adjusted by the distance between the discs; disc distances of 0.1, 0.2, 0.3, 0.6, 1.0, 1.5, and 2.0 mm were applied at a rotating speed of 3000 rpm. Heating of biomass and blocking of the milling system were prevented by adding water to the seaweed during the processing. The resulting dry matter of all milling slurries were determined after drying at 105 °C overnight, and dry matter of all slurries was adjusted to 7.5 % by weight by addition of water prior to enzymatic saccharification. After milling, the samples were analyzed directly by microscopy (see below). Some sample aliquots were stored frozen at −20 °C until enzymatic treatment.

Particle size determination

A KEYENCE digital microscope (VHX-500FD) along with its integrated software was used for evaluation of the surface area of milled particles. The image analysis software was first set to mark all particles by a process based on color differences. Subsequently, all unnecessary markings, such as background noise, were removed by filtering. Finally, holes in the marking were filled using the filler function in the VHX-500FD software. For each milling fraction investigated, the surface areas of $n \geq 120$ particles were determined.

Additionally, the available surface area was predicted from the increase in viscosity of the particle volumes over the different milling degrees. Viscosities of all slurries were recorded on a Rapid Visco Analyser (Newport Scientific, UK) from two replicate runs of each sample at a dry matter level of 7.5 % by weight (wet, milled seaweed). Each viscosity measurement was based on $n = 21$ measurement points at an impeller mixing of 150 rpm at 25 °C.

Enzymatic treatment

Enzymatic hydrolysis was conducted on thawn, wet seaweed material at 5 % (*w*/*w*) dry matter substrate concentration at 40 °C, pH 5, in 0.2 M phosphate 0.1 M citrate buffer at 60 rpm on a horizontal roller mixer. Treatment was performed with 2 % E/S (Enzyme/Substrate level in % by weight) of alginate lyase (EC 4.2.2.3) from *Flavobacterium multivorum* (Sigma-Aldrich, Germany) and 5 % E/S (enzyme/substrate % *w/w*) of the cellulase preparation Cellic®CTec2 (Novozymes A/S, Denmark). As a benchmark for the effect of milling on the enzymatic deconstruction of the wet milled slurries, a single piece non-milled fresh alga was used. Samples were taken at the following intervals in minutes: 0, 15, 30, 60, and 90 min and after 24 h during the enzymatic hydrolysis. Further on, enzyme dosage studies were accomplished on the slurry which had been milled at a disc distance of 2.0 mm. In the enzyme dosage study, enzyme concentrations varied from 0 to 2 % E/S for alginate lyase and between 0 to 20 % E/S for Cellic®CTec2 with sampling after (0), 4, 6, and 8 h. Studies on pure laminarin were conducted in 1.5-mL Eppendorf tubes in a thermomixer at 1000 rpm with similar reactions conditions as those used for the other enzymatic hydrolysis experiments. Enzyme concentrations were set corresponding to the available glucan (i.e., 51 % by weight) as in the dosage studies of fresh seaweed to 4 % E/S alginate lyase and 20 % E/S Cellic®CTec2. Reactions were terminated by addition of 5 M NaOH. Samples were then centrifuged at 5400× *g* for 10 min and filtered through a 0.2-μm syringe tip filter prior to assessment of glucose release (see below).

Enzymatic glucose determination assay

Glucose contents in enzymatic hydrolysates were determined with the Megazyme HK/G6P-Dh D-glucose kit using a 96-well microplate reader (TECAN Infinte 200) with data collection by the TECAN *i-control*® software for absorbance spectroscopy measurements.

Results and discussion

Mechanical particle size reduction

As expected, the milling generated seaweed particles, i.e., lamina pieces, of decreasing sizes (100–0.1 mm^2) over increasing milling degree assessed as disc distance between the rotating discs in the refiner mill (the smaller the disc distance, the higher the milling degree) (Fig. 1). At the very short disc distances of 0.2 and 0.1 mm, the mean particle sizes of the seaweed pieces were below 0.25 mm^2 averaging 0.19 and 0.12 mm^2, respectively (Fig. 1a). However, the boxplots illustrate that a large span of particle sizes was obtained within each type of milling severity, and the larger disc distances did in particular produce some lamina pieces which had large particle size areas (Fig. 1a). The obtained mean particle surface area (*dashed line*) was thus strongly affected by the bigger lamina pieces and was always above the median of 50 % of the particles (Fig. 1a). The data imply that even though the smaller particles outnumbered the larger ones, the fewer bigger pieces of lamina present in the milled samples dominated the surface area of the particle population.

Particle size response to milling degree

The logarithm of the particle size response could be fitted to milling degree by a polynomial correlation (R^2 ~0.6). This correlation is likely due to the dominance of a few large pieces in each particle population (Fig. 1b). The unusual response of the seaweed to milling is proposed to be ascribable to the morphology of the seaweed material; the seaweed material thus consists of elongated flat blades producing only a two-dimensional disruption, i.e., scission of the seaweed blades, with milling at the larger disc distances. The apparent lack of a three-dimensional defibrillation of the seaweed, which is obtained with refiner milling of lignocellulosic materials such as straw, wood, or pulp, indicates that the morphology and soft state of the seaweed blades result in the refiner milling merely cleaving the seaweed blades into smaller pieces. This two-dimensional scission of the seaweed blades in essence resembles the cutting of a sheet of paper into smaller pieces, i.e., the material is cut in two dimensions (width and length) with no effect on the thickness. In turn, this scission does not produce a significant increase in surface area with diminution of the size of the pieces as

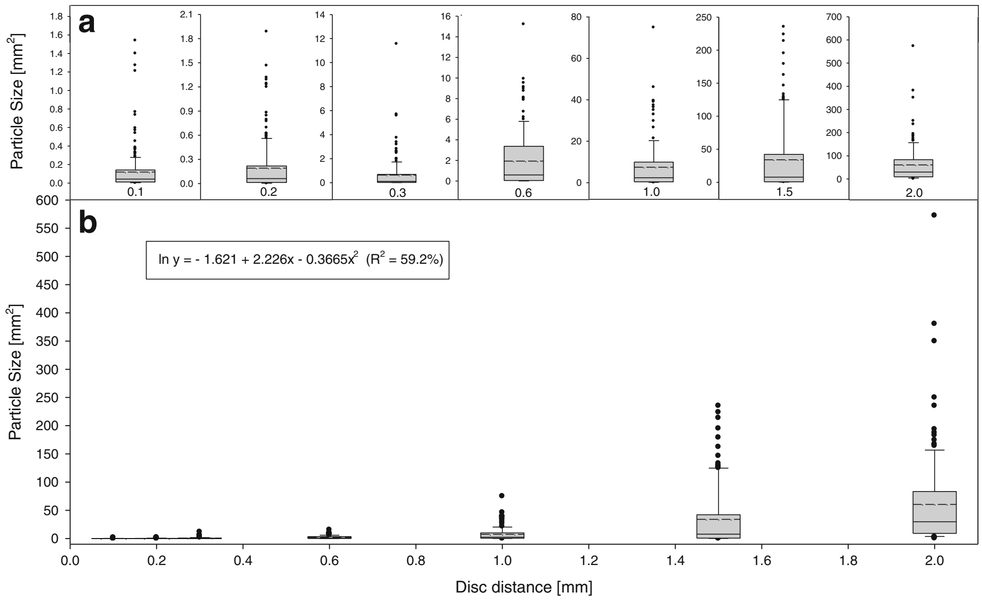

Fig. 1 Boxplots of particle size distribution after refiner milling with decreasing severities (*left* to *right*; i.e., increasing disc distances from 0.1 to 2.0 mm) of each individual milling batch (**a**) and over milling severity (**b**) of wet *Laminaria digitata*. The *boundaries* of the box represents 50 % of the data ($n_i > 122$), the *solid line* within the box marks the median, and the *dashed line* the mean. *Whiskers* (*error bars*) above and below the box indicate the 90th and 10th percentiles, and the outliers are displayed as *circles*. Polynomial regression analysis ($\alpha < 0.05$) as correlation between disc distance and generated particle size was estimated using the fitting plot tool of analysis software Minitab 14.

compared to what occurs from three-dimensional disruptions of, e.g., lignocellulosic materials, which, when calculated as a reduction of the radius, r, of spheres, increase the surface area to weight of the material dramatically, in accord with the area/volume ratio for spheres being $3/r$ ($[4\pi{\cdot}r^2]$ / $[4/3\pi{\cdot}r^3]$)

To estimate the mass of the seaweed pieces per unit area, ten randomly picked pieces of wet *L. digitata* biomass were weighed and the surface areas of the flat blades were measured; the surface area was calculated for the flat blades (area on both sides, data not shown). This resulted in an estimation of the average seaweed biomass weight per area of 0.081 g cm^{-2} ± 0.011. Hence, assuming a density of 1 g cm^{-3}, the average thickness of the (milled) brown seaweed blades was estimated to be ~0.8 ± 0.1 mm. Consequently, a three-dimensional disruption due to milling, and therefore a significant increase in available surface area of this flat material, can only occur via milling or refining at disc distances below this thickness. The refiner milling disc distances of 2.0 to 1.0 mm used for the *L. digitata* material were thus bigger than the thickness of the *L. digitata* blades, which could explain why the milling with these disc distances only resulted in two-dimensional scission of the seaweed blades producing no significant increase in the surface area for enzyme attack.

Milling has been applied previously on brown seaweeds such as *S. latissima* (after cutting the blades into smaller pieces of ~5 cm^2) (Adams et al., 2009; 2011), *Laminaria hyperborean* (Horn et al., 2000), *Undaria pinnatifada* (Lee et al., 2011), and, e.g., *Alaria crassifolia* (Yanagisawa et al., 2011) employing different types of milling technologies from blending to ultra-centrifugal milling—the latter producing uniform seaweed biomass particles of less than 0.5 mm in diameter (Yanagisawa et al., 2011). Although advanced milling regimes such as ultra-centrifugal milling may be useful for lab-scale research, this kind of high-intensity milling is too energy consuming for large-scale seaweed biorefining.

Viscosity versus particle size distribution of milled seaweed samples

For kelp seaweed biomass, standardized methodologies for particle size distribution assessment are not available. In order to achieve a better understanding of the correlation between refiner milling degree, true biomass material disruption, and resulting surface area, an evaluation of the viscosity response to the milled *L. digitata* particle area measurements of the refiner slurries was conducted (Fig. 2). The disc distances of 2.0 and 1.5 mm produced particle volume fractions with mean particle sizes of 60 and 34 mm^2 (Fig. 1) and relatively low slurry viscosities averaging approximately 400 cP (Fig. 2). However, millings at disc distances of 1.0, 0.6, and 0.3 mm, i.e., at disc distances lower or equal than the thickness of the algae blades, produced particles with mean sizes of 7.47, 1.9,

