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Enzymatic saccharification of the polysaccharides in *Saccharina latissima* (L.)

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Master's degree in Chemistry

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Abstract

In a time of great dependence on fossil fuels and rising need for sustainable, renewable resources, seaweeds can be a valuable asset to the new and emerging bioeconomy. Norway is in a unique position because its particularly long coastline can provide an alternative to the use of terrestrial biomass, as brown seaweeds are more environmentally friendly to exploit. The polysaccharides in brown seaweed can be processed enzymatically to release fermentable sugars, which can be used further as a carbon source in biorefineries. Biorefineries process biomass in order to produce fuel, energy, chemicals and value-added products. To utilize as much of the carbohydrates in brown seaweed as possible, successful enzymatic degradation of these components is a key step in the process. In this study, the aim was to combine an enzyme cocktail for the degradation of alginate, laminarin and cellulose in *Saccharina latissima* (L.), a brown seaweed harvested from the Trondheim fjord. Endolytic and exolytic alginate lyases AMOR_PL7A and AMOR_PL17A, laminarinases (or β-1,3-glucanases) GH16 and GH5, and cellulases GH6.4 and therm3 were combined on ground *S. latissima* for the release of sugars, and compared to the effect of commercial cellulase cocktail Cellic® CTec2. The abovementioned enzymes were added to 15% DM (w/v) ground seaweed, and degradation yield measured both as reducing sugars with the 3,5-Dinitrosalicylic acid (DNS) assay and as glucose with high-performance liquid chromatography (HPLC). Reaction products were measured after 48 hours incubation in a Thermomixer at 50°C, and the enzyme cocktail successfully released oligosaccharides and monomeric sugars from the main carbohydrates in the seaweed. Reaction product analysis from degradation of sodium alginate with AMOR_PL7A and AMOR_PL17A was conducted with MALDI-TOF mass spectrometry and ^{1}H NMR, which showed that these enzymes combined releases monomeric DEHU from alginate. The results from this thesis indicate that an enzyme cocktail can be applied to *S. latissima* for saccharification of its polysaccharides, which is a very essential step towards more efficient processing of macroalgae in a biorefinery in the future.

Samandrag

I ei tid der avhengnaden av fossile ressursar er stor og høvet for berekraftige, fornybare ressursar veks, kan tare bli verdifulle i den nye veksande bioøkonomien. Norge er i ein unik posisjon grunna den store kystlinja som omgjev landet, fordi utnytting av tare kan gi eit meir miljøvennleg alternativ til bruken av landbasert biomasse. Polysakkarida i bruntare kan bli prosessert med enzym for å sleppe ut fermenterbare sukker, som kan brukas vidare som karbonkjelde i bioraffineri. Bioraffineri prosesserer biomass for produksjon av brensel, energi, kjemikaliar og høgverdi-produktar. For å nytta så stor del som mogleg av karbohydrata i bruntare er det naudsynt med suksessfull enzymatisk nedbryting av desse. I denne studien vart målet å kombinere ein enzymblanding for nedbryting av alginat, laminarin og cellulose i *Saccharina latissima* (L.), ein bruntare hausta frå Trondheimsfjorden. Dei endolytiske og eksolytiske alginat lyasane AMOR_PL7A og AMOR_PL17A, laminarinasane (eller β-1,3-glucanasar) GH16 og GH5, og cellulasane GH6.4 og therm3 vart kominert på malt *S. latissima* for å sjå på sukkerutslepp, og samanlikna med den kommersielle cellulaseblandinga Cellic® CTec2. Dei nemnde enzyma blei tilført til 15% tørrstoff (i vekt/volum) malt tare, og nedbrytingsutbyttet målt både som reduserande sukker med 3,5-Dinitrosalicylsyre (DNS) analyse og som glukose med HPLC. Reaksjonsprodukta vart målt etter 48 timars inkubering i ein Thermomixer på 50°C, og enzymblandinga resulterte i utsleppet av oligosakkarid og monomerisk sukker frå dei største karbohydratkomponenta i taren. Produktanalyse etter nedbryting av sodium alginat med AMOR_PL7A og AMOR_PL17A vart gjennomført med MALDI-TOF massespektrometri og ^{1}H NMR, og dette viste at desse enzyma kombinert braut ned alginat til monomerisk DEHU. Resultata frå denne oppgåva indikerer at ei enzymblanding kan bli tilsett for sakkarifisering av polysakkarida i *S. latissima*, noko som er eit svært essensielt steg mot ei meir effektiv prosessering av makroalgar i eit bioraffineri i framtida.

Abbreviations

CBH	Cellobiohydrolase
CBM	Carbohydrate binding module
CD	Catalytic domain
CV	Column volume
DM	Dry matter content
DNS	3,5-Dinitrosalicylic acid
DP	Degree of polymerization
EG	Endoglucanase
G	Guluronic acid
GG	Polyguluronic acid
GH	Glycoside hydrolase
HPLC	High-performance liquid chromatography
IMAC	Immobilized metal affinity chromatography
M	Mannuronic acid
MALDI-TOF	Matrix assisted laser deionization – time of flight
MG	Heteropolymeric sequences of mannuronic and guluronic acid
MM	Polymannuronic acid
NMR	Nuclear magnetic resonance
w/v	Weight/volume

Contents

1. Introduction

1.1 Sustainability and marine biomass

71% of the Earth's surface is made up of ocean (1). In a time where the world population is growing rapidly and a transition from fossil to renewable resources is necessary, the marine environment can offer new potential for sustainable biomass production. Terrestrial biomass production is limited to available land area and requires addition of freshwater. Thus, the use of marine biomass is highly beneficial for production, also because such large areas are available. An extensive coastline surrounds Norway and makes it unique in terms of marine biomass production. Aquatic resources are being exploited considerably when it comes to seafood in Norway, but there is also a growing industry of seaweed cultivation (2). This presents an excellent alternative for increasing the use of renewable resources and moving away from our dependency on fossil fuel. In addition, seaweed cultivation helps reduce eutrophication due to the growing nutrient discharge from salmon production (3). Norway harvests annually 130 000-180 000 tons of wild seaweed, mainly in the form of the brown seaweed *Laminaria hyperborea* (4,5). With the recent developments in seaweed cultivation, the biomass harvest could increase with another 16 000 tons (2). Developing technology and efficient processing methods for valorization of seaweed is an important and necessary aspect of this emerging industry.

1.2 Seaweed

Seaweeds, or marine macroalgae, are photosynthetic eukaryotic organisms that live attached to rocks or other hard surfaces along the coastline. Cyanobacteria, or blue-green algae, are sometimes also counted as seaweeds, although they are in fact a free-living photosynthetic bacteria. The majority of seaweeds live in the intertidal belt and upper littoral zone. Because they absorb green light of medium wavelength they are able to live as far down as a depth of 30-50 m (6). They are divided into four main groups on the basis of color: blue-green algae (Cyanophyta), red algae (Rhodophyta), brown algae (Ochrophyta) and green algae (Chlorophyta) (7). The North Atlantic Ocean is home to over 400 species of red, green and brown seaweed (2). An exceptionally long coastline makes

Norway a common habitat for kelp species of the order Laminarales. This is a large brown alga which can be cultivated or wild-harvested and processed for use as chemicals, in food, or in value-added products such as cosmetics and pharmaceuticals. Seaweed is a sustainable biomass because it has no requirement for terrestrial land area and does not need addition of fertilizer. Furthermore, its cultivation puts low pressure on the environment as it is a carbon neutral and renewable resource, along with a biomass output that is potentially higher than that of terrestrial plants (8). As seaweeds are submerged in water they do not need an internal transport of nutrients and water.This saves energy and results in high productivity (6). The biomass yield of North Atlantic coasts is reported to be approximately 2 kg carbon per m^2, compared to a productivity of less than 1 kg carbon per m^2 on temperate tree plantations or grasslands (8).

Wild harvesting in Norway consists primarily of forest kelp (*Laminaria hyperborea*) and to a lesser extent knotted wrack (*Ascophyllum nodosum*), whereas the most cultivated seaweed is sugar kelp (*Saccharina latissima*) (2). From seaweed biomass, fermentable sugars can be obtained through enzymatic saccharification. Brown seaweeds are composed of several polysaccharides, and therefore require a combination of enzymes targeted for degradation of the different components. While commercial cellulase cocktails such as Cellic® CTec2 can hydrolyse seaweed to a certain extent, they are originally developed for lignocellulosic biomass (9). An enzyme cocktail for efficient seaweed degradation is therefore yet to be developed (10). A minimal enzyme cocktail that can degrade seaweed with little or no pretreatment would be of great interest, and the obtainment of fermentable sugars would also provide a potential basis for e.g. single cell protein production (11).

Brown seaweed is mainly being harvested from wild stock at specific locations in Norway for the production of alginate. However, with the recent developments in seaweed cultivation, large areas along the Norwegian coast can be utilized for seaweed production. If seaweed can be successfully processed with a novel enzyme cocktail, this could facilitate increased exploitation of seaweed in the emerging bioeconomy, where there is a growing marked of seaweed-derived products.