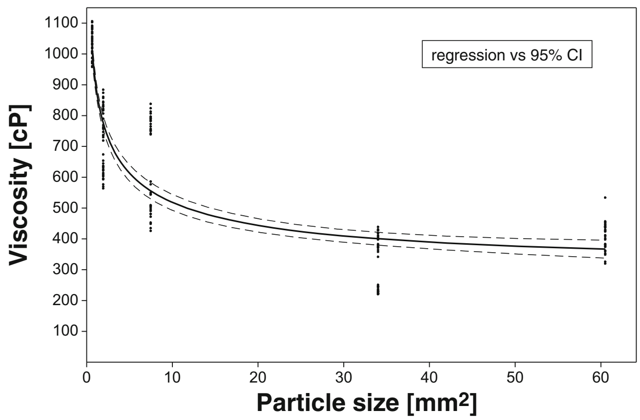

Fig. 2 Fitted plot of received viscosities at shear rate of 150 rpm of the refiner slurries (*filled circles*) milled at disc distances of 0.1, 0.2, 0.3, 0.6, 1.0, 1.5, and 2.0 mm (dry matter for all 7.5 %) over mean particle sizes (polynomial regression analysis with confidence interval (CI) of 95 % excluding the data for 0.1- and 0.2-mm disc distances): $\ln y = 912.0 - 505.8x + 112.1x^2$ ($R^2 = 82.6$ %). Data from two individual samples with total data points of $n = 42$ was used for each slurry

and 0.64 mm^2 (Fig. 1), and the viscosities of these particle volume fractions were high, reaching 800–1050 cP, highest with shorter disc distance at milling (Fig. 2). The viscosity response to particle size for the milling data with disc distances down to 0.3 mm^2 thus followed a steep polynomial function (Fig. 2). In general, the viscosity response to particle size diminution of suspensions of homogenous solid particles is mainly influenced by the so-called particle volume fraction, which is correlated to the particle size; in other words, the viscosity increases with particle size reduction because the particle volume fraction increases (Mueller et al., 2009). It is tempting to conclude that the viscosity increase at low particle size (Fig. 2) was in accord with this solid particle volume fraction theory. However, for the brown seaweed particles, the correlation may be more complex; first, the viscosities obtained for the slurries having been subjected to the very harsh milling at refiner disc milling distances of 0.1 and 0.2 mm were low, namely, ~320–480 cP (Fig. 2); second, it was observed that a carbohydrate-rich exudate was released from the seaweed material during harsher milling, with the amount of the exudate dry matter increasing at disc distances below 1.0 mm (data not shown). The drop in the slurry viscosity with the smallest refiner disc distances, i.e., at intensified milling, could be caused by the agglomeration of small particles or be a result of breakage of a gel network in the exudate carbohydrates (notably alginate) which was released spontaneously from the *L. digitata* biomass during milling. Although exudate release would be expected to increase the slurry viscosity as the milling degree was intensified, the RVA recording of the aqueous fraction of the slurry viscosity was unable to pick up any viscosity of the exudates, which all had viscosities lower than 100 cP (data not shown). The high content in minerals of brown seaweed (Manns et al., 2014) may enable agglomeration between small particles due to ionic exchange. Hence, it is likely that in addition to particle size, forces such as ionic bonding may have a direct influence on the rheological properties of milling slurries of brown seaweed.

Effect of milling on enzymatic glucose release

A positive influence of particle size reduction on enzymatic biomass deconstruction has been observed for both cellulose and various types of lignocellulosic biomass (Silva et al., 2012; Yeh et al., 2010). For lignocellulosic biomass, the effect of the substrate particle diminution has been explained as being a result of increasing the accessible surface area for enzymatic attack as well as a shortening of the entry and exit paths for the enzymes and hydrolysis products with decreased particle size of porous substrate particles (Pedersen and Meyer, 2009). However, in the present work, reduction of the particle size did not improve enzymatic decomposition of the *L. digitata* biomass after refiner milling (Fig. 3, Table S1). Neither was a positive effect of substrate milling on glucose yields compared to the non-milled starting material observed since all available glucose was enzymatically released after 24 h (1440 min) both with and without milling pre-treatment (Fig. 3, Table S1).

The initial glucose release rates were measured over the first 90 min of enzymatic treatment. There was no statistically significant difference within the range of

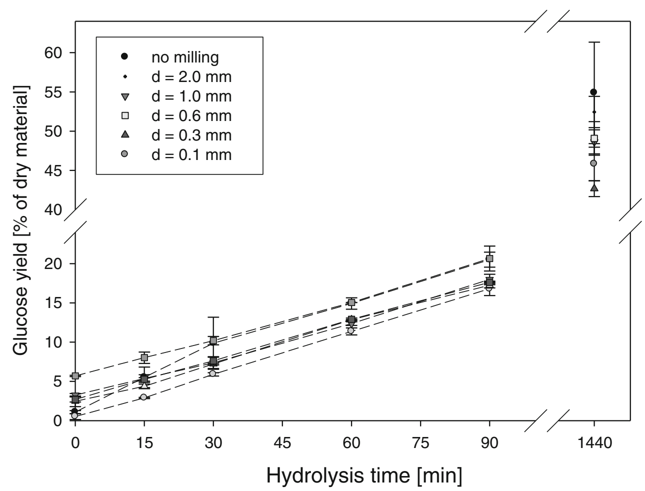

Fig. 3 Glucose yields in % of dry slurry for refiner milled wet *Laminaria digitata* at different degrees and non-milled *L. digitata* over hydrolysis time. Each data point represents the average value of independent duplicates; *vertical bars* indicate the standard deviation. All values are given as hydrated monomers (ANOVA of the data is detailed in Table S1 in the Supplementary material)

release rates (Fig. 3, Table S1), but a tendency to higher initial glucose release rates with lower milling severity was evident:~120 $mg_{glucose}g_{dry\ material}^{-1} \times h^{-1}$ for non-milled material, ~110 $mg_{glucose}g_{dry\ material}^{-1} \times h^{-1}$ for the mildest milling (2.0-mm disc distance), and ~100 $mg_{glucose}g_{dry\ material}^{-1} \times h^{-1}$ for the harshest milling (0.1-mm disc distance).

That the initial release rates tended to be lowest for the samples that had been subjected to the harshest milling could be a result of the findings that at timepoint zero, the deconstruction of the cell wall due to milling increased the presence of free glucose monomers up to 6.4 % (timepoint zero for milling with 0.1-mm disc gap). In contrast, the glucose monomers in the non-milled samples and in the samples milled at higher disc gap were released only during the enzymatic treatment (Fig. 3). The presence of free glucose monomers in the raw material thus affected the release rate and confirmed the finding of Ostgaard et al. (1993) that autumn harvested brown seaweed contains free glucose monomers. The release rate data and the yields obtained were in accord with those we obtained in a prior study on the same material, although with a substrate concentration of 2 % (*w/w*) and higher cellulase dosage (20 % (*v/w*) Cellic®CTec2 on the substrate) (Manns et al., 2014).

Effect of enzyme dosage on enzymatic glucose release

The slurry having been subjected to the lowest milling intensity at disc distance 2.0 mm was studied further to investigate the effect of enzyme dosage and alginate lyase addition on the enzymatic glucose release from the seaweed.

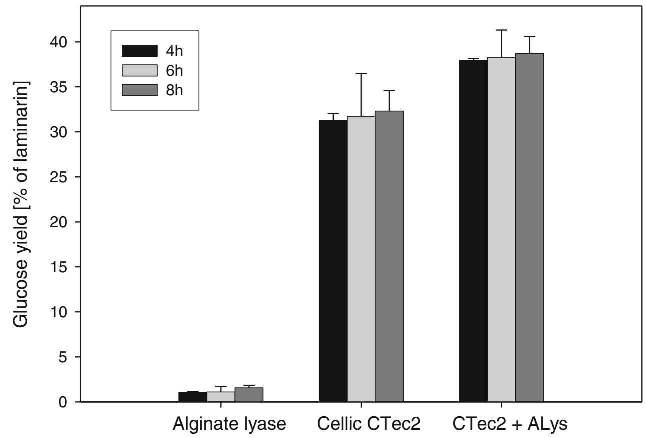

Fig. 5 Glucose yields in % of pure laminarin after enzymatic hydrolysis with alginate lyase (4 % *w/w*), Cellic® CTec2 (20 % *v/w*), and both in a mixture with measurements at timepoints 4, 6, and 8 h. Each data point represents average values of independent duplicates; *error bars* indicate the standard deviation. All values are given as hydrated monomers

Increased dosage of cellulase (Cellic®CTec2) with 2 % E/S alginate lyase produced a steady increase in glucose yield after reactions of 4, 6, and 8 h, respectively (Fig. 4). The statistical analysis of the data revealed that a cellulase (Cellic®CTec2) concentration of 10 % and a reaction time of 8 h was required to achieve release of all the glucose present in the seaweed (Fig. 4, Table S2). Further increase in cellulase dosage to 15 and 20 % E/S did not give an increase of glucose yield after 8 h (data not shown).

Adams et al. (2011) used laminarinases at 2 % *w/w* on ground *L. digitata* for 2 h to estimate the concentration of laminarin dependence on the season. A maximum of about 20 % laminarin was determined for material harvested in August which was much lower than the content of glucose determined in our sample. Laminarinase is believed to be ac-

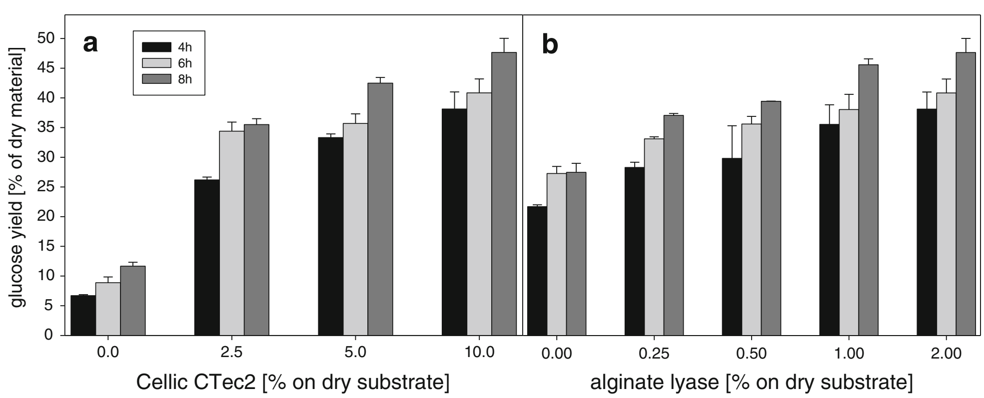

Fig. 4 Glucose yields in % of dry material for refiner milled wet *Laminaria digitata* with disc distances of 2.0 mm. Enzymatic hydrolysis yields of glucose displayed **a** over Cellic®CTec2 concentration at fixed alginate lyase (2 % *w/w*) and **b** over alginate lyase concentration at fixed Cellic® CTec2 (10 % *v/w*) with measurements at timepoints 4, 6, and 8 h. Each data point represents the average value of independent duplicates; *vertical bars* indicate the standard deviation. All values are given as hydrated monomers (ANOVA of the data is detailed in Tables S2, S3, S4 and S5 in the Supplementary material)

tive only on β-1,3 glucan neither on cellulose nor the β-1,6 linkages of laminarin. Yanagisawa et al. (2011) treated brown seaweed *A. crassifolia* with a commercial cellulase preparation derived from *Trichoderma viride* for 120 h. After the first day, glucose release almost leveled out, and after 5 days, 82.3 % of the potential glucose could be released. A mixture of commercial Celluclast 1.5 L and Viscozyme L (β-glucanase and endo-glucanase activity) performed best on *L. japonica* releasing 72.4 % of sugars of the theoretical yield post–acid treatment (Kim et al., 2011b). In contrast, a prior analytical study on the composition of brown seaweeds gave a total glucose release of *L. digitata* using only Cellic®CTec2treatment (Manns et al., 2014). Cellic® CTec2 is known as a cellulase preparation which contains at least the two main cellobiohydrolases EC 3.2.1.91 (Cel6A and Cel7A), five different endo-1,4-β-glucanases EC 3.2.1.4 (Cel7B, Cel5A, Cel12A, Cel61A, and Cel45A), β-glucosidase EC 3.2.1.21, β-xylosidase EC 3.2.1.37, and particular proprietary hydrolysis-boosting proteins. The preparation was also proven to have activity on pure laminarin (Fig. 5).

Alginate lyase addition alone, without Cellic® CTec2, facilitated the release of glucose as glucose yields increased with time in the control experiments (Fig. 4a, point 0.0). This effect of the alginate lyase must be a result of the material containing some free glucose monomers embedded in the matrix, which were released upon alginate degradation. The presence of free glucose is supported by the findings that some initial free glucose was detected directly after milling, especially for the most harshly milled samples (Fig. 3). The findings that alginate lyase treatment alone on pure laminarin did in fact release a little glucose of 1–2 % glucose suggest a minimal activity on the seaweed glucan (Fig. 5).