Additionally, macroalgae are considered a beneficial food supplement as they contain proteins, carbohydrates, lipids, minerals, vitamins and enzymes. They are comparable to oats in terms of protein and carbohydrate content (12). However, brown seaweeds have a protein content of 3-15%, which is lower than red and green seaweeds (13). Although the seaweed composition varies according to season, water temperature, geography and species, all seaweeds are especially rich in carbohydrates (2). The ash content of brown seaweeds is also significant, typically constituting 15-35 % of the dry weight (13–15).

1.3. Structural composition of brown seaweed

The structural composition of brown seaweed varies according to seasonality. Light intensity, temperature, depth and sea currents have been shown to influence the content of components like ash, carbohydrates, protein and minerals (13,14,16). Kelps are reported to have a high carbohydrate content of about 50-60% on dry weight basis (8), which makes these seaweeds very suitable for microbial conversion processes. The fiber content of brown seaweeds are generally higher than that of green and red seaweeds, which are reported to contain 29-67% and 10-59% respectively (17,18). The brown seaweed cell wall is rigid, with a cellulosic backbone that attach alginate and phenol components. Proteins are also found in the cell wall structure associated with sulphated fucans and phenols (19).

1.3.1 Moisture and salt content

Macroalgae have a high moisture content of about 80-90%, and consequently water removal can be a considerable cost in seaweed processing (20). They also have a relatively high salt content where e.g. unwashed *Sargassum muticum* has been reported to contain 15% sodium chloride on a dry weight basis (21).

1.3.2 Carbohydrates in brown seaweed

The main carbohydrates found in kelps are alginate, cellulose, laminarin, mannitol and fucoidan. The polysaccharide laminarin and sugar alcohol mannitol are storage carbohydrates, whereas alginate and cellulose function as structural compounds. The cell wall is organized with cellulose microfibrils around an

alginate network, with xylo-fuco-glucans, glucuronans and homofucans embedded in between (Figure 1). Storage carbohydrates are accumulated during the season with high solar radiation (spring to fall), and are consumed during the dark winter season (6).

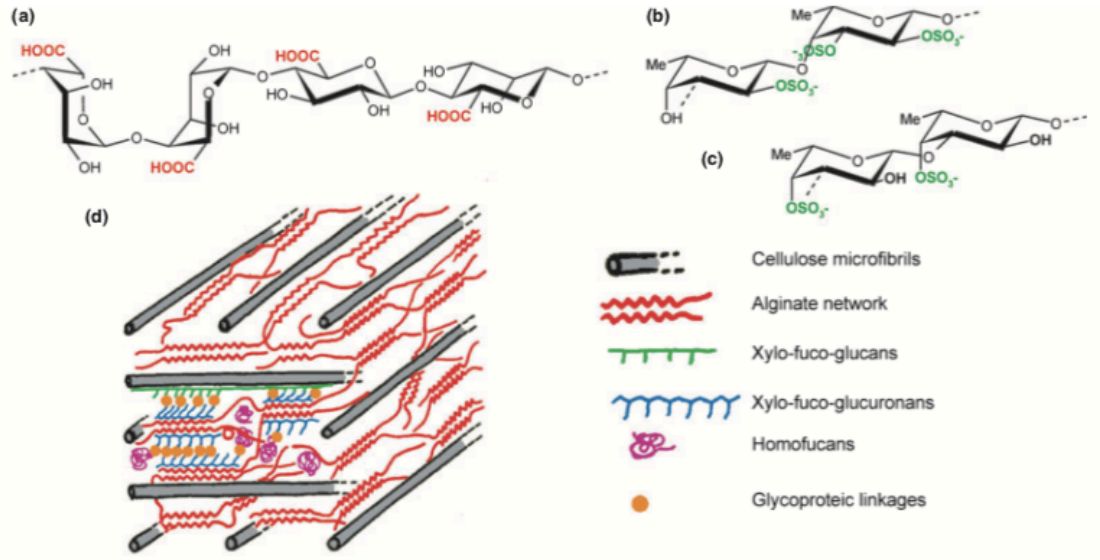

Figure 1. Illustration of the structural organization of polysaccharides in brown seaweeds (a) alginate with G- and M-blocks; (b) sulfated fucan (Fucales); (c) sulfated fucan (Ectocarpales): (d) proposed model of biochemical organization of the cell walls in brown seaweed. Figure source: Michel et al., 2010 (22).

1.3.2.1 Alginate

Alginate constitutes the main structural carbohydrate of brown seaweeds. The polysaccharide is present in the cell wall and largely makes up the main skeletal component of the intercellular matrix. It forms a three-dimensional network around the algal cells that both strengthens the seaweed mechanically and makes it flexible. It has been reported to account for up to 40% of the dry matter of *L. hyperborea* (23), or over 80 % of the organic matter in the intercellular matrix (6).

Alginate is a linear copolymer of two alternating uronic acids: α-1,4-L-guluronic acid (G) and β-1,4-D-mannuronic acid (M) (6). The polymer is arranged into C4-linked homopolymeric polyguluronate (G) and polymannuronate (M) blocks (Figure 2), as well as sequences of heteropolymeric and random GM-blocks. It functions as a gelling agent due to its rheological and physiological properties. The block structure of the algae determines its physical properties, and in the

presence of divalent cation, it can form structural gel elements (24). Especially G-blocks can cause binding of divalent metal ions and form gels (6). This gel is rigid and strong, whereas alginate with a higher proportion of M- or MG-blocks will result in a weaker and more elastic gel (25).

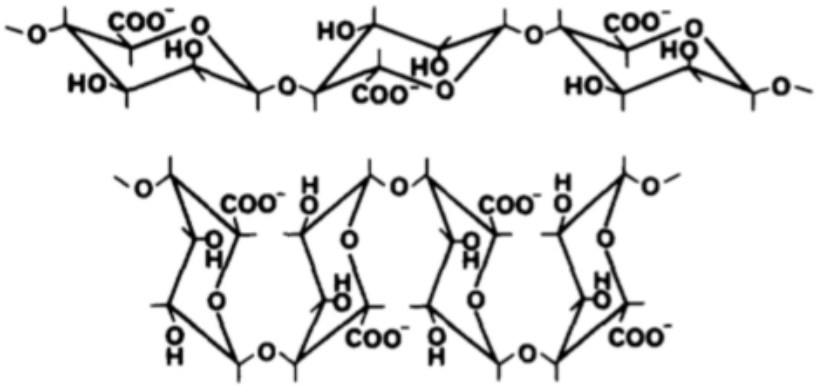

Figure 2. Conformation of poly-D-mannuronic acid chains (top) and poly-L-guluronic acid chains (bottom) that make up alginate. Figure source: Gacesa (1988) (26).

1.3.2.2 Cellulose

Contrary to land plants where cellulose is the main skeletal component, cellulose is a minor structural element of brown seaweeds. Alginic acids and sulfated fucans make up the majority (up to 45% of dry weight) of the cell wall in brown seaweed, while cellulose only constitutes between 1-8% of the wall (19). Cellulose is a linear chain of thousands of anhydrous β-D-glucopyranose units linked by β-1,4-glycosidic bonds. Cellobiose is the repetitive unit of cellulose, as each alternating glucose residue in one chain is rotated 180° (Figure 3). Cellulose has a non-reducing end with a closed ring structure and a reducing end with a carbonyl group, making cellulose a polarized molecule (Figure 4) (27). It is insoluble in water, as the hydrogen bonding environment influences both physical and chemical properties of cellulose. This can result in aggregation or at least incomplete solubilization, as is common with polysaccharides (28). Highly strengthened microfibrils hold the cell wall together as hydroxyl groups on the glucose form intra- and interchain hydrogen bonds with oxygen atoms on a neighboring chain. Cellulose microfibrils produced by algae are substantially different from that of higher plants, with a spongy or porous structure due to a different biosynthetic process (28). While microfibrils found in the primary wall

of plant cells are usually 36 glucan chains (Figure 3), the size of algal microfibrils may be large and contain over 200 chains (29).

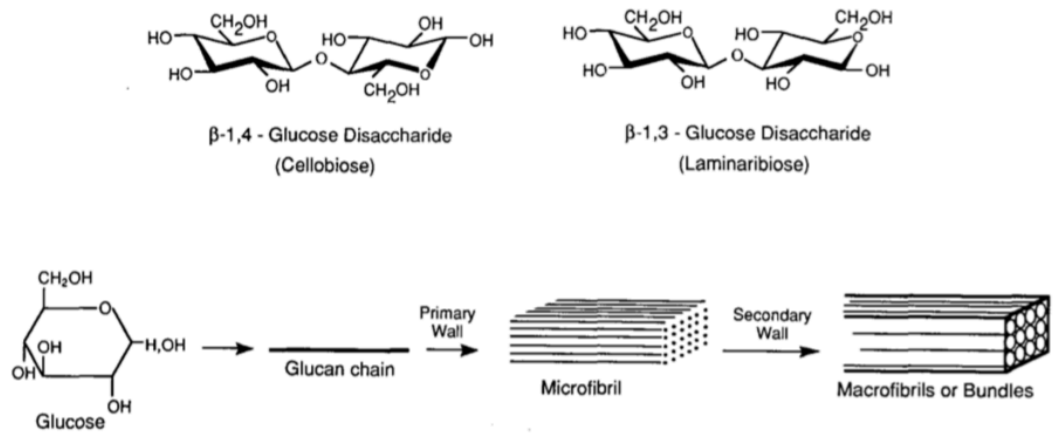

Figure 3. The assembly of cellulose in the plant cells. Cellulose consists of the repeating unit cellobiose which has each glucose residue rotated 180°. The elementary microfibril of the primary cell wall of plants is made up of ~36 glucan chains, which are further assembled into macrofibrils or bundles in the secondary cell wall. Figure source Delmer & Amor, 1995 (29).

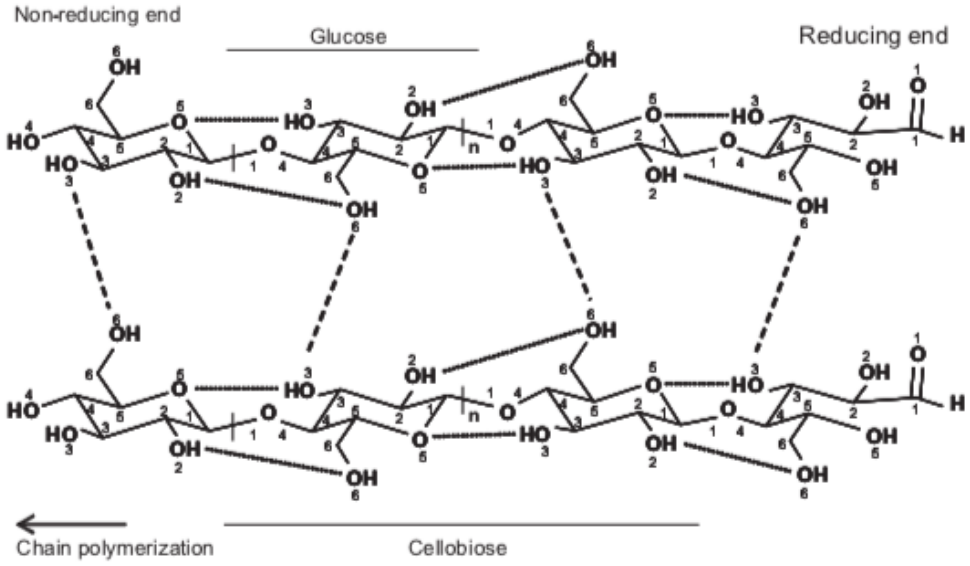

Figure 4. The assembly of cellulose I with intra- and interchain hydrogen bonds. Cellulose consists of the repeating unit cellobiose which has each glucose residue rotated 180°, and assembles into chains with an intra- and interchain bonding structure

and a reducing and a non-reducing end on opposite sides. Figure source: Festucci-Buselli et al., 2007 (27).

Cellulose is a crystalline component with several known structures depending on the network organization of hydrogen bonds in the microfibrils. The cell wall of higher plants contains both a microfibrillar, crystalline cellulose phase and a non-crystalline cell wall matrix, consisting of various polysaccharides and compounds like proteins and phenols (27). Algae, however, only contain a small amount of crystalline microfibrils. There are six different known crystalline structures of cellulose: cellulose I, II, III_I, III_{II}, IV_I and IV_{II}. Only cellulose I and II are found in nature, whereas the other structures must be constructed *in vitro* (27). Cellulose I crystal is the main structure found in nature and has chains aligned parallel to each other (29). Cellulose I exists in the two different allomorphs, I_α and I_β, which can be distinguished by ^{13}C-NMR (30). The main difference between the two allomorphs is the stagger direction of the hydrogen bonded sheets between chains, which is unidirectional in I_α and multidirectional in I_β (31). Algal cellulose I_α is converted to I_β by way of high-temperature annealing, and the I_α-like chain synthesized by higher plants is similar to the algal I_α-chain, although the hydrogen bonding environment differs between the two (32). The number of glucose residues that constitute each cellulose chain determines the degree of polymerization of the chain, and it varies between 2,000-25,000 glucose residues depending on the organism (33).

1.3.2.3 Mannitol

In brown seaweed, mannitol is a storage carbohydrate and has important functions as an osmoprotectant and antioxidant. While it usually constitute less than 10% of the whole seaweed, it can, especially in the *Laminarales,* make up as much as 20-30% of the fronds (34,35). It is the sugar alcohol of mannose and is commonly used as a sweetener or in medication as a diuretic or to reduce intracranial and intraocular pressure (36). Mannitol is synthesized in macroalgae by way of two main enzymatic steps. Fructose-6P is directly reduced to mannitol-1P, and subsequently the phosphoric ester of mannitol-1P is hydrolyzed in order to produce mannitol (37). This biosynthesis involves the

two enzymes mannitol-1-phosphate dehydrogenase (M1PDH) and mannitol-1-phosphate specific phosphatase (M1Pase). Although the complete mannitol biosynthesis in macroalgae is somewhat unclear, these two enzymes have been purified from a red algae, *Caloglossa continua*, and high activity has been detected in several brown algae species, indicating that they constitute a main photosynthetic product (37).

1.3.2.4 Laminarin

Laminarin is a small storage glucan of 20-25 glycosyl residues found in brown algae, and it consists of β-1,3-D-glucan with β-1,6-D-glucan intrachain branching (38) (Figure 5). The degree of branching has direct effect on its solubility in water, and the polysaccharide can be found in both soluble and insoluble forms in cold and hot water, respectively (38). Laminarin can be split into two types depending on the chain terminal sugar, the M-chains and the G-chains. M-laminarin is characterized by a non-reducing end 1-linked D-mannitol residue, whereas G-laminarin is terminated by a reducing end 3-linked D-glucose residue (39,40). M-chains may also contain an additional glycosyl group, or be completely absent in laminarin altogether (41). Laminarin does show structural heterogeneity to a certain extent with a polydisperse composition, because the M- and G-chains as well as the β-1,3-linked glycosyl backbone have different degrees of polymerization (39). Laminarin chain length is reported to vary across seasons and species (42,43).

1.3.2.5 Fucoidan

Fucoidan is a sulphated α-L-fucose polysaccharide commonly found in brown algae. The polysaccharide has a complex structure with fucose units being the main constituents (Figure 5). In fucoidan, fucose has a backbone of α-1,3-L-fucopyranosyl or alternating α-1,4- and α-1,3-linked fucopyranosyl residues (44). Galactofucans are also included in the fucoidan family and are often isolated from brown seaweed. These consist of an equal portion of L-fucose and D-galactose and are found to be dominated by 3-linked α-fucopyranosyl residues as well as 4-linked β-galactose(45,46). The main constituents have added sulphate ester groups, and contain smaller amounts of monosaccharides, such as

D-galactose, D-xylose, D-glucose, D-mannose and D-uronic acids (46,47). Fucoidan is well known for its biological functions in therapeutic use, which includes anticancer effects, antithrombotic and antioxidant activity (44,46,48,49). As with other polysaccharides, the fucoidan content of brown seaweed fluctuates throughout the season. The component has been found with different degrees of branching, sulphation and chain length at different times of the year (16).

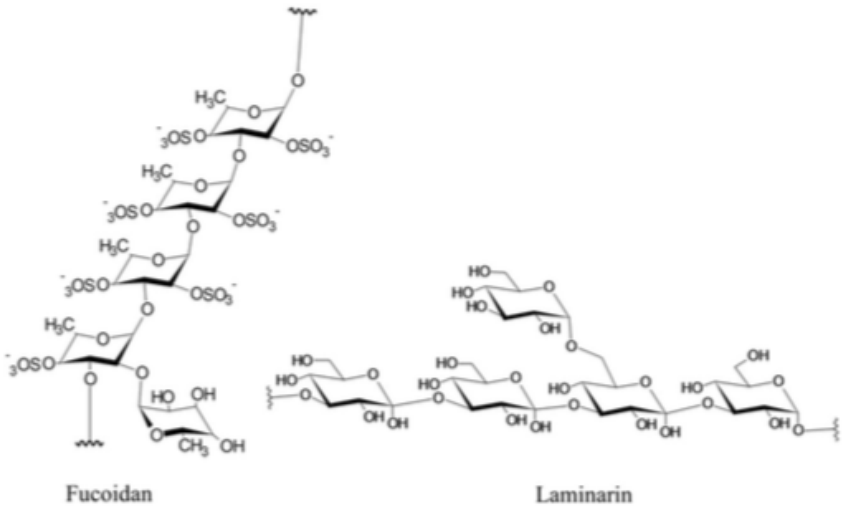

Figure 5. Chemical structure of fucoidan and laminarin as suggested by Cumashi et al., 2007 (50) and Adamo et al., 2011 (51). Figure source: Garcia-Vaquero et al., 2017 (44).