When varying the alginate lyase concentration on a base of 10 % E/S of Cellic®CTec2, the glucose yields from the refiner-milled slurry of *L. digitata* increased over both hydrolysis time and enzyme concentration of alginate lyase (Fig. 4b, Table S4 and Table S5). Statistically, the alginate lyase dosage effect was significant at all hydrolysis times up to a concentration of 1 % (*w*/*w*) lyase on the substrate (Fig. 4b, Table S4 and Table S5). The enzymes apparently attacked the substrate surface directly. The linear alginate chains in brown seaweed are made up of different blocks of G and M and are referred to as MM blocks or GG blocks, but MG blocks may also occur (Indergaard et al., 1990; Kloareg & Quatrano, 1988; Percival & McDowell, 1967). Alginate lyases exhibit a preferred but not highly selective activity on either MM-, GG-, or MG-blocks (Kim et al., 2011a; Wong et al., 2000).

Hence, during the treatment with cellulase and alginate lyase, it is presumed that the alginate lyase action catalyzes the cleavage of alginate by endo-action on the substrate, which both decreases the viscosity of the substrate matrix and catalytically solubilizes the alginate to provide access for the endo-glucanases to the laminarin and cellulose in the brown seaweed cell wall matrix. This perception of the alginate lyase action is in accordance with the described embedding matrix and an inner cell wall skeleton of brown seaweed (Kloareg & Quatrano, 1988; Schiewer & Volesky, 2000).

Conclusions

Wet refiner milling as physical pretreatment of glucose-rich brown seaweed *L. digitata* led to particle size reduction with the degree of milling. Although the milling decreased the size of the brown seaweed blade pieces, the milling was in essence a two-dimensional disruption, which did not increase the overall surface area for enzymatic attack. However, the data obtained showed that there is no need for milling pre-treatment as glucose was enzymatically released also on non-milled material. Enzyme dosage of 1 % (*w*/*w*) alginate lyase and 10 % (*v*/*w*) Cellic® CTec2 released the potential glucose during 8 h, and less glucose was released with lower enzyme loading (i.e., by either lowering the alginate lyase or the Cellic® CTec2 dosage), and the enzymes apparently attacked the substrate surface directly. Alginate lyase improved the enzymatic glucose release, presumably by improving laminarin and cellulose accessibility by catalyzing alginate degradation. Nevertheless, in order to guarantee a homogenous process, a particle size reduction may be advisable. In addition to being of interest in relation to using brown seaweed for bioprocessing applications, the academic main novelty points are that (1) the size diminution of the brown seaweed did not increase the surface area for enzyme attack due to the milling being a two-dimensional scission of the seaweed blades (lamina) and that (2) the fungal cellulases developed for saccharification of terrestrial cellulosic plant material were able to catalyze the degradation of the brown seaweed laminarin structure.

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References

Adams JM, Gallagher JA, Donnison IS (2009) Fermentation study on *Saccharina latissima* for bioethanol production considering variable pre-treatments. J Appl Phycol 21:569–574

Adams JMM, Ross AB, Anastasakis K, Hodgson EM, Gallagher JA, Jones JM, Donnison IS (2011) Seasonal variation in the chemical composition of the bioenergy feedstock *Laminaria digitata* for thermochemical conversion. Bioresour Technol 102:226–234

Alvira P, Tomás-Pejó E, Ballesteros M, Negro MJ (2010) Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresour Technol 101:4851–4861

Black W (1950) The seasonal variation in weight and chemical composition of the common British Laminariaceae. J Mar Bioll Ass U K 29:45–72

Brown A, Tustin J (2010) Algae – The future for bioenergy? Summary and conclusions from the IEA Bioenergy ExCo64 Workshop. IEA Bioenergy: ExCo 2010:02

Davis TA, Volesky B, Mucci A (2003) A review of the biochemistry of heavy metal biosorption by brown algae. Wat Res 37:4311–4330

Horn SJ, Aasen IM, Østgaard K (2000) Ethanol production from seaweed extract. J Indust Microbiol Biotechnol 25:249–254

Indergaard M, Skjåk-Bræk G, Jensen A (1990) Studies on the influence of nutrients on the composition and structure of alginate in *Laminaria saccharina* (L.) Lamour. (Laminariales, Phaeophyceae). Bot Mar 33:277–288

John RP, Anisha GS, Nampoothiri KM, Pandey A (2011) Micro and macroalgal biomass: a renewable source for bioethanol. Bioresour Technol 102:186–193

Kim H, Lee C-G, Lee E (2011a) Alginate lyase: Structure, property, and application. Biotech Bioproc Eng 16:843–851

Kim N-J, Li H, Jung K, Chang HN, Lee PC (2011b) Ethanol production from marine algal hydrolysates using *Escherichia coli* KO11. Bioresour Technol 102:7466–7469

Kloareg B, Quatrano RS (1988) Structure of the cell walls of marine algae and ecophysiological functions of the marix polysaccharides. Oceanogr Mar Biol 26:259–315

Kumar P, Barrett DM, Delwiche MJ, Stroeve P (2009) Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. Ind Eng Chem Res 48:3713–3729

Lee S, Oh Y, Kim D, Kwon D, Lee C, Lee J (2011) Converting carbohydrates extracted from marine algae into ethanol using various ethanolic *Escherichia coli* strains. Appl Biochem Biotech 164: 878–888

Lee JY, Kim YS, Um BH, Oh K (2013) Pretreatment of *Laminaria japonica* for bioethanol production with extremely low acid concentration. Renew Energy 54:196–200

Mabeau S, Kloareg B (1987) Isolation and Analysis of the Cell Walls of Brown Algae: *Fucus spiralis, F. ceranoides, F. vesiculosus, F. serratus, Bifurcaria bifurcata and Laminaria digitata*. J Exp Bot 38:1573–1580

Manns D, Deutschle A, Saake B, Meyer AS (2014) Methodology for quantitative determination of the carbohydrate composition of brown seaweeds (Laminariaceae). RSC Adv 4:25736–25746

Michel G, Tonon T, Scornet D, Cock JM, Kloareg B (2010) Central and storage carbon metabolism of the brown alga *Ectocarpus siliculosus*: insights into the origin and evolution of storage carbohydrates in Eucaryotes. New Phytol 188:67–81

Mueller S, Llewellin EW, Mader HM (2009) The rheology of suspensions of solid particles. Proc Roy Soc 466:1201–1228

Ostgaard K, Indergaard M, Markussen S, Knutsen SH, Jensen A (1993) Carbohydrate degradation and methane production during fermentation of *Laminaria saccharina* (Laminariales, Phaeophyceae). J Appl Phycol 5:333–342

Pedersen M, Meyer AS (2009) Influence of substrate particle size and wet oxidation on physical surface structures and enzymatic hydrolysis of wheat straw. Biotech Progr 25:399–408

Percival E, McDowell RH (1967) Chemistry and enzymology of marine algal polysaccharides. Academic Press, London

Rioux L-E, Turgeon SL, Beaulieu M (2010) Structural characterization of laminaran and galactofucan extracted from the brown seaweed *Saccharina longicruris*. Phytochem 71:1586–1595

Roesijadi G, Jones SB, Snowden-Swan LJ, Zhu Y (2010) Macroalgae as a Biomass Feedstock: A Preliminary Analysis. Pacific Northwest National Laboratory. Richland, Washington, p 50

Schiener P, Black K, Stanley M, Green D (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

Schiewer S, Volesky B (2000) Biosorption by Marine Algae. In: Valdes J (ed) Bioremediation. Springer, Dordrecht, pp 139–169

Schultz-Jensen N, Thygesen A, Leipold F, Thomsen ST, Roslander C, Lilholt H, Bjerre AB (2013) Pretreatment of the macroalgae *Chaetomorpha linum* for the production of bioethanol – Comparison of five pretreatment technologies. Bioresour Technol 140:36–42

Siddhanta AK, Prasad K, Meena R, Prasad G, Mehta GK, Chhatbar MU, Oza MD, Kumar S, Sanandiya ND (2009) Profiling of cellulose content in Indian seaweed species. Bioresour Technol 100:6669–6673

Silva GGD, Couturier M, Berrin J-G, Buléon A, Rouau X (2012) Effects of grinding processes on enzymatic degradation of wheat straw. Bioresour Technol 103:192–200

Wargacki AJ, Leonard E, Win MN, Regitsky DD, Santos CNS, Kim PB, Cooper SR, Raisner RM, Herman A, Sivitz AB, Lakshmanaswamy A, Kashiyama Y, Baker D, Yoshikuni Y (2012) An engineered microbial platform for direct biofuel production from brown macroalgae. Science 335:308–313

Wong TY, Preston LA, Schiller NL (2000) Alginate lyase: review of major sources and enzyme characteristics, structure-function analysis, biological roles, and applications. Annu Rev Microbiol 54:289–340

Yanagisawa M, Nakamura K, Ariga O, Nakasaki K (2011) Production of high concentrations of bioethanol from seaweeds that contain easily hydrolyzable polysaccharides. Process Biochem 46:2111–2116

Yeh A-I, Huang Y-C, Chen SH (2010) Effect of particle size on the rate of enzymatic hydrolysis of cellulose. Carbohydr Polym 79:192–199

Table S1: Glucose yields at different time points and reaction rates of the first 90 minutes of enzymatic treatments displayed in Figure 3. Mean and standard deviation of the calculated reaction rates are displayed. Different roman superscript letters indicate significant differences ($\alpha < 0.05$) column-wise per group.

Material	Glucose yield [% of dry material]						Glucose release rate
	0 min	15 min	30 min	60 min	90 min	24 h	[$mg_{glucose} \times g_{dry\ material}^{-1} \times hour^{-1}$]
d = 0.1 mm	5.7^{a} ±0.1	8.0^{a} ±0.7	10.2^{a} ±0.5	15.0^{a} ±<0.1	20.7^{a} ±1.6	45.8^{a} ±2.1	$99.2^{a} \pm 7.1$
d = 0.3 mm	2.7^{b} ±0.3	5.3^{b} ±0.2	7.6^{a} ±0.5	12.9^{b} ±0.2	17.6^{ab} ±0.2	42.7^{a} ±1.0	$99.9^{a} \pm 1.7$
d = 0.6 mm	2.5^{b} ±0.7	4.4^{bc} ±0.3	7.2^{a} ±0.1	12.9^{b} ±<0.1	17.2^{ab} ±0.3	49.1^{a} ±2.1	$101.5^{a} \pm 5.8$
d = 1.0 mm	3.3^{ab} ±0.2	5.4^{b} ±0.5	7.4^{a} ±0.7	12.4^{bc} ±0.2	18.0^{ab} ±0.7	48.6^{a} ±1.5	$98.3^{a} \pm 6.4$
d = 2.0 mm	0.5^{c} ±0.4	2.9^{c} ±0.1	5.9^{a} ±0.2	11.4^{c} ±0.4	16.8^{b} ±0.9	52.4^{a} ±2.0	$109.5^{a} \pm 8.3$
no milling	1.1^{c} ±0.2	5.5^{b} ±1.3	9.9^{a} ±3.3	14.9^{a} ±0.7	20.5^{a} ±1.0	54.9^{a} ±6.5	$119.1^{a} \pm 11.6$

Table S2: ANOVA-analysis through the different glucose yields over concentration of Cellic®CTec2 within the given timepoints of hydrolysis (see also Fig. 4A). Different roman letters indicate significant differences ($\alpha < 0.05$; $\alpha < 0.1$) row-wise per group.

			Cellic®CTec2 (v/w) on dry substrate			
			0.0%	2.5%	5.0%	10.0%
Hydrolysis time	4h	$\alpha < 0.05$	c	b	a	a
		$\alpha < 0.1$	d	c	b	a
	6h	$\alpha < 0.05$	c	b	ab	a
		$\alpha < 0.1$	c	b	b	a
	8h	$\alpha < 0.05$	d	c	b	a
		$\alpha < 0.1$	-	-	-	-

Table S3: ANOVA-analysis through the different glucose yields over hydrolysis time within the given enzyme concentrations of Cellic®CTec2 (see also Fig. 4A). Different roman letters indicate significant differences ($\alpha < 0.05$; $\alpha < 0.1$) row-wise per group.