1.4 Seaweed biorefining

Due to their biochemical composition, brown seaweeds can be processed in a biorefinery to extract valuable chemicals and materials. The biorefinery concept is defined as a way of integrating processes and tools for biomass conversion in order to produce fuel, energy, value-added products and chemicals similarly to the present petroleum refineries, where products are extracted from crude petroleum (52). As seaweeds are a renewable resource, development and optimization of biorefinery processes is an integral part of the shift towards a biobased economy.

Conventional biorefinery processes are frequently employed on brown seaweeds to solely extract alginate, a high value polysaccharide, using alkaline conditions (53). Today, there are limited methods for concurrent extraction and purification

of polysaccharides like laminarin and fucoidan. However, a new two-step process developed in a recent study allows for acid extraction followed by alkaline extraction of alginate, fucoidan and laminarin (53). This biorefinery process utilizes acid extraction with HCl to achieve both a fucoidan/laminarin fraction and an acid extractable alginate fraction. The following alkaline extraction with $NaCO_3$ produces an additional fraction of alkaline extractable alginate, yielding three polysaccharides to a higher total compared to the conventional alkaline alginate extraction (53). The new biorefinery improvement shows that multiple biopolymers can be extracted in the same process, which is a valuable asset in the expanding exploitation of seaweeds as a feedstock.

To further process the extracted biopolymers to fermentable sugars and to convert biomass in an efficient, environmentally friendly manner, enzymes can be of great help in a biorefinery. By combining endo- and exolytic activities of cellulases and laminarinases for glucose release and alginate lyases for uronic acid release, the polysaccharides can be further degraded to yield fermentable sugars.

1.5 Enzymatic processing of seaweed

Total degradation of seaweed using only enzymes constitutes a challenge since seaweeds are composed of a range of biopolymers. Alginate is dominant in brown seaweed and as the main structural component, degradation of this compound is key for seaweed processing. As the polymer composition of brown algal cell walls differ between species (16), the optimal enzymatic treatment could vary. In order to obtain fermentable sugars, an efficient hydrolysis of laminarin and cellulose is important. Brown seaweed carbohydrates can be hard to access for enzymes when applied individually, because of the complex combination of alginate, laminarin and cellulose in the tissue. The combination of cellulases and alginate lyases with endolytic and exolytic action should ideally work synergistically to make substrate more available for degradation.

1.5.1 Storage and preservation

Seaweeds will typically have to be stored after harvest for later use, and this requires efficient preservation methods. Drying, either by using solar energy or drying facilities, is a good preservation method. Due to high ash and moisture content, both drying and further transportation and processing can be costly, and it has been suggested that drying seaweeds to a moisture content of <22% is sufficient before storage (54). This would improve shelf life and in addition reduce transportation costs (20).
Wild *L. hyperbora* harvested in Norway today is preserved with formaldehyde, a highly toxic chemical (55). This method is not a viable option for further processing, especially if the seaweed is to be used for food purposes, but it is currently the most common method for presevation of very large biomasses.
Ensiling is another preservation method that apply acidification by acid addition or by anaerobic microorganisms such as lactic acid bacteria, and this prevents growth of unwanted spoilage microorganisms. The method is more challenging for seaweeds due to their high buffering capacity caused by high anionic acid content, but inoculation with lactic acid bacteria has showed best result in obtaining the wanted pH drop for brown seaweed compared to red and green (56).

1.5.2 Pretreatment

Pretreatment of algal biomass prior to enzymatic saccharification have been found to increase glucose release in some species (57,58). Brown seaweed biomass differs significantly from lignocellulosic biomass with regards to the importance of pretreatment, because the seaweeds do not contain lignin. Preatreatment makes it less energy-demanding to process glucose? and polysaccharide fractions are more available to hydrolytic enzymes.
Mechanical pretreatment methods such as size reduction, beating, washing or sonication improves enzyme access prior to hydrolysis by affecting the physical structure of the seaweed (59). The preferred method of pretreatment varies and can depend on seaweed species, its structural composition, harvest time and location. Size reduction, by chopping or milling the biomass in either wet or dry condition, is generally a good method for increasing the surface area and thus directly improving access for hydrolytic enzymes.

It is common to wash the seaweed before processing to remove contaminants such as gravel or sand stuck in the biomass. Furthermore, it removes salt that may have an inhibitory effect on downstream processing like ethanol production (60). However, in a study by Adams, Schmidt and Gallagher (2015), washing decreased ethanol production from *L. digitata*, probably because it removed water-soluble carbohydrates (61).

Thermal pretreatment by autoclave, microwave or steam explosion is commonly used to release polysaccharides from seaweed (59). Heating the biomass and exposing the fibrous parts, potentially makes the enzymatic processing more efficient. Autoclave treatment at 121 °C does not affect all parts of the seaweed structure, and thus the structural composition of species may influence the relative effects of thermal pretreatment on complete and efficient hydrolysis (59). Microwave heating has been successful with seaweeds due to their high moisture content and consequently quick heating. It is mostly used for extraction of value-added products such as agar and carrageenan (59).

Chemical pretreatment involving alkali, acid or peroxide can result in higher solubilization and consequently higher yield of reducing sugars from seaweed (62,63). NaOH is commonly used for alkali, and H_2SO_4 or HCl for acidic, pretreatment which aid the degradation process by hydrolyzing storage carbohydrates (59).
Dilute acid pretreatment has been found to change the G:M ratio in alginate as MM blocks are more exposed to the pretreatment (57) and could be useful by employing a G-specific alginate lyase that makes cellulose fractions available to cellulases. Acid treatment is however criticized due to the high costs of recycling and handling, especially with concentrated acid (64).

1.5.3 Alginate lyase

Alginate lyases are enzymes that catalyze the depolymerization of alginate. They can be either mannuronate-specific (EC 4.2.2.3) or guluronate-specific (EC 4.2.2.11) lyases, referring to different regions in the copolymer alginate. The enzymes are called poly mannuronate (polyM) lyases and poly guluronate

(polyG) lyases when acting on MM and GG bonds, respectively (65). They are usually specific but some also recognize GM and MG bonds (66). Alginate lyases have been isolated from a variety of sources, including algae, marine mollusks, bacteria, fungi and some viruses (65). They are commonly grouped into seven polysaccharide lyase groups: PL5, PL6, PL7, PL14, PL15, PL17 and PL18, as well as a group of unclassified lyases (67). The groups are distinguished mainly on the structure of alginate lyases. PL5, PL15 and PL17 have an $(\alpha/\alpha)_n$ toroid fold structure, whereas PL6 has a β-helix fold structure and PL7, PL14 and PL18 have β-jelly roll fold structures (67). Alginate lyases can work endolytically or exolytically. Most are endolytic and cleave alginate blocks internally, requiring a significant amount of reaction time to cleave the substrate down to shorter oligomers (67).

The reaction mechanism of alginate lyases is a β-elimination reaction. Endolytic alginate lyase cleaves the alginate polymer to oligomers, resulting in a new reducing end and an unsaturated uronic acid at the non-reducing end (68). D-mannuronate is cleaved by mannuronate-specific lyase, and L-guluronate is cleaved by guluronate-specific lyase (Figure 6). Exolytic lyase further degrades oligomers into unsaturated monosaccharides, which are spontaneously converted into 4-deoxy-L-erythro-hexoseulose uronic acid (DEHU) (Figure 6). The reaction mechanism for alginate lyases is proposed to consist of three steps: (i) removing the negative charge of the carboxylate anion, (ii) a base-catalysed abstraction of the C5 proton and (iii) the β-elimination of the 4-O-linked glycosidic bond (69). To completely degrade alginate, an endo-lyase followed by an exo-acting lyase should ultimately release the monomer 4-deoxy-L-*erythro*-hex-4-ene pyranosyluronate.

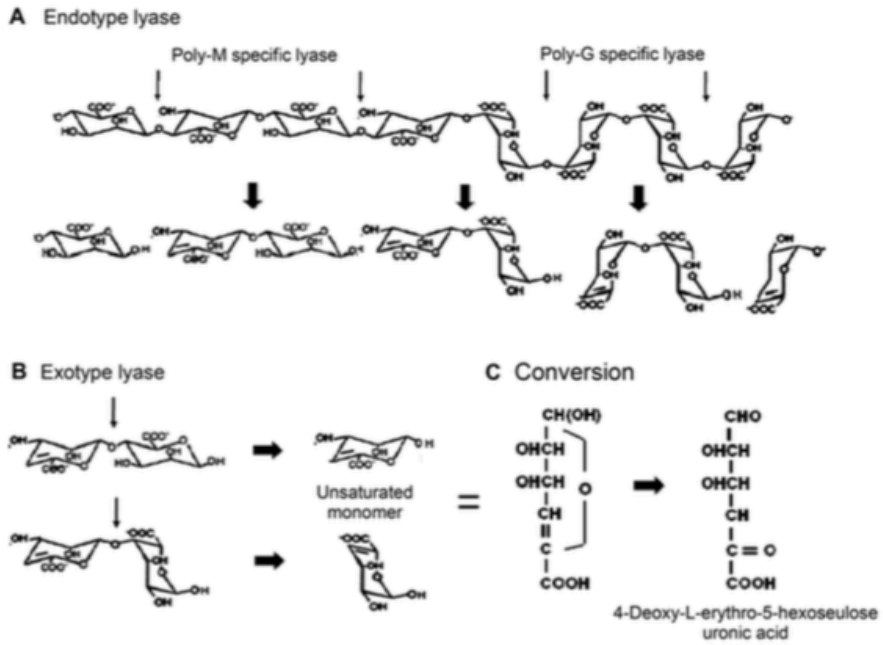

Figure 6. Illustratrion of alginate degradation by endo- and exotype alginate lyases. Arrows indicate the cleavage site for each type of lyase. Endotype lyase (A) release oligomers with an unsaturated uronic acid at the non-reducing. Exotype lyase (B) cleaves these products into unsaturated monomers, which converts spontaneously into 4-deoxy-L-erythro-5-hexoseulose uronic acid (DEHU) (C). Figure source: Kim et al., 2011 (68)

Alginate lyases normally have a lower temperature optimum than typically commercial cellulase cocktails, which means that combining such enzymes would require a two-step reaction to accommodate for both the lyases and the cellulase cocktail requirements (9). Recently, a novel thermostable, endo-type alginate lyase have been used in combination with the commercial cellulase cocktail Cellic® CTec2 for saccharification of brown seaweed in a single step reaction (10). AMOR_PL7A from a metagenomic dataset sampled in the Arctic Mid-Ocean Ridge successfully reduced viscosity of whole seaweed reaction mixtures and allowed for increased efficiency of the commercial cellulase cocktail at one single temperature (50 °C) (10). This allows for development of a simpler process at a higher temperature, lowering the risk of bacterial contamination of the samples.

1.5.4 Cellulases

Cellulases are commonly used for converting lignocellulosic biomass to ethanol, which is a major aim in the production of second-generation biofuels. This process utilizes biochemical conversion with a first step typically involving size reduction and a mild thermochemical pretreatment (70). The second step is enzymatic hydrolysis of cellulose to sugars. Cellulolytic microorganisms use different mechanisms to degrade cellulose, where cellulases are the key enzymes in all the approaches (71). Cellulases are produced by microorganisms in the kingdom Fungi and the domain Bacteria, but also by some insects, mollusks, nematodes and protozoans (72,73). The fungi *Trichoderma reesei* is the preferred organism for production of industrial cellulases, due to its high capacity for secreting cellulolytic enzymes.

Cellulases catalyze the hydrolysis of the β-1,4-linked glycosidic bond between two glucose molecules. The enzyme diffuses to the substrate to move a segment of cellulose from the insoluble part and into its active site, in contrast to a soluble substrate which diffuses to the enzyme's active site by itself (72).

Three classes of cellulases are involved in enzymatic degradation of cellulose, which can work synergistically to degrade cellulose as illustrated in Figure 7. Endocellulases or endoglucanases (EG) (EC 3.2.1.4.) catalyze cleavage of internal glycosidic bonds randomly along the glucose chain, producing oligomers of various degree of polymerization. Exocellulases or cellobiohydrolases (CBH) act on the terminal glucose units with cellobiose as the main product. There are two classes of CBHs; CBHI releases cellobiose from the reducing end of the cellulose molecule (EC 3.2.1.176), and CBHII cleaves from the non-reducing end (EC 3.2.1.91). The third cellulase class is β–glucosidases (EC 3.2.1.21) and these enzymes catalyze the hydrolysis of cellobiose units into glucose.

Recently, a new class of cellulose degrading enzymes was discovered, the lytic polysaccharide monooxygenases (LPMOs) which introduce a new way of depolymerizing recalcitrant polysaccharides (74,75). This group of enzymes catalyzes oxidative cleavage of the glycosidic bonds in crystalline regions of cellulose, thus generating new chain ends available for further degradation by cellobiohydrolases (76).

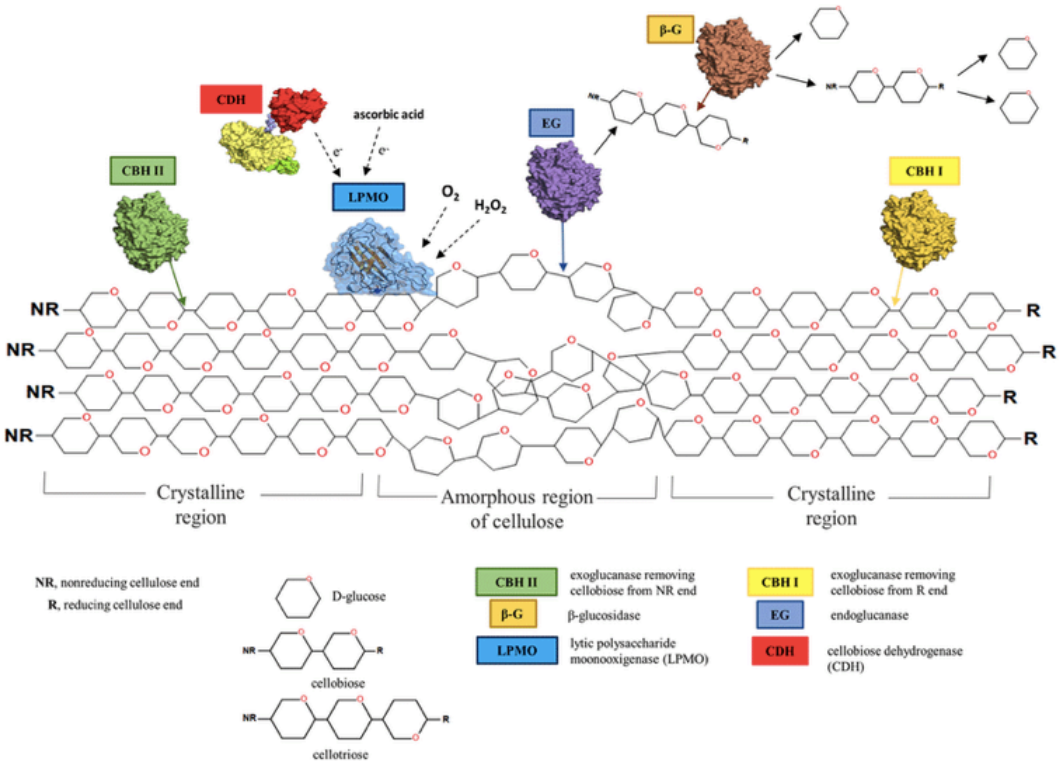

Figure 7. Schematic overview of enzymatic cellulose degradation. The figure shows synergistic reaction of the different classes of cellulases; endo- and exoglucanases, β–glucosidases and LPMOs. Figure source: Andlar et al., 2018 (77).

Glycosyl hydrolases (GH) that catalyze cleavage of the glycosidic bond are categorized into 161 GH families based on substrate specificity and occasionally molecular mechanism (78).

1.5.5 Laminarinases

Laminarinases, or 1,3-β–glucanases (EC 3.2.1.39), are enzymes that catalyze the hydrolysis of β–1,3- or β–1,4-glycosidic linkage in β–D-glucans. Most known are endoglucanases which hydrolyze internal glycosidic linkages, but there are also exoglucanases (EC 3.2.1.58) that facilitate successive hydrolysis of β–D-glucose units from the non-reducing end, releasing glucose. Laminarinases are often named so because of their activity on laminarin, but 1,3-β–glucanases also have activity on substrates like curdlan, lichenan, pachyman, pustulan and cellulosic derivatives. Some show strict substrate specificity towards β–1,3-linkages, but combined β–1,3- and β–1,4-linkage activity is also found (79–81).

Laminarin is one of the more complex substrates to degrade, as it consists of both β–1,3-linkages and intrachain β–1,6-branching compared to substrates with only one type of linkage. Laminarinases that hydrolyze β–1,6-glycosidic linkages (EC 3.2.1.75) are usually found as endo-acting enzymes and are separate from 1,3-β–glucanases. 1,6-β-glucanases are largely produced by fungi and act by randomly cleaving internal glycosidic bonds, releasing β–1,6-oligosaccharides (82). However, a new GH family, GH131, has recently been discovered with enzymes that hydrolyze β–1,3- and 1,6-linkages combined as exo-acting and β–1,4-linkages as endo (83).

When degrading laminarin, the aim is to get as much glucose as possible, which calls for the synergistic action of both an endo-acting and an exo-acting laminarinase. A combined exo-1,3-1,6-β–glucanase needed for complete degradation to monomeric glucose is rare, but have in one case been discovered and purified from the mushroom *Flammulina velutipes* (84). However, this study suggested that the exo-action of such an enzyme still needs the added effect of endo-acting 1,3-β–glucanase for best degradation efficiency.

Commercial cellulase cocktails like Cellic® CTec2 contains enzyme activities for laminarin hydrolysis and successfully releases glucose from the laminarin component of brown seaweed (9,85). However, this cocktail is specialized for cellulose-based substrates and likely contains several enzymes that are unnecessary for brown seaweed degradation. Although it can be combined with alginate lyase for hydrolysis of seaweed polysaccharides (9), a lower glucose release from laminarin due to lyases with high poly-M activity has been observed (85). For brown seaweed, an enzyme cocktail needs to contain both the lyase activity on alginate, as well as successful hydrolytic enzymes targeting laminarin and cellulose, but without any inhibitory effects of enzyme interaction.