			Hydrolysis time		
			4h	6h	8h
Cellic®CTec2 (v/w) on dry substrate	0.0%	$\alpha < 0.05$	b	ab	a
		$\alpha < 0.1$	c	b	a
	2.5%	$\alpha < 0.05$	b	a	a
		$\alpha < 0.1$	b	a	a
	5.0%	$\alpha < 0.05$	b	b	a
		$\alpha < 0.1$	c	b	a
	10.0%	$\alpha < 0.05$	b	b	a
		$\alpha < 0.1$	b	b	a

Table S4: ANOVA-analysis through the different glucose yields over concentration of alginate lyase within the given timepoints (see also Fig. 4B). Different roman letters indicate significant differences ($\alpha < 0.05$; $\alpha < 0.1$) row-wise per group.

			Alginate lyase (w/w) on dry substrate				
			0.0%	0.25%	0.50%	1.0%	2.0%
Hydrolysis time	4h	$\alpha < 0.05$	b	ab	ab	a	a
		$\alpha < 0.1$	b	ab	ab	a	a
	6h	$\alpha < 0.05$	c	bc	ab	ab	a
		$\alpha < 0.1$	d	c	cd	bc	a
	8h	$\alpha < 0.05$	c	b	b	a	a
		$\alpha < 0.1$	c	b	b	a	a

Table S5: ANOVA-analysis through the different glucose yields over hydrolysis time within the given enzyme concentrations of alginate lyase (see also Fig. 4B). Different roman letters indicate significant differences ($\alpha < 0.05$; $\alpha < 0.1$) row-wise per group.

			Hydrolysis time		
			4h	6h	8h
Alginate lyase (w/w) on dry substrate	0.0%	$\alpha < 0.05$	b	a	a
		$\alpha < 0.1$	b	a	a
	0.25%	$\alpha < 0.05$	c	b	a
		$\alpha < 0.1$	-	-	-
	0.50%	$\alpha < 0.05$	a	a	a
		$\alpha < 0.1$	a	a	a
	1.0%	$\alpha < 0.05$	a	a	a
		$\alpha < 0.1$	b	ab	a
	2.0%	$\alpha < 0.05$	b	b	a
		$\alpha < 0.1$	-	-	-

PAPER IV

Impact of different microbial alginate lyases on combined cellulase-lyase saccharification of brown seaweed

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Two bacterial polysaccharide lyase (PL) family 7 alginate lyases (EC 4.2.2.-) from *Sphingomonas* sp. (SALy) and *Flavobacterium* sp. (FALy), respectively, were selected for heterologous, monocomponent expression in *Escherichia coli*. The thermal stability, pH and temperature reaction optima and substrate preferences of the enzymes on different alginate polymers were assessed and compared to the properties of a commercially available microbial alginate lyase (SigmALy). The optimal pH range for SALy was pH 5.5-7.0, for FALy and SigmALy it was pH 7.5. The investigated reaction temperatures of 30-50 °C had no influence on the activity of any of the enzymes, but thermal stability was reduced above 50 °C. The FALy preferred poly-mannuronic acid as substrate, but also exhibited activity on poly-guluronic acid, whereas SALy had highest activity on poly-guluronic acid, and the SigmALy was only active on poly-guluronic acid. When applied together with a fungal cellulase preparation Cellic®CTec2 at pH 6 and 40 °C on a glucan rich brown seaweed *Laminaria digitata* viscosity decrease took place in the initial minutes while alginate degradation occurred primarily within the first 1-2 hours of reaction. Whereas FALy and SALy degraded up to one third more alginate in *L. digitata* only the SigmAly enabled release of 90 % of the available glucose within 8 hours of combined enzyme treatment. The level of mannuronic acid moieties released was inversely proportional to the glucose release indicating that the degradation of mannuronic acid blocks inhibited the cellulase catalyzed glucose release from *L. digitata.* Nevertheless, combined alginate lyase and cellulase treatment for 24 hours released all potential glucose regardless of the applied lyase. Moreover, the enzymatic treatment appeared to also induce liberation of proteins from the seaweed solids as well as solubilization of sulfated fucoidan.

1 Introduction

There is a growing interest in using macroalgae, i.e. seaweeds, as a potential new biomass resource for bioenergy and biomass derived chemicals production.[1,2] Brown seaweed *Laminaria digitata* harvested in late summer in the Danish North Sea (off Hanstholm) has been found to have a low ash content and a very high glucan content with glucose moieties constituting 51 % by weight of the dry matter.[3] *L. digitata* is therefore considered a particularly suitable brown seaweed glucose feedstock when harvested at the right time and place.[3]
Brown seaweeds are also rich in alginate, and application of alginate lyases (EC 4.2.2.-) for brown seaweed saccharification[4] and alginate lyase addition on enzymatic glucose release has been evaluated recently.[5,6] When combined with fungal cellulases, alginate lyase addition apparently induce alginate removal from the cell wall matrix and viscosity decrease of brown seaweed to help cellulase catalyzed saccharification of glucan (laminarin and cellulose) to enhance glucose release.[5]
Alginate polysaccharides consist of 1,4-glycosidically linked α-L-guluronic acid (G) and β-D-mannuronic acid (M) in varying proportions forming linear chains with M/G ratio ranges of 1.2 to 3. The linear chains are made up of alternating long blocks of guluronic (GG) and mannuronic (MM) acids with DP 90-100, but less crystalline and much shorter MG/GM blocks may also occur in brown seaweed alginates.[7,8] Alginate lyases catalyze depolymerization of alginate through a β-elimination reaction. Alginate lyases are classified within EC number EC 4.2.2.-. Preference towards G-blocks (poly-guluronate lyase) is classified as EC 4.2.2.11 and specifity towards M-blocks (poly-mannuronate lyase) as EC 4.2.2.3.[4,9] Alginate lyases are mainly divided into two polysaccharide lyase (PL) families, PL 5 and 7 with preference for poly-(M) and poly-(G), respectively, but enzymes categorized in PL family 6, 14, 15, 17, and 18 have also been categorized to have alginate lyase activity (with different substrate specificities including "MG-specific").
Although an alginate lyase is classified as either M or G specific catalytic degradation of alternating blocks and activity towards the other homopolymer may take place. Additionally, alginate lyases with high activity on both homopolymers have been isolated from varies sources.[10] Endolytic alginate lyases were reported to have higher activity than exolytic lyases making them promising catalysts for alginate degradation.[11,12]
The objective of this study was to examine substrate specificity and substrate viscosity impact of microbial alginate lyases to improve the knowledge base for their use in seaweed biorefining to support glucose release.

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2 Experimental

2.1 Alginate lyase cloning, expression and purification

Genes encoding two alginate lyases (EC 4.2.2.3) A1-II' from *Sphingomonas* sp. (SALy) and Alg2A from *Flavobacterium* sp. (FALy) were selected from literature search.[13,14] Protein Data Bank accession number for SALy is 2CWS and GenBank accession number for FALy is JF412659. For both alginate lyases, DNA constructs were engineered to also encode an N-terminal His_6-tag linked via a thrombin recognition site to the ORF. Constructs were codon optimized for expression in *Escherichia coli* (*E. coli*), synthesized and inserted into the vector pET21b (T7 promotor) and pET21a by DNA2.0 (Menlo Park, CA, USA), respectively. *E. coli* BL21 (DE3), *E. coli* C41 and C43 (DE3) and *E. coli* Tuner (DE3) were transformed with the resulting plasmids and selected for ampicillin resistance. Overnight cultures from single colonies were used for IPTG- and autoinduction of alginate lyase expression.

For IPTG induction, lysogenic broth (LB) supplemented with 50 µg/mL ampicillin, inoculated with an overnight culture to a starting OD_{600} of 0.1 was grown at 37 °C. When reaching an OD_{600} of 0.6, the temperature was reduced to 25 °C and expression induced by addition of IPTG to a final concentration of 1 mM. Cells were grown overnight post induction before harvest. Expression with autoinduction was done as follows: an overnight culture was used to inoculate the autoinduction medium (6 g Na_2HPO_4, 3 g KH_2PO_4, 20 g tryptone, 5 g yeast extract, 5 g NaCl, 0.06 % v/v glycerol, 0.05 % w/v glucose and 0.04 % w/v α-lactose, pH7.2) to a starting OD_{600} of 0.1, cells were grown overnight at 25 °C, and harvested. The expression of the lyases with the two expression strategies was evaluated by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE, BioRad, CA, US). The most promising expression strategy was selected for scaled-up expression.

Post scaled-up expression, the cells were centrifuged and the pellets resuspended in Ni^{2+}-column binding buffer (20 mM Na-phosphate buffer, 500 mM NaCl and 20 mM imidazole, pH 7), followed by sonication to open the cells and centrifugation to remove cell debris. The supernatant containing the lyases was passed through a 0.45 µm filter before being loaded on a 1 mL Ni^{2+}-Sepharose HisTrap HP column (GE Healthcare, Uppsala, Sweden), equilibrated with binding buffer, using an ÄKTA purifier (GE Healthcare, Uppsala, Sweden). Unbound material was washed off the column with 5 column volumes (CV) of binding buffer. The alginate lyases were eluted with elution buffer (binding buffer with 500 mM imidazole) in a gradient from 0-100% elution buffer in 15 CV. Protein purity was confirmed by SDS-PAGE and the concentration determined by the lambert beer law using baseline corrected absorption at 280 nm and extinction coefficients of 43890 $M^{-1}\cdot cm^{-1}$ (SALy) and 55350 $M^{-1}\cdot cm^{-1}$ (FALy), respectively.[15]

The benchmark alginate lyase, SigmALy, was purchased from Sigma-Aldrich (Steinheim, Germany) (undisclosed microbial origin, but previously Sigma-Aldrich displayed this enzyme as derived from *Flavobacterium multivorum*).

2.2 Seaweed

Laminaria digitata was harvested from the Danish North Sea coast end of August 2012. Prior to processing the material was washed successively four times with water to remove residual sand and salt. After washing, the biomass was stored at -20 °C until use. The dry matter content was determined after thawing. By weight, the dry biomass consisted of 57.1 % glucose, 8 % mannitol, 17.2 % mannuronic and 5.7 % guluronic acid, and ~4.5 % of other carbohydrate moieties (hydrated monomers).[3] The seaweed was processed through a lab refiner mill as described earlier.[5] In this study the slurry milled at disc distance of 0.3 mm was investigated. Characterization of the milled *Laminaria digitata* revealed glucose and mannitol concentrations of 46.6 and 6.7 % of dry weight. The contents for the other carbohydrates remained constant.

2.3 Alginate lyase characterization

To determine the optimum pH and temperature a statistical design of the Jump® 9 program (SAS) was used. The temperature was varied between 30 and 50 °C and the pH from 4.5 to 8.5. For the thermal stability studies the enzyme solution was incubated at different temperatures (40 to 60 °C) at pH 6 and 7 for 0, 15, 45, 90, 240 and 480 min before the activity was measured. For pH and temperature optima and the thermal stability the activity was measured on sodium alginate (Sigma-Aldrich, Steinheim, Germany).

For the substrate specifity assessment, activity was measured on sodium alginate, poly-mannuronic acid (>5000 kDa and <5000 kDa) and guluronic acid, respectively, at pH 7. Pure substrates were purchased from Carbosynth Ltd., Berkshire, UK. All activity measurements were conducted on substrate concentrations of 0.2 % w/v dissolved at the particular pH in phosphate-citrate buffer. Except for temperature optimization the temperature was set to 40 °C. Activity was determined online over time in an Infinite200 microplate reader (TECAN, Salzburg, Austria) with continuous data collection (Tecan i-control v 1.5.14.0, TECAN, Salzburg, Austria). Activity was quantified as formation of double bonds at absorbance of 235 nm caused by lyase induced β-elimination.

2.4 Enzymatic decomposition of brown seaweed

Enzymatic seaweed saccharification was conducted on 650 mg by dry weight in 13 mL of slurry (5 % substrate concentration). Temperature was 40 °C in a buffer system at pH 6 with 51 mM phosphate 14 mM citrate buffer. Treatment was performed with 1 % E/S (Enzyme/Substrate level in % by weight) of the selected alginate lyase and 10 % E/S (v/w) of the cellulase preparation Cellic® CTec2 (Novozymes A/S, Bagsværd, Denmark) in a horizontal roller mixer at 60 rpm. Samples of 250 µL were taken at 0, 0.5, 1, 2, 4, 6 and 8 hours during the enzymatic liquefaction.

Viscosity assessment during enzymatic treatment was done on 1500 mg dry material (5 % substrate concentration) in a Rapid Visco analyser RVA (Newport, UK) every 8 seconds for a total of 60 minutes at 60 rpm. Subsequently, samples were transferred to the roller mixer and extended to a total of 24 hours. Samples were taken at 2, 4, 8, 14 and 24 hours. Reactions were stopped by addition of 5 M NaOH. After reaction the liquefied fraction was decanted from the insoluble pellet remaining after centrifugation for 30 min. at 14,000×g. Enzymatic treatments on pure laminarin (Sigma-Aldrich) and poly-mannuronic acid (>5000 kDa) were conducted in Eppendorf tubes in a thermomixer at 1400 rpm. For these sequential enzymatic treatments the poly-mannuronic acid was first enzymatically treated with the particular alginate lyase for one hour and the reaction stopped by heat (95 °C for 10 min), then the slurry was mixed with laminarin. Reaction conditions were set as those used for the other enzymatic experiments described above. Ratio of substrate concentrations of 2.4 corresponded to the available glucan (*i.e.* 51 % by weight) to alginic acid (*i.e.* 20.8 % by weight) as present in the fresh seaweed. Laminarin was deconstructed by treatment with Cellic®CTec2 for 30, 60 and 120 minutes and the reaction stopped by heat (95 °C for 10 min).

2.5 Sulfuric acid hydrolysis

After lyophilization a 2-step sulfuric acid hydrolysis was applied on the milled slurry (disc distance 0.3 mm), the post enzymatic treatment insoluble residues and the enzymatically released sugar solutions according to Manns *et al.*[3].

2.6 Carbohydrate analysis

Enzymatic glucose assay. Glucose contents in enzymatic liquefactions were determined with the Megazyme HK/G6P-Dh D-glucose kit using a 96-well microplate reader (TECAN Infinte 200) with automatic data collection by the TECAN *i-control®* software.
Alginate degradation assay. Unsaturated uronic acid residues released were measured at 235 nm in a Tecan microplate reader (TECAN, Salzburg, Austria) with continuous data collection. The amount (weight) of unsaturated uronic acids released was determined via the molar extinction coefficient of 8500 $M^{-1}\cdot cm^{-1}$.[16]
Carbohydrate analysis by HPAEC-PAD. Monomeric sugars, mannitol and uronic acids in the sulfuric acid hydrolysates were separated by high performance anion exchange chromatography with pulsed amperometric detection as described in detail previously.[3]

2.7 Elemental analysis

C, H, N and S contents in the seaweed were measured using a Vario EL cube elemental analyzer (Elementar Hanau, Germany).

2.8 Statistics

One-way analyses of variances (one-way ANOVA): 95% confidence intervals were compared as Tukey–Kramer intervals calculated from pooled standard deviations (Minitab Statistical Software, Addison-Wesley, Reading, MA).

3 Results and Discussion

3.1 Enzyme characterization

Sphingomonas sp. strain A1 encodes three endotype alginate lyases (A1-I, A1-II [family PL-7], and A1-III [family PL-5]) and additionally a transformant of A1-II (A1-II') has been reported.[12] Based on the enzyme's high activity on alginate the application potential of the A1-II' (SALy) has been proposed.[13,17] Recently, another alginate lyase, derived from *Flavobacterium* sp., was discovered and also proposed to have application potential for alginate degradation and biochemicals and biofuels production from brown seaweeds.[14] This *Flavobacterium* derived alginate lyase (FALy) was successfully overexpressed in *E. coli* BL21 with IPTG induction. [14] However, we found this enzyme to express best in *E. coli* C41 with autoinduction (figure A.1 in the appendix). The *Sphingomonas* sp. (SALy) was purified from a cell extract of *E. coli* transformant of BL21 (figure A.1 in the appendix).[12,13,18] In terms of mode of action both SALy and FALy were described previously as being endolytic and belonging to family-7 of the Polysaccharide Lyases (PL).[12-14,17] FALy was previously found to mainly release tri-saccharides, but also di- and tetra-, and penta-and hexa-oligosaccharides in lesser amounts.[14] The depolymerization pattern of alginate after treatment with SALy was reported to result in final products of unsaturated uronic acids of tri- and tetrasaccharides.[18]
In order to assess the possible use of these alginate lyases in brown seaweed saccharification, the pH and temperature activity responses, thermal stabilities, and substrate specificities of SALy and FALy were investigated and compared to those of the commercially purchased alginate lyase (SigmALy).

3.1.1 pH and temperature optimum

When compared as the increase of absorbance at 235 nm after 4 hours of reaction the highest absorbance for FALy and SigmALy was achieved within the range of pH 6.5 to 8, whereas the pH range for the maximal activity of SALy was lower, namely from pH 5.5-7 (active between pH 5-7.5), regardless the temperature 30-50 °C (figure 1). Optima for the initial rates with linear increase over the first 30 min were determined with equal findings (data not shown). Alginate lyases from *Flavobacterium* sp. were of the same pH optimum of about 7.5 but strongly related to temperature when tested in the range 20-35 °C.[16,20] When comparing only the modeled responses for FALy and SigmALy the purchased lyase showed more activity towards lower pH (down to pH 6) and FALy more towards higher pH (figure 1). Originally, optimal pH was 8.5 (optimal temperature: 40-45 °C) with 80 % relative activity at pH 8 or 9.5, respectively.[14] 40 % remained at pH 7.5 (optimum in this study) and only 10 % with pH 6 while figure 1 illustrates about 1/3 of its max activity at this pH. Enzyme loading of FALy was 3/10 of that of SigmALy, but the activity of SigmALy was still of more than half of the maximum activity at pH 6 (figure 2). Other Family 7 lyases have been reported to have pH optimum from pH 7-8.5 and optimal temperature at ~50 °C.[10,19]
Due to its lower pH optimum, SALy could be an appropriate candidate to combine with fungal cellulases to increase glucose release from brown seaweeds. The findings for SALy (figure 1) contrasted those reported by Miyake *et al.*[12] They reported this enzyme to have highest activity at pH 7.5, and moreover found the enzyme to have temperature optimum at 40 °C and a fast decrease in activity above 45 °C.[12] Yoon *et al.*[18] also expressed the alginate lyase SALy (A-II) from *Sphingomonas* sp. (though not the transformant) and found it to be most active at pH 8 and 70 °C. Recently, another alginate lyase derived from *Sphingomonas* sp. was characterized with identical optima for pH and temperature as the present SALy of pH 6.5 and 50°C, respectively.[21]

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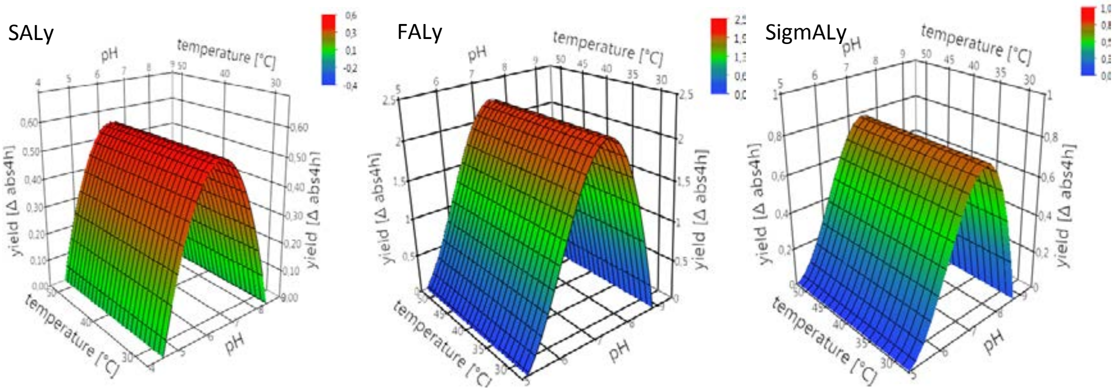

Figure 1: Surface response as a function of temperature and pH on the activity of alginate lyases with borders of pH 4.5-8.5 and T 30-50 °C. $\Delta absorbance_{\lambda=235\,nm}$ after 4h of reaction on sodium-alginate (S/V = 0.2 %), Modeled pH optima left to right: SALy pH 6.45; FALy pH 7.49, purchased lyase SigmALy: pH 7.46. No influence of temperature in the tested range for any of the enzymes.

3.1.2 Thermal stability

The lyase from *Sphingomonas* sp. (SALy) was found to remain stable at temperatures up to 50 °C during more than eight hours of incubation (figure 2a). At 55 °C the activity of SALy was 70 % compared to incubation at 50 °C and 15/45 min. Over the extended period of incubation the remaining activity decreased logarithmically to 5 % compared to the activity at timepoint 0 min. 60 °C let to further decrease in activity (figure 2a). Previously, the SALy was reported to be less thermally stable, and to loose activity already after a few minutes of incubation, with only 50 % activity remaining at 45 °C and with complete inactivation above temperatures of 55 °C.[12,18]

Likewise, in the present work, FALy remained active at elevated temperatures, i.e. retained 30-40 % activity at 55 °C for 4 hours but lost its activity drastically at 60 °C (figure 2b). This enzyme has been described previously to be stable only up to 45 °C.[14,20] The purchased lyase SigmALy was only stable at the lowest incubation temperature of 40 °C. Temperatures of ≥50 °C led into an immediate loss of activity, the higher temperature and the longer the incubation time the lesser the remaining activity (figure 2c). Additionally, the thermal stability experiment was conducted at pH 7, and enzyme activity readings revealed equal thermal stabilities as described for pH 6 (data not shown). SALy was generally more active at pH 6 while it was the other way around for FALy and SigmALy. The data are in accordance with the determined optima for pH and temperature (see section 3.1.1). Principally, stability depends not only on chosen pH and temperature but also on the choice of buffer system.[22]

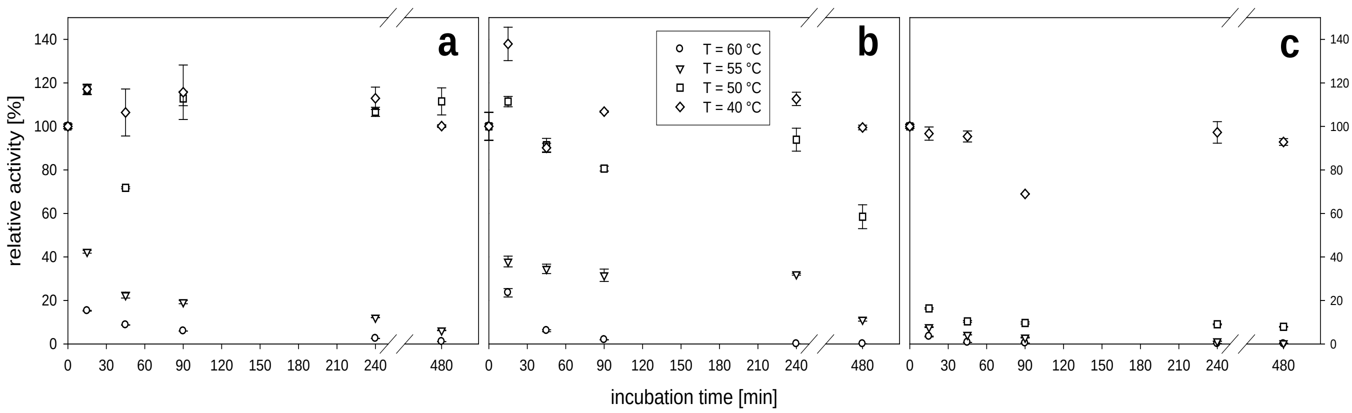

Figure 2: Remaining relative activity of alginate lyases SALy (a), FALy (b) and SigmALy (c) over time of thermal incubation. Incubation at pH 6 and temperatures (T) of 40, 50, 55 and 60 °C for 0, 15, 45, 90 min and 4 and 8 h. Activity was taken as the initial rate recorded within the first 30 min of reaction on sodium alginate at S/V 0.2 %, pH 6 and 40 °C after incubation. Activity at timepoint 0 min was set to 100 % activity.