1.6 Aim of study

Brown seaweeds are a rich source of carbohydrates that could be converted to fermentable sugars using enzymes. However, no enzyme cocktail exists for this purpose. The main aim of this study is to combine an enzyme cocktail for degradation of the polysaccharides in the brown seaweed *Saccharina latissima*, which ultimately can work on other brown seaweeds in the future. Furthermore,

the nature of the alginate monomeric product will be assessed. In-house enzymes and minimize pretreatment of the substrate will be used when possible. In the experimental development of the enzyme cocktail, I will try to (1) degrade as much as possible of the different carbohydrate components of *S. latissima*, and (2), obtain a high yield of monomeric sugars for fermentation.

A combination of endo- and exocellulases will be used to degrade the cellulosic component *S. latissima*, together with alginate lyases AMOR_PL7A and AMOR_PL17A for alginate hydrolysis and laminarinases for laminarin hydrolysis. All groups of enzymes consist of endo- and exo-acting enzymes in order to fully hydrolyze the substrate down to monomers. The effect of the different enzymes will be assessed with DNS assay for estimating reducing sugars and HPLC analysis. First, the assay will be tested on individual substrates (alginate and laminarin) before finally combining the cocktail for whole seaweed degradation.

2. Materials

2.1 Laboratory equipment and consumables

Table 1. Laboratory equipment and consumables with supplier

Equipment	Supplier
Allegra X-30R Centrifuge	Beckman Coulter
Autoclave 120°C	Certoclav
Automated pipettes	Thermo Scientific
Avanti™ J-25 Centrifuge	Beckman Coulter
Bunsen burner	Usbeck, Germany
Cellstar® Centrifuge Tubes, 15 ml	Greiner Bio-One
Cellstar® Centrifuge Tubes, 50 ml	Greiner Bio-One
Centrifuge bottles, Nalgene® wide-mouth with sealing caps, style 3141, 500 ml	Sigma-Aldrich
Centrifuge tubes, Nalgene® Oak Ridge style 3119, 50 ml	Sigma-Aldrich
Corning® 96-well UV-transparent microplate	Corning
Cuvettes, disposable	Eppendorf
Drying oven, Heratherm OGS60, 105°C	Thermo Scientific
Eppendorf tubes, 1.5 ml	Axygen
Filtropur membrane, 0.45 µm PES	VWR
Foam Racks, 1.5/2.0 ml	Heathrow Scientific
Freezer, -20°C	Bosch
Freezer, -80°C	Sanyo
Heat block	IKA®
Heraeus™ Pico™ 21 Centrifuge	Thermo Scientific
His-Trap™ HP column, 5 mL	GE Healthcare Life Sciences, USA
HPLC analytical column, Rezex™ ROA Organic Acid H^+ (8%) 7.8 x 300 mm	Phenomenex
HPLC vials	VWR
Incubation chamber, 37°C	Termaks

Inoculation loop, blue, 10 µl	Sarstedt
JA-10 Fixed-angle aluminium rotor	Beckman Coulter
JA-25.50 Fixed-angle aluminium rotor	Beckman Coulter
Laboratory glass bottles	VWR + DURAN®
LEX-48 Bioreactor	Epiphyte Three, Harbinger Biotech
Magnetic stirring bar, teflon	SP Science ware
Magnetic stirrer	VELP Scientifica
Measuring cylinder	VWR
Mega Star 1.6R Centrifuge	VWR
Micro tubes, screw cap, 2.0 ml	Sarstedt
MilliporeSigma™ Multiscreen$_{HTS}$ Durapore™ 96-well filter plates, 0.45 µm	Merck Millipore
Milli-Q® Direct Water	Merck Millipore
Mini-PROTEAN® Tetra Vertical Electrophoresis System	Bio-Rad
Multiscreen®$_{HTS}$ Vacuum manifold	Merck Millipore
Nunc™ MicroWell™ 96-well microplate	Thermo Scientific
PA BBO 400S1 BBF-H-D-05 Z SP probe for NMR	Bruker (MA, USA)
pH-meter S1400	Sentron
pH-meter inoLab® 7110	WTW
Pipettes, automated	Thermo Scientific
Pipette refill tips	VWR
PowerPac™ 300 power supply	Bio-Rad
Refrigerator, 4°C	Bosch
Scale, 0.1 mg accuracy	Sartorius, Germany
Sealing tape, Nunc™	Thermo Scientific
Spatula, stainless steel	
Stain-free Sample Tray	Bio-Rad
Thermomixer™ C	Eppendorf
TX-400 Anti-friction rotor	Thermo Scientific
Vacuum pump/compressor, 230 V/ 50/60 Hz	Merck Millipore

Vibra-Cell™ V-750 Ultrasonic Liquid Processor	Sonics & Materials, Inc.
Vivaspin® 20, 10000 MWCO PES Vacuum Filtration Systems	Sartorius
Vivaspin® 20, 3000 MWCO PES Vacuum Filtration Systems	Sartorius
Volumetric flask, 2000 ml	VWR
Vortex minishaker	Fisher Scientific
Water bath, 100°C	Stuart
10-well comb	Bio-Rad

2.2 Chemicals and solutions

Table 2. Chemicals and solutions with supplier

Chemical/solution	Supplier
10X Tris/Glycine/SDS Buffer (TGS)	Bio-Rad
2,5-Dihydroxybenzoic acid	Sigma-Aldrich
3,5-Dinitrosalicylic acid (DNS), $C_7H_4N_2O_7$	Sigma-Aldrich
Acetonitrile, 100%	VWR
Antifoam 204	Sigma-Aldrich
Any kD™ Mini-PROTEAN® TGX Stain-Free™ Protein Gel	Bio-Rad
BD Bacto™ Tryptone	BD Biosciences
BD Bacto™ Yeast extract	BD Biosciences
BenchMark™ Protein Ladder	Invitrogen
Dipotassium phosphate, K_2HPO_4	Merck
Ethanol absolute, C_2H_6O	VWR
Fucose, $C_6H_{12}O_5$	Sigma-Aldrich
Glacial acetic acid, 100%	Merck
Glucose, D(+), $C_6H_{12}O_6$, anhydrous	VWR
Glycerol, $C_3H_8O_3$, 85%	Merck

Guluronic acid, $C_6H_{10}O_7$	Sigma-Aldrich
Hydrochloric acid, 32%	Merck
Imidazole, $C_3H_4N_2$, ≥99%	VWR
Isopropyl β-D-1-thiogalactopyranoside (IPTG), $C_9H_{18}O_5S$	Sigma-Aldrich
Kanamycin sulfate, $C_{18}H_{36}N_4O_{11}$	Sigma-Aldrich
Mannitol, $C_6H_{14}O_6$	Ida Steine Oma, NMBU
Methanol, anhydrous	Merck
NuPAGE™ LDS Sample buffer (4X)	Invitrogen
NuPAGE™ Sample reducing agent (10X)	Invitrogen
Potassium phosphate monobasic, KH_2PO_4	Merck
Potassium sodium tartrate tetrahydrate, $C_4H_4KNaO_6 \bullet 4H_2O$	Sigma-Aldrich
Sea water	NFS
Sodium acetate trihydrate	Sigma-Aldrich
Sodium chloride	VWR
Sodium hydroxide, NaOH pellets	VWR
Sulfuric acid, H_2SO_4, 98%	Merck
Trizma® base, ≥99.9%	Sigma-Aldrich
Xylose, $C_5H_{10}O_5$	Ida Steine Oma, NMBU

2.3 Substrates and enzymes

Table 3. Substrates and enzymes presented with sources

Substrate/enzyme	Source
AMOR_PL7A	Produced in this study
AMOR_PL17A	In-house, KBM, NMBU
Albumin	Sigma-Aldrich
Cellic® CTec2	Novozymes, Denmark
GH16, endo-1,3(4)-β-glucanase (laminarinase)	In-house, KBM, NMBU

GH3 therm3, β-glucosidase	Produced in this study
GH48.3, cellobiohydrolase	In-house, KBM, NMBU
GH5, exo-1,3-β-D-glucanase (laminarinase)	Megazyme
GH6.1, endoglucanase	In-house, KBM, NMBU
GH6.4, cellobiohydrolase	Produced in this study
Laminarin	Sigma-Aldrich
Seaweed, *Saccharina latissima*	Seaweed Energy Solutions, Trondheim
Sodium alginate	Sigma-Aldrich