3.1.3 Substrate specificity

Huang *et al.*[14] reported a preference of the enzyme FALy on poly-(G) as a substrate over poly-(M). In contrast, in this study (figure 3) FAly preferably degraded poly-(M), in accord with data reported for another *Flavobacterium* sp. derived lyase.[20] Moreover, the initial rates were almost double as high on poly-(M) than on poly-(G) (table 1). In contrast, SALy performed better on guluronic than on mannuronic acid (figure 3). The preference of SALy on poly-(G) rather than on poly-(M) and its activity on its heteropolymer was in agreement with data reported earlier.[13,17,18] However, the activity for SALy was with 63 % towards poly-(M) vs. on poly-(G) (table 1) higher than the relative difference of 20 % reported earlier.[13]

Table 1: Initial rates of alginate lyases on mannuronic acid (poly-M, purity ≥85 %) <5000 kDa, >5000 kDa, guluronic acid (poly-G, purity ≥85 %) and sodium alginate. Rates calculated from figure 3 for the linear increase of substrate recorded within the first 90 min. Brackets indicate activity on residual impurities of poly-G.

enzyme	Initial rate [% of total substrate/min]			
	poly-M <5000kDa	poly-M >5000kDa	poly-G	Na-alginate
SALy	0.013	0.014	0.022	0.036
FALy	0.111	0.102	0.058	0.093
SigmALy	(0.034)	(0.046)	0.122	0.063

Differences in the specific activity on the different substrate types agree with another investigation where it was found that SALy was similarly active towards mannuronic and guluronic acid.[12] Presumably, different assay conditions for alginate lyase activity affect the substrate preferences; an interesting detail that should be further investigated.[20]

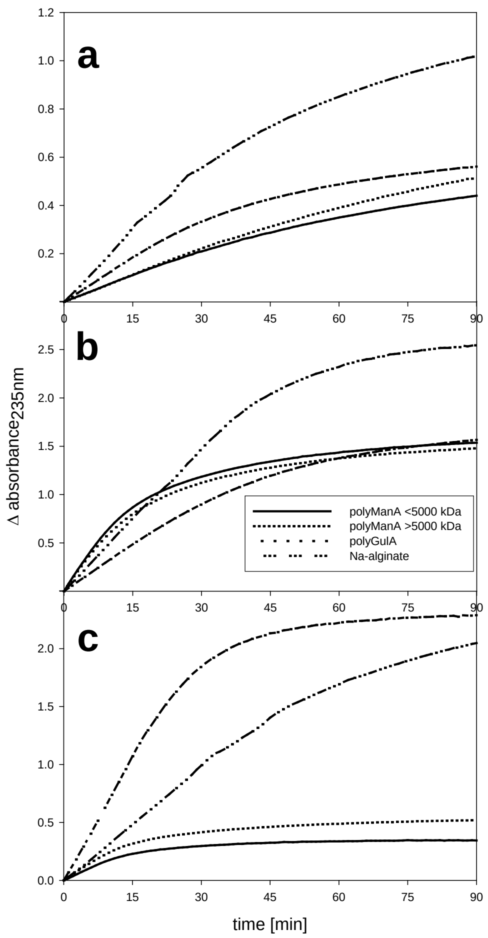

Figure 3: Substrate specify of alginate lyases SALy (a), FALy (b) and the purchased lyase (SigmALy; c) on poly-mannuronic acid < 5000 kDa, poly-mannuronic acid > 5000 kDa, poly-guluronic acid and sodium alginate at S/V 0.2 %, pH 7 and 40 °C recorded as Δ absorbance$_{\lambda=235\ nm}$ over the first 90 min of reaction. Enzyme dosages E/S were 0.1 % for SALy and SigmALy, respectively 0.03 % for FALy.

After an initial active cleavage period of about 5 min of poly-(M) the reaction of the purchased lyase SigmALy almost ceased (figure 3). It is most likely that the initial cleavage was due to impurity of the substrate with guluronic acid residues (approx. 15 % of dry weight determined by HPAEC-PAD post sulfuric acid treatment; data not shown). Presumably, the SigmALy was substrate specific towards guluronic acid as described elsewhere.[14] This distinct substrate selectivity is rare as hitherto reported alginate lyases usually display at least a moderate processivity for the other heteropolymer.[9]

Overall, FALy had the highest decomposition ability taking into account the lowest enzyme loading of 0.03 % enzyme per substrate (E/S) (figure 3). As E/S for SALy and SigmALy were set to 0.1 % SALy performed with the lowest activity. Furthermore, after 30 min of reaction, *i.e.* the initial reaction, SALy created per release of unsaturated M-unit (>5000 kDa) 1.5 unsaturated G-units while FALy 0.8 units of unsaturated G-blocks (figure 3).

3.2 Application on brown seaweed

Combined application of alginate lyase and cellulase was reported to be superior over cellulase application alone on the release of glucose from *Laminaria digitata*.[5,6] The symbiotic benefit of the two enzymes can be explained when considering that the matrix polysaccharides (fucose-containing sulfated polysaccharides, alginates and cellulose) are presumed to be tightly associated in the brown seaweed cell walls as recently proposed by Deniaud-Bouët *et al.*[23]

3.2.1 Enzymatic degradation

Brown seaweed *L. digitata* was subjected to refiner milling (disc distance 0.3 mm) and the milled seaweed slurry consisted of 46.6 % dry weight hydrated glucose. Subsequently, the seaweed was enzymatically treated with the cellulase preparation Cellic® CTec2 together with one of each characterized alginate lyase (figure 4). The data obtained (Figure 4a) confirmed our earlier findings with application of the cellulase preparation Cellic® CTec2 and the alginate lyase from Sigma-Aldrich, although at a lower rection pH of pH 5.[5] A glucose yield of 40.8 % of dry weight milled seaweed corresponds to 87.6 % of the potential available glucose after 8 hours of treatment (figure 4a). Application together with the lyases FALy and SALy was assumed to perform better. As expected, the lyases showed activity towards both homopolymers, poly-(M) and poly-(G), and the pH optimum for SALy suited better with application of pH 6 (section 3.1). Surprisingly, the release of glucose was significantly lower then expected. After 2 hours FALy released 14.9 % and SALy 18.4 % of glucose from total seaweed by dry weight. This corresponded to 58 %, respectively 71 % compared to what has been released by the cellulase preparation applied together with SigmALy (figure 4a). The glucose yield for treatment with SigmALy started to bend off after 2 hours. Hence, the comparative relative yields of FALy and SALy rose to 65 % (26.7 % w/w biomass), respectively 80 % (32.7 % w/w biomass) after 8 hours of treatment.

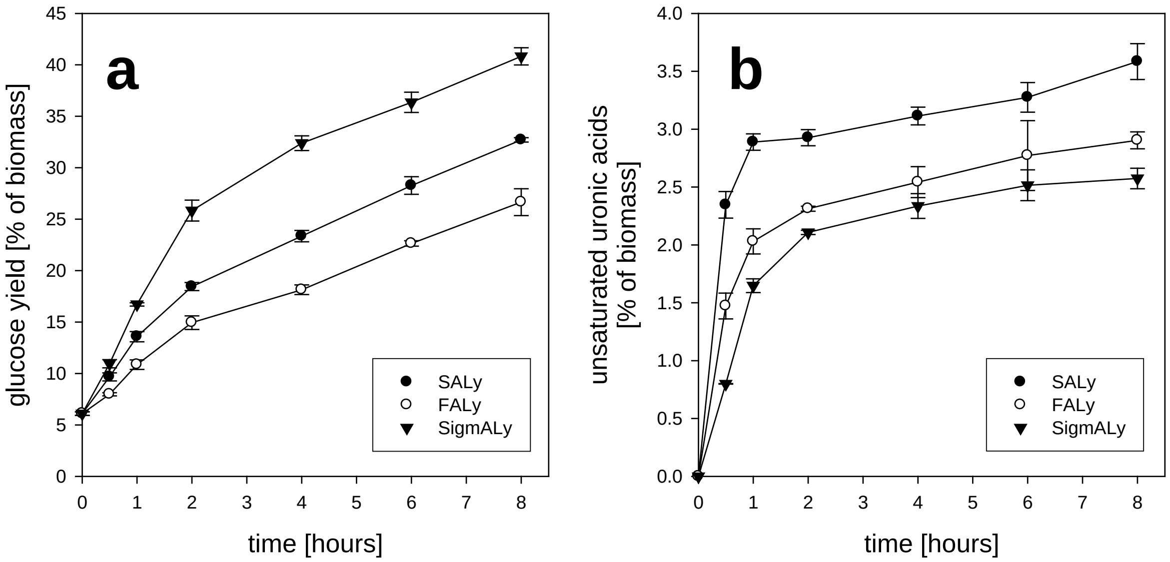

Figure 4: Yields over time of enzymatic treatment of glucose in % of dry biomass (a) and alginate degradation products of unsaturated uronic acid residues due to β-elimination in % of dry biomass (b) of dry material of refiner milled wet *Laminaria digitata* with disc distance at 0.3 mm. Enzymatic saccharification with Cellic® CTec2 concentration of 10 % v/w and alginate lyase (SALy, FALy and purchased lyase SigmALy) of 1 % w/w over time with measurements at timepoints 0, 0.5, 1, 2, 4, 6 and 8 hours. Each data point represents the average value of independent duplicates; vertical bars indicate the standard deviation.

Lower lyase dosis (of the purchased lyase from Sigma-Aldrich) but also lower substrate loading with maximum glucose recovery of 80 % (~25 % after 8 hours) was achieved previously on dried and milled material after 24 hours of enzymatic treatment.[6] Equally overall sugar recovery (glucose and mannitol) of over 90 % was reached with a substrate concentration of 15 % w/v but was 78 % with increased solid loading (25 %) after 29 hours with no change over treatment extension until 48 hours.[24] Both investigations were conducted on dried material using the cellulase preparation Celluclast 1.5L and Cellobiase 188 (Novozymes) at about pH 5. The preparation Celluclast 1.5L released less reducing sugars than another commercially available cellulase from *L. digitata*.[25] Furthermore, drying was shown to hinder glucose release, although from lignocellulosic material.[26,27] In regard to the pH, Celluclast remained still 80 % of the activity at pH 6 (optimum was pH 5.2) when applied on brown seaweed *Macrocystis pyrifera*. In contrast, the activity for alginate lyases, including the endo-type lyase from Sigma-Aldrich, the activity was of <10 % at pH 6 and about one third at pH 7 compared to pH 7.5.[28] With respect to temperature, glucose release could be enhanced by raising the temperature as yields were doubled by a temperature increase from 37 °C to 50 °C.[28] However, to allow suitable conditions for all alginate lyases temperatures in the experiment of figure 5 were set to 40 °C. The purchased lyase from Sigma-Aldrich (SigmALy) was tested to have significant activity losses for temperatures ≥50 °C (section 3.1.2).

For saccharifying the alginate within the seaweed only the first two hours of reaction were crucial. There were unsaturated uronic acid residues (UA) of 2-3 % by dry biomass and only slightly more (2.6 to 3.6 % UA) at the end of reaction (figure 4b). This reaction pattern (leveling off) on alginate degradation with lyase concentration of ≥1 % per substrate has been seen before.[29] Moreover, figure 4b showed that the initial fast degradation of the alginate was achievable already within one hour with the application of SALy (2.9 % unsaturated uronic acid residues per dry biomass). Potentially, this was a result of higher enzyme activity due to the more suitable pH conditions (pH optimum for SALy was 6.5; section 3.2.1). Likewise, Thomas *et al.*[16] reported an intermediate initial degradation to larger oligosaccharides (DP 4 to 20) following by a further slower chopping of the alginate into a DP of 2 over several hours.