2.4 Software and instruments

Table 4. Software and instruments with supplier

Software/instrument	Supplier
ÄKTA pure protein purification system	GE Healthcare Life Sciences, USA
BioPhotometer® D30 spectrophotometer	Eppendorf
Bruker Ascend™ 400 NMR instrument	Bruker (MA, USA)
Chromeleon™ 7 Chromatography Data System	Thermo Scientific
Gel Doc™ EZ Gel Imaging System	Bio-Rad
Gen5™ 2.0 Data Analysis Software	BioTek
HPLC system UltiMate 3000	Dionex™
MestReNova version 9.1.0.14011	Mestrelab Research
Multiscan™ FC Microplate	Thermo Scientific
Synergy™ H4 Hybrid Multi-Mode Microplate Reader	BioTek
Topspin version 3.7	Bruker (USA)
ultrafleXtreme™ MALDI-TOF/TOF	Bruker (Daltonics, Germany)
Unicorn™ Chromatography Control Software	GE Healthcare Life Sciences, USA

3. Methods

3.1 Buffers

50 mM Sodium acetate buffer pH 6 was prepared by using stock solution of 0.1 M glacial acetic acid and 0.1 M sodium actetate trihydrate. 0.1 M glacial acetic acid was obtained by diluting glacial acetic acid 1:10 with dH_2O to a 1000 ml stock solution in a 1 L blue-cap laboratory bottle, and likewise for 0.1 M sodium acetate trihydrate. Table 5 was used to prepare the sodium acetate buffer to a concentration of 50 mM and pH 6. The 100 ml 0.1 M sodium acetate buffer was obtained by mixing 5,6 mL 0.1 M acetic acid and 94,8 mL 0.1 M NaOAc in a 300 mL blue-cap laboratory bottle, and diluted 1:2 with 100 ml dH_2O to a total of 200 ml 50 mM buffer.

pH was adjusted to exactly 6.0 with 1 M NaOH and 32% HCl.

Table 5. Preparation of 50 mM sodium acetate buffer.

50mM sodium acetate buffer			
Glacial acetic acid:			
MW	60,05	g/mol	
Sodium acetate trihydrate:			
MW	136,08	g/mol	
Make up 0.1M solution of each and mix;			
pH	Acetic acid 0.1M (ml)	NaOAc 0.1M (ml)	Total volume (ml)
4	84,7	15,3	100
5	35,7	64,3	100
6	5,2	94,8	100
*Dilute to 50mM			

For the ÄKTA™ pure protein purification system, buffer A and B were prepared. The buffers are 50 mM Tris-HCl buffer with pH 8 and 500 mM NaCl, containing 5 mM imidazole for buffer A and 500 mM imidazole for buffer B.

1 M Tris-HCl pH 8 was prepared in advance following the recipe in Table 6. 121.14 g Tris base was weighed out and added to a 1 L blue-cap laboratory bottle, then mixed with 800 mL dH_2O on a magnetic stirrer. pH was measured and adjusted with concentrated HCl with a pipette until reaching 8.0, and the buffer volume was adjusted with dH_2O to a total of 1 L.

Table 6. Preparation of 1 M Tris-HCl buffer.

1 L 1 M Tris-HCl pH 8	
121.14 g Tris base	800 mL dH_2O
32% HCl	Adjust slowly with pipette to pH 8

Buffer A was made according to the recipe in Table 7, combining 0.34 g imidazole, 50 mL 1 M Tris-HCl pH 8 and 29.42 g NaCl with dH_2O to a total buffer volume of 1 L on a magnetic stirrer. Buffer B was made according to the same recipe, but increasing the imidazole amount to 34.02 g for a final concentration of 500 mM. The buffers were filtered through a 0.45 µM filter before use.

Table 7. Preparation of buffer A for protein purification.

Buffer A (1 L, pH 8)	
5 mM imidazole	0.34 g
50 mM Tris-HCl pH 8	50 mL 1 M
500 mM NaCl	29.42 g
Buffer B (1 L, pH 8)	
Substitute for 500 mM imidazole	34.02 g

For HPLC analysis of samples from enzymatic saccharification experiments, 5 mM H_2SO_4 was used as eluent, and 20 % MetOH as wash solution. The 5 mM H_2SO_4 eluent was made by first preparing a stock solution of 25 mM H_2SO_4 in a 2 L blue-cap laboratory bottle and diluting this 1:5 with dH_2O. The stock solution was made by adding x ml H_2SO_4 to a total of 1000 ml dH_2O in a 1 L blue-cap laboratory bottle.

3.2 Enzyme production with *E. coli* BL21™ Star

The working stocks of enzymes AMOR_PL7A, therm3 and GH6.4 were prepared as part of this work using in-house glycerol stocks of *E. coli* carrying the relevant genes. The genes for all these enzymes contain a His tag for the purification step. The same protocol for protein production as described for *E. coli* BL21™ Star was followed for all enzymes. The enzymes AMOR_PL17A, GH6.1, GH48.3 and

GH16 were produced in other projects at the KBM faculty at NMBU, while the exo-laminarinase GH5 was supplied by Megazyme.

3.2.1 Glycerol stocks

All *E. coli* stocks containing cloned enzymes were stored in 2.0 ml screw-cap tubes at -80°C. Cell growth was initiated by lightly scraping some content from the stock with an inoculation loop and transferring this to liquid growth medium.

3.2.2 Growth medium

LB-medium was prepared according to the recipe in Table 8. 5 g Bacto™ Tryptone, 5 g NaCl and 2.5 g Bacto™ Yeast extract was mixed with dH_2O in a 500 mL blue-cap laboratory bottle to a total of 500 mL medium using a magnetic stirrer.

Table 8. Preparation of LB-medium for enzyme purification.

LB-medium	
Bacto™ Tryptone	5 g
NaCl	5 g
Bacto™ Yeast extract	2.5 g
dH_2O	To 500 mL

Terrific broth (TB-medium) was prepared according to the recipe in Table 9. 12 g Bacto™ Tryptone, 24 g Bacto™ Yeast extract and 4 mL Glycerol were mixed in a 1 L blue-cap laboratory bottle with dH_2O to a total of 900 mL on a magnetic stirrer. 100 mL 85% glycerol was autoclaved in a 100 mL blue-cap laboratory bottle at 121°C for 20 minutes before adding to the TB-medium. The content was then split between two 500 mL blue-cap laboratory bottles containing 450 mL TB-medium each.

All medium bottles were autoclaved at 121°C for 20 minutes before use.

Table 9. Preparation of Terrific broth (TB) medium for enzyme purification.

Terrific broth (TB-medium)	

Bacto™ Tryptone	12 g
Bacto™ Yeast extract	24 g
Glycerol	4 mL
dH_2O	To 500 mL

After autoclaving, a phosphate solution was added to the TB-medium. The solution was prepared following the recipe in Table 10. 23.14 g KH_2PO_4 and 125.4 g K_2HPO_4 was mixed in a 1 L blue-cap laboratory bottle on a magnetic stirrer with filtered dH_2O to a total of 1000 mL, making the final concentration 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4. The bottle was autoclaved at 121°C for 20 minutes before adding 50 mL to each of the two TB-medium bottles.

Table 10. Preparation of phosphate solution for TB-medium.

Phosphate solution	
0.17 M KH_2PO_4	23.14 g
0.72 M K_2HPO_4	125.4 g
dH_2O filtered	To 1000 mL

3.2.3 Enzyme production with *E. coli* BL21™ Star

Enzyme production was initiated by growing competent *E. coli* BL21™ Star with the relevant genes from a glycerol stock solution in sterile LB medium overnight at 37°C (two batches of 5 ml). Meanwhile, TB medium was prepared and autoclaved as described in Section 3.2.2.

A kanamycin solution of 50 mg/mL was prepared by adding 500 mg powdered kanamycin to 10 ml dH_2O while using a magnetic stirrer. The solution was filtered through a 0.22 µm filter into aliquots before use.

Each of the two cultures in LB medium were checked for visible growth after 24 hours and 5 mL subsequently added to the two 500 mL TB medium bottles. 500 µL of a 50 mg/mL kanamycin solution was added to each the TB cultures as well as 150 µL antifoam. Cultures were grown for another 24 hours in these bottles at 22 °C using a LEX-48 Bioreactor system (Harbinger Biotech), with a lid and autoclaved, sterile tubes providing a continuous air flow.

The next day, OD_{600} was measured for both cultures, and 100 µL 1M IPTG was added when the OD_{600} measurement yielded values of above 1.000. The cultures were then left to grow for another 24 hours, and OD_{600} was measured until the growth appeared to stagnate. Cell harvesting took place when reaching this stationary stage.

3.3 Harvesting of intracellular *E. coli* enzymes

The two cultures were allocated to two 500 mL centrifuge bottles for the JA-10 centrifuge. The centrifuge bottles were were balanced to the same mass ± 0.01 g in the sterile cabinet. The cultures were then centrifuged in an Avanti™ J-25 centrifuge (Beckman Coulter) using the JA-10 rotor for 15 minutes at 4 °C on 5000 xg.

40 mL supernatant from each JA-10 centrifuge bottle was transferred to two 50 mL Nunc tubes. The remaining supernatant was discarded as GMO waste, and the collected supernatants (40 mL) was poured back into its respective JA-10 centrifuge bottle. The cells were suspended in the supernatant using a spatula and the whole suspension poured back to the two 50 mL Nunc tubes. These were adjusted to equal weight ± 0.01 g in the fume hood, and then centrifuged in an Allegra X-30R centrifuge (Beckman Coulter) for 15 minutes at 4 °C on 5000 xg. Following this, the supernatant was discarded and the pellet left to sit in the freezer at -80°C overnight before going through the protein purification procedure.