Whereas nearly complete glucose release was achieved from just milled seaweed (figure 5a) a harsher pretreatment for decomposition of alginate from brown seaweed was proposed elsewhere.[28] A 5-fold increase of uronic acids after 2 hour treatment with exo- and endo-alginate-lyases was reported post a sulfuric acid pretreatment compared to none.[28] However, the seaweed *Macrocystis pyrifera* was dried and cut prior to use.[28]

In general, yields of unsaturated uronic acids were lower with FALy and SigmALy than compared to SALy (figure 4b). First, pH 6 was closer to the optimum of SALy. However, SigmALy was still efficient when pH 5 applied.[5] Second, SigmALy was not active on to poly-mannuronic acid unlike the other lyases.

Unsaturated monosaccharides, such as products from exolytic alginate lyases, convert non-enzymatically to the stable 5-keto structure and are not UV-visible. Hence, after an increase of A_{235} a decrease followed shortly after.[16] There was no decrease observed (figure 4b). Hence, the applied lyases act endolytically like all PL 7 family enzymes which appeared sufficient for the release of glucose (figure 5a). Furthermore, as the lyase activity was only important within the initial phase reaction conditions should be further investigated for best cellulase activity still ensuring sufficient alginate catalysis. Nonetheless, addition of exolytic oligoalginate lyase to produce monosaccharide units of alginate was recently demonstrated.[21,29] Further, these monosaccharides were shown to be available for ethanol production by a newly discovered organism.[30]

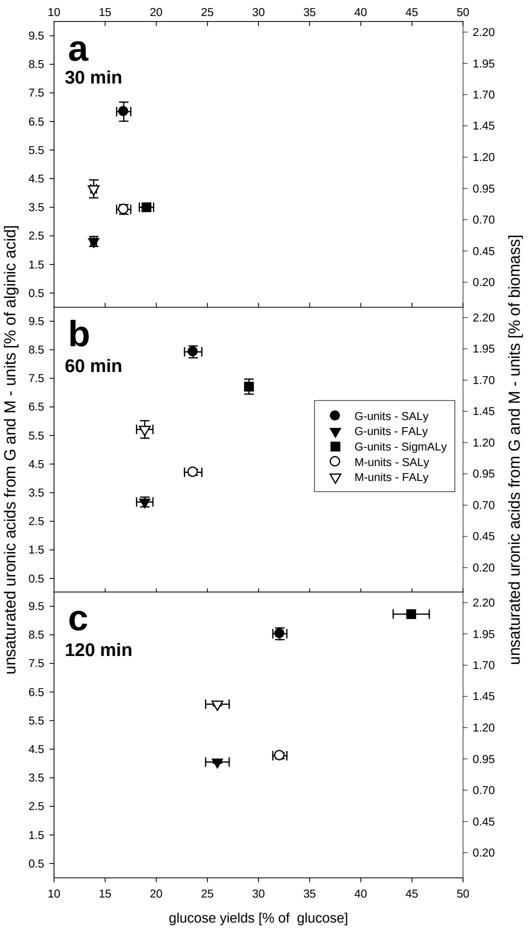

Figure 5: Yields of unsaturated uronic acids deriving from poly-guluronic acids (G-units, left y-axis) and poly-mannuronic acids (M-units, right y-axis) as % of potential total alginic acid of *Laminaria* over glucose yields (% of potential glucose); after 30 (**a**), 60 (**b**) and 120 min (**c**) of enzymatic saccharification with Cellic® CTec2 and alginate lyase (SALy, FALy and purchased lyase SigmALy). Potential total content of glucose and alginic acid monomers determined by HPAEC post sulfuric acid hydrolysis.[3] Each data point represents the average value of independent duplicates, bi-dimensional bars indicate the standard deviation.

Regardless of the mannuronic acid content an efficient disruption of alginate requires a lyase with high activity on G-G linkages.[31] Based on the initial rates derived from the lyase activity on pure substrates (table 1) ratios of G-cleavages to M-cleavages (G:M cleavage ratio) were calculated. G:M cleavage for SALy was 1.5:1 and for FALy 0.6:1. SigmALy was not active on mannuronic acid. Subsequently, these ratios were transferred to calculate if the amount of unsaturated uronic acids derived from seaweed alginate saccharification (figure 4b) whether could be attributed to the cleavage of G-units or M-units (figure 5). The unsaturated M- and G-units were plotted over the glucose yields for the crucial first 2 hours (30, 60 and 120 min) of alginate degradation. Hence, the more unsaturated M-units were released the lower the glucose yields were obtained (figure 5).

The M/G ratio in the present *L. digitata* was 3:1 with a total amount of guluronic acid of 5.7 % (w/w).[3] Taking into account the fact that the purchased lyase was almost only active on poly-(G) approx. 37 % of all present guluronic acid did undergo a ß-elimination leading to unsaturated uronic acid at the reducing end with an average DP of 2-3 (figure 5c). The mode of action of the purchased lyase from Sigma-Aldrich (SigmALy) was described as endolytic, releasing mainly trimers.[14] In the same study FALy was found to release oligomers of DP 5-7 within the first 20 h of reaction. Hence, the presence of longer oligomers could describe the lower yields of unsaturated uronic acids deriving from G-units of FALy compared to the other two lyases (figure 5). For FALy the yield stabilized at 18 % after 2 hours of reaction and would have produced oligomers of average DP 5-6 (figure 5c). SALy was described to release tri- and tetrasaccharides.[14,18] This corresponded with the release of unsaturated (G)-units from seaweed using SALy of 8.6 % of the total content of alginate (equal to 34 % of guluronic acid). Hence, G-unit trimers were achieved already after 60 min of reaction (figure 5b). Further, a significant consumption from of guluronic acid after 120 min could not be observed (figure 5c). However, also for (M)-units no significant further increase over reaction time over 2 hours was achieved, both poly-(M) active lyases (SALy and FALy) similarly only released unsaturated M-units of 4-6 % of the total mannuronic acid (figure 5c).

Potentially, poly-(M) and poly-(G) interacted competitively with the lyases active on both substrates exhibiting increased binding affinity towards poly-(G). Iwamato *et al.*[22] indicated a strong reduced production of unsaturated mannuronates from poly-(M) by the presence of poly-(G), the higher the concentration of (M) the higher the reduction.

At a concentration of 0.1 % for both of poly-(M) and poly-(G) the production of unsaturated UA was halved compared to (M) as the only product and only one third at increased concentration of poly-(M) to 0.2 % S/V.[22] Conclusively, as the total (M) in the present reaction volume of *L. digitata* was about 0.9 % of (M) the presence of approx. 0.3 % of poly-(G) might inhibited any further activity of the enzyme on the mannuronic acid blocks in the brown seaweed. Furthermore, a product inhibition was emphasized on the degradation of alginate by *Sphingomonas* sp. deriving lyases, also when exo- and endolytic alginate lyases were acting together.[29] This could also indicate that by addition of exolytic lyase a faster release of glucose is most unlikely as a further increase in enzyme loading did not enhance the decomposition of brown seaweed.[5]

Nonetheless, degradation of poly-mannuronic acid led to an inhibition of glucose release from seaweed. The more activity towards M-blocks the more the glucose yields decreased (figure 5). Analogously, release of glucose from isolated commercially available laminarin mixed with pretreated poly-(M) decreased with respect to activity on poly-(M) (figure 6). Pretreatment with the lyases SALy and FALy inhibited the glucose release significantly after 2 hours (figure 6b). For FALy the inhibition was significant already after 1 hour of reaction (figure 6b). Treatment with the purchased lyase (SigmALy) did yield in similar amounts as the control containing no lyase (figure 6b). Hence, non-activity towards poly-(M) apparently protected the cellulase catalyzed glucan degradation.

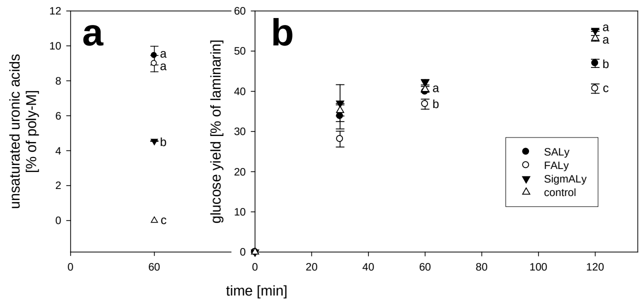

Figure 6: (**a**) Poly-mannuronic acid (poly-M) degradation products of unsaturated uronic acid residues due to β-elimination in % of poly-M with alginate lyase (SALy, FALy, SigmALy) and a control without lyase after 1 hour of reaction at 40 °C pH 6. (**b**) Glucose yield from an artificial mix of laminarin and the degraded poly-M) over 2 hours of enzymatic treatment with Cellic®CTec2 at 40 °C pH 6. Each data point represents the average value of independent triplicates, bars indicate the standard deviation. Roman letters indicate significant differences (α < 0.05). Poly-M consisted approx. 15 % of guluronic acid impurities.

3.2.2 Viscosity decrease and post enzymatic treatment insoluble residues

With application of alginate lyases the viscosity dropped rapidly indicating the endo-type action of the ALys. The endo-action is in agreement with previous data achieved on alginate lyases.[20,22] The addition of the SALy to the cellulase preparation acted the fastest, and the SigmALy the slowest when added at equal protein level of 1 % enzyme concentration per dry seaweed biomass (figure 7). Even though the specific reaction viscosity decreased quickly in the early phase of reaction the formation of unsaturated UA still increased as the reaction proceeded (Fig. 4b). Regarding the enzyme catalyzed release of glucose, the data did not unequivocally reveal whether the initial viscosity decrease affected the initial glucose release rate.

A 24 hours enzymatic treatment with the FALy or SALy supplemented to the cellulase preparation Cellic®CTec2 left behind an insoluble residue constituting about 20 % by weight of the original (insoluble) seaweed substrate dry matter (table 2). This residue could be separated from the liquid by centrifugation. Interestingly, treatment with the SimALy, which appeared to have high affinity for poly-guluronic acid (figure 3c) catalyzed the liquefaction of more of the seaweed material and left behind 12.4 % by weight only (table 2). The achieved degrees of enzymatic saccharification were significantly higher than those achieved by 48 h-saccharification at pH 5 at 50 °C of 26 % of the original *L. digitata* biomass.[6]

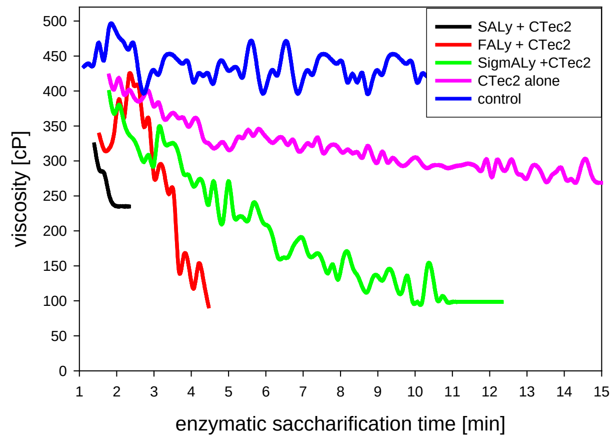

Figure 7: Evolution of viscosities at shear rate of 60 rpm over 15 min of enzymatic treatment with Cellic®CTec2 and alginate lyase (SALy, FALy, SigmALy); Cellic®CTec2; and the control without any enzyme addition.

Compared to the raw material 70-80 % by weight of nitrogen was recovered in the solid residue indicating that the majority of the seaweed protein was left in this fraction (table 2).

Table 2: Yields of insoluble residues, including the nitrogen recovery and carbohydrate monomers of the liquefied fraction after enzymatic treatment of refiner milled wet *Laminaria digitata* for 24 hours with Cellic®CTec2 and alginate lyase (SALy, FALy and purchased lyase SigmALy), as well as treatments with alginate lyase (SigmALy), respectively CTec2 alone. Separation by centrifugation at 14,000×g for 30 min.

	residue		liquefaction	
treatment	amount	N-recovery[1]	mannitol[2]	glucose[3]
	[% of original biomass]		[% of original biomass]	
SALy + CTec2	19.3	80.1	3.4	51.8
FALy + CTec2	20.3	83.5	5.9	48.2
SigmALy + CTec2	12.4	71.4	5.0	52.7
CTec2 alone	51.8	68.4	3.9	46.0
SigmALy alone	28.9	78.5	2.3	17.6

[1]after elemental analysis; raw material N=0.73 %[3]

[2]hydrated monomers after HPAEC analysis

[3]hydrated monomers after determination with enzyme assay

The data (table 2) are in accord with the recently published findings on the same seaweed material that a protein enriched residue having similar amino acid profile as the raw material remained insoluble after extensive saccharification.[6]

In addition to nitrogen, the residue also contained a mixture of carbohydrates at a level equivalent to approx. 4 % of the total seaweed carbohydrates by dry weight (data not shown). The separated liquefied seaweed fraction contained glucose levels of 51.8, 48.2 and 52.7 % by weight of the dry matter (table 2), corroborating that nearly all the potential glucose was released.

Carbohydrate analysis by high performance anion exchange chromatography (HPAEC-PAD) showed that the liquefied fraction also contained mannitol besides glucose. The mannitol levels measured (3.4 to 5.9 % of the original milled seaweed biomass; table 2) only made up about 80 % of the original content of mannitol in the biomass. Sulfuric acid treatment of the liquefied fraction released fucose along with minor monosaccharides (data not shown). The fucose indicated that the fucoidan was dissolved but not hydrolyzed during the enzymatic treatment. Fucoidan were described as water soluble sulfated polysaccharide.[32]

In conclusion, the application of alginate lyase and cellulase preparation Cellic®CTec2 for 24 hours enabled almost complete release of the fermentable sugar monomers glucose and mannitol harbored in the brown seaweed *L. digitata*. New yeast strains demonstrated the ability to convert mannitol into ethanol.[33] 8 h treatment was enough to release 90 % of the glucose if the guluronic acid specific SigmALy along with the cellulase preparation was applied and 14 h were sufficient with the use of SALy whereas 24 hours with FALy and cellulase were required for complete glucose release (data not shown).

4 Conclusions

Expression of and endolytic bacterial alginate lyase from *Sphingomonas* sp. (SALy) was feasible with high yields of 12.8 g/L cell extract. Furthermore, the characterization of activity with pH range from 5.5 to 7 and thermal stability up to 50 °C made it a promising candidate to support glucose release of brown seaweed catalyzed by the commercial, fungally derived cellulase preparation Cellic®CTec2. Like the endolytic lyase from *Flavobacterium* sp (FALy, optima pH 7.5), the SALy was active on both alginate epimers poly-mannuronic acid and poly-guluronic acid. In contrast, a purchased lyase (SigmALy) was only endolytically active towards poly-(G). M/G ratio of the investigated brown seaweed *Laminaria digitata* was 3:1. Consistently, SALy and FALy showed higher activity on the alginate of the seaweed. Precisely, the guluronic acid was assumingly degraded to smaller oligomers of DP 3 for SALy, DP 5-6 for FALy and DP 2-3 for SigmALy. However, only 4-6 % of the mannuronic acid present in the seaweed was epimerized to unsaturated uronic acids. Moreover, a degradation of poly-(M) led into a decreased release rate of glucose from *L. digitata* by the cellulase preparation. In conclusion, not only the binding activity of the lyase towards poly-(G) was higher, the decrease of poly-(M) was directly linked to an inhibition of the glucose release.

Nevertheless, enzymatic treatment for 24 hours was sufficient to release all potential glucose from the glucan rich *L. digitata* (51 % moieties) regardless the applied lyase. Viscosity deduction occurred primarily in the first minutes of the reaction. This emphasized that the alginate lyases were rather required to decompose the cell wall in order to guarantee access for the cellulase to the glucan. Furthermore, in the solid pellet after enzymatic treatment 70-80 % of the nitrogen was recoverable and molecules containing fucose were present in the liquefied fraction. Assumingly, after treatment with cellulase and alginate lyase the highly valuable by-products of sulfated polysaccharides (fucoidan) and proteins can be extracted from the soup of mannitol and glucose monomers.

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References

1 A. Brown and J. Tustin, *Algae – The Future for Bioenergy? Summary and conclusions from the IEA Bioenergy ExCo64 Workshop. IEA Bioenergy: ExCo: 2010:02*, 2010.
2 A. Dave, Y. Huang, S. Rezvani, D. McIlveen-Wright, M. Novaes and N. Hewitt, *Bioresource Technology*, 2013, **135**, 120-127.
3 D. Manns, A. L. Deutschle, B. Saake and A. S. Meyer, *RSC Advances*, 2014, **4**, 25736-25746.
4 H. Kim, C.-G. Lee and E. Lee, *Biotechnology and Bioprocess Engineering*, 2011, **16**, 843-851.
5 D. Manns, S. Andersen, B. Saake and A. Meyer, *Journal of Applied Phycology*, 2016, available on-line: DOI 10.1007/s10811-015-0663-9.
6 X. Hou, J. H. Hansen and A.-B. Bjerre, *Bioresource technology*, 2015, **197**, 310-317.
7 E. Percival and R. H. McDowell, *Chemistry and enzymology of marine algal polysaccharides*, Academic Press Inc. (London) Ltd., 1967.
8 O. A. Aarstad, A. Tøndervik, H. Sletta and G. Skjåk-Bræk, *Biomacromolecules*, 2011, **13**, 106-116.
9 B. Zhu and H. Yin, *Bioengineered*, 2015, **6**, 125-131.
10 T. Y. Wong, L. A. Preston and N. L. Schiller, *Annual review of microbiology*, 2000, **54**, 289-340.
11 O. Miyake, W. Hashimoto and K. Murata, *Protein Expression and Purification*, 2003, **29**, 33-41.
12 O. Miyake, A. Ochiai, W. Hashimoto and K. Murata, *Journal of Bacteriology*, 2004, **186**, 2891-2896.
13 M. Yamasaki, K. Ogura, W. Hashimoto, B. Mikami and K. Murata, *Journal of Molecular Biology*, 2005, **352**, 11-21.
14 L. Huang, J. Zhou, X. Li, Q. Peng, H. Lu and Y. Du, *Journal Industrial Microbiology Biotechnology*, 2013, **40**, 113-122.
15 M. R. Wilkins, E. Gasteiger, A. Bairoch, J. C. Sanchez, K. L. Williams, R. D. Appel, D. F. Hochstrasser, *Methods Molecular Biology*, 1999, **112**, 531-52.
16 F. Thomas, L. C. E. Lundqvist, M. Jam, A. Jeudy, T. Barbeyron, C. Sandström, G. Michel and M. Czjzek, *Journal of Biological Chemistry*, 2013, **288**, 23021-23037.
17 K. Ogura, M. Yamasaki, B. Mikami, W. Hashimoto and K. Murata, *Journal of Molecular Biology*, 2008, **380**, 373-385.

18 H.-J. Yoon, W. Hashimoto, Y. Katsuya, Y. Mezaki, K. Murata and B. Mikami, *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, 2000, **1476**, 382-385.
19 H. Kim, H.-J. Ko, N. Kim, D. Kim, D. Lee, I.-G. Choi, H. Woo, M. Kim and K. Kim, *Biotechnology Letters*, 2012, **34**, 1087-1092.
20 A. Inoue, K. Takadono, R. Nishiyama, K. Tajima, T. Kobayashi and T. Ojima, *Marine Drugs*, 2014, **12**, 4693.
21 H. Park, N. Kam, E. Lee and H. Kim, *Marine Biotechnology*, 2012, **14**, 189-202.
22 Y. Iwamoto, R. Araki, K.-i. Iriyama, T. Oda, H. Fukuda, S. Hayashida and T. Muramatsu, *Bioscience, Biotechnology, and Biochemistry*, 2001, **65**, 133-142.
23 E. Deniaud-Bouët, N. Kervarec, G. Michel, T. Tonon, B. Kloareg and C. Hervé, *Annals of Botany*, 2014, **114**, 1203-1216.
24 M. Alvarado-Morales, I. B. Gunnarsson, I. A. Fotidis, E. Vasilakou, G. Lyberatos and I. Angelidaki, *Algal Research*, 2015, **9**, 126-132.
25 C. H. Vanegas, A. Hernon and J. Bartlett, *International Journal of Ambient Energy*, 2015, **36**, 2-7.
26 X. Luo and J. Y. Zhu, *Enzyme and Microbial Technology*, 2011, **48**: 92-99.
27 X. L. Luo, J. Y. Zhu, R. Gleisner and H. Y. Zhan, *Cellulose*, 2011, **18**, 1055-1062.
28 M. C. Ravanal, R. Pezoa-Conte, S. von Schoultz, J. Hemming, O. Salazar, I. Anugwom, O. Jogunola, P. Mäki-Arvela, S. Willför, J.-P. Mikkola and M. E. Lienqueo, *Algal Research*, 2016, **13**, 141-147.
29 M. Ryu and E. Y. Lee, *Journal of Industrial and Engineering Chemistry*, 2011, **17**, 853-858.
30 A. J. Wargacki, E. Leonard, M. N. Win, D. D. Regitsky, C. N. S. Santos, P. B. Kim, S. R. Cooper, R. M. Raisner, A. Herman, A. B. Sivitz, A. Lakshmanaswamy, Y. Kashiyama, D. Baker and Y. Yoshikuni, *Science*, 2012, **335**, 308-313.
31 K. Formo, O. A. Aarstad, G. Skjåk-Bræk and B. L. Strand, *Carbohydrate Polymers*, 2014, **110**, 100-106.
32 L. Pereira, S. F. Gheda and P. J. A. Ribeiro-Claro, *International Journal of Carbohydrate Chemistry*, 2013, **2013**, 7.
33 A. Ota, S. Kawai, H. Oda, K. Iohara and K. Murata, *Journal of Bioscience and Bioengineering*, 2013, **116**, 327-332.

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Supplementary Material

Appendix

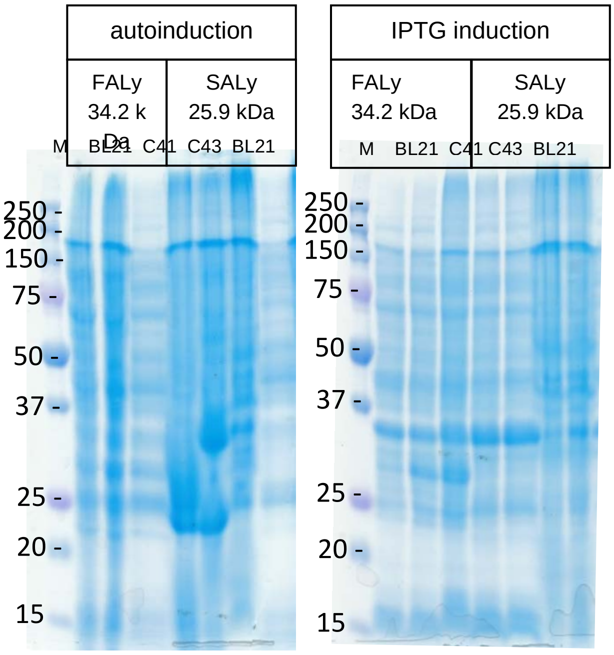

Figure S.1: SDS-Page of alginate lyase clones deriving from *Flavobacterium* sp. (FALy) and *Sphingomonas* sp. (SALy) expressed in the different *E. coli* strains BL21, C41, C43 and Tuner via autoinduction and IPTG induction. Protein markers (M [kDa]; lane 1).