The frozen pellets were retrieved and left to thaw on ice a few hours before carrying out the enzyme purification protocol. After thawing, pellets were resuspended in 30 mL pre-cooled buffer A in each Nunc tube for protein purification. The cells were then sonicated on ice with Vibra-Cell™ V-750 sonicator (Sonics & Materials, Inc.) for 3 minutes using a cycle of 5 seconds on and 5 seconds off with an amplitude of 30%.
The cells were subsequently transferred to two JA-25.5 centrifuge bottles marked A and B, adjusted to equal weight ± 0.01 g, and centrifuged with a JA-25.5 rotor in an Avanti™ J-25 centrifuge (Beckman Coulter) centrifuge for 15

minutes at 4 °C on 5000 xg. The lysate from each bottle was then filtered into two clean 50 mL Nunc tubes using a 0.45 µM filter.

3.3.1 Protein purification with ÄKTA™ pure protein purification system

The method employed for purifying protein was immobilized metal affinity chromatography (IMAC) using an ÄKTA™ pure chromatography system and a HisTrap HP 5 mL column with Ni^+. The method separates proteins according to their affinity, so that proteins lacking the affinity for Ni^+ wash out whereas proteins with a terminal His-tag and affinity for Ni^+ bind to the column resin. The ÄKTA™ system was prepared with a pump wash using dH_2O to remove debris and potential contamination from the previous run, and then a pump wash for both buffer A and B. Following this, the column itself was washed with 5 column volumes (CV) dH_2O using a flow rate of 1.0 mL/min and a pressure limit of 0.3 bars.

Using the Unicorn™ control software, a run with dH_2O was done prior to the samples at a 1.0 mL/min flow rate to ensure the pressure did not exceed the limit and that UV signal was not abnormally high. The sample valve was then washed with dH_2O for 10 minutes to remove possible contaminations, and the method run for sample purification was started as shown in Table 11.

The system pump was primed with 100% buffer A for 2.0 CV and then washed with 10.0 CV buffer A in order to elute all proteins or other material that did not have affinity for the column. Following this, the method went on to elution and fractionation, increasing buffer B from 0-100% over 20.0 CV resulting in a linear gradient of 5-500 mM imidazole in 50 mM Tris-HCl pH 8, 500 mM NaCl. This step ensured proper elution of the His-tagged proteins, which lose their affinity towards the column resin due to the gradual increase in imidazole concentration. The eluted material was collected in fractions of 5 mL each using a fraction collector. Larger fractions were collected during sample application at first to ensure none of the protein were lost if it were to pass straight through the column. The setting “fixed fractionation” was used in the program when collecting material, making the fraction collector continuously collect 5 mL fractions throughout the entire elution. Fractions corresponding to the highest

peaks as shown by the UV/Vis detector were saved for protein concentration measurement, and the rest discarded as GMO waste. After elution, the pump was again primed with 100% buffer A, this time for 5.0 CV.

Table 11. Method run setup for Äkta purification.

1. Prime and equilibration	Prime pump with 100% buffer A for 2.0 CV
2. Sample	25 mL sample volume at 1.0 mL/min
3. Washout unbound	10.0 CV 100% buffer A until all non-binding proteins are eluted
4. Elution and fractionation	0-100% buffer B linear gradient, increasing over 20.0 CV
5. Prime and equilibration	Prime pump with 100% buffer A for 5.0 CV

The ±10 fractions with highest A280 values as seen by the UV/Vis detector on Unicorn™, and measured on a BioPhotometer® D30 spectrophotometer (Eppendorf) after elution, were selected for upconcentration with Vivaspin and protein measurement. The content of the remaining vials was thrown away as GMO waste. The ÄKTA™ system (sample valve, column and fractionation tube) was then washed with dH_2O and subsequently with 20% EtOH in the same order as when starting up the system. The column was removed and filled with 20% EtOH for storage until next use, and sample and buffer tubes left to sit in 20% EtOH.

3.3.2 SDS-PAGE (Sodium dodecyl sulfate-Poly Acrylamid Gel Electrophoresis)
Fractions with high A280 values collected after protein purification were analysed with SDS-PAGE to find overexpressed, soluble protein that could be upconcentrated. A 10-well gel plate was used for SDS-PAGE and a BenchMark™ ladder of 10-220 kDa was applied for reference.
A 10 µL sample of each protein-containing fraction was combined with 10 µL 2x SDS loading solution, boiled for 10 minutes and centrifuged. SDS loading buffer

was prepared by mixing together 4x SDS sample buffer, 10x reducing agent and dH_2O to a final concentration of 2x.

10 µL of each boiled sample was applied to the stain free gel plate and the gel was run in a chamber with 10X TGS running buffer at 270 V for 20 minutes using a PowerPac™ 300 power supply (Bio-Rad). The gel was analyzed with Gel Doc™ EZ Gel Imaging System (Bio-Rad) for purity and possible contamination by other proteins, and fractions containing the correct, purified enzyme were saved. Contaminated fractions or fractions with very weak bands were discarded as GMO waste.

3.3.3 Up-concentration of enzymes

Enzyme-containing fractions collected after protein purification were combined in two Vivaspin ultrafiltration units. For enzymes <10 000 Da, a 3000 MWCO were used, and for enzymes >10 000 Da, a 10 000 MWCO cutoff. The Vivaspin® ultrafiltration units up-concentrate protein solutions by filtering molecules larger than the pore size through a membrane, and keeping molecules that are too large to pass through.

The tubes were centrifuged in a Mega Star 1.6R Centrifuge (VWR) at 4°C on 5000 xg for 20 minutes or until all but <5 mL of the lysate was filtered through. The filtered lysate was discarded and storage buffer 20 mM Sodium acetate pH 8 (including 300 mM NaCl for AMOR_PL7A) was added to both tubes to a total volume of about 20 mL. The centrifugation was repeated until the first buffer wash was filtered through with <5 mL lysate left, and the process repeated once, with storage buffer added a second time and centrifuged through the filter until containing <1 mL purified enzyme. The enzyme was then transferred to a 2 ml screw-cap tube, labeled and kept at 4°C.

3.3.4 Protein concentration measurement

Protein concentration was measured by recording absorbance at 280 nm using a BioPhotometer® D30 spectrophotometer. Samples were measured in single-use 1.5 mL cuvettes, first calibrating to zero by measuring a blank sample without

protein, and then measuring the sample. The spectrophotometer was calibrated with a blank measurement after each sample was measured.
Aromatic amino acids absorb light at 280 nm and their absorption value has a linear relationship with the concentration within the range 0.1-1 A280 units. The protein concentration was calculated using the Beer-Lambert law below,

$A = \varepsilon lc$,
Where A is the absorbance at 280 nm, ε is the extinction coefficient, l is the length of the cuvette in cm (set to the standard 1 cm length), and c indicates protein concentration in M.
Concentration was found by converting with the theoretical extinction coefficient for the purified enzyme in question. The extinction coefficient retrieved from web.expasy.org/protparam using the amino acid sequence of the enzyme.

3.4 Enzymatic saccharification experiments

Saccharification experiments of *Saccharina latissima* biomass were carried out using different combinations of hydrolytic enzymes, all except one prepared from glycerol stocks. Both an endolytic and an exolytic enzyme were chosen to target each of the major polysaccharide components of the brown seaweed: alginate, cellulose and laminarin. Cellic® CTec2 was used as a control to compare the yield from cellulases produced from glycerol stocks to a conventional cellulase cocktail.

For alginate degradation, endolytic alginate lyase AMOR_PL7A and exolytic AMOR_PL17A were used in saccharification experiments. Both enzymes were produced from glycerol stocks and purified using the ÄKTA™ protein purification system (Section 3.3.1).
Initially, all cellulases GH48.3, GH6.1 and GH6.4 were tested for activity. Further on, cellulases GH48.3 and GH6.4, two cellobiohydrolyases that cuts from the reducing and non-reducing end of the glucose chain, respectively, were combined with β-glucosidase GH3 from *Thermotoga*, therm3 to target the cellulosic part of brown seaweed. GH6.4 and therm3 were produced from

glycerol stocks and purified before proceeding to enzymatic saccharification experiments. In later experiments, GH48.3 was omitted and only GH6.4 used to cleave cellulose endolytically.

Laminarin hydrolysis was targeted with endolytic GH16 1,3(4)-β-glucanase (*Coprothermobacter proteolyticus*), also known as laminarinase, previously produced from glycerol stocks at KBM, NMBU. This enzyme was combined with exolytic GH5 1,3-β-D-glucanase (*Aspergillus oryzae*), or exo-laminarinase, which was purchased by Megazyme.

Enzymes were first tested on their respective target substrates to confirm activity and optimize enzyme loading and substrate concentration, before doing experiments with *S. latissima*. Cellic® CTec2 was added to a final concentration of 0.045 mg/g DM when used as a comparison, whereas the in-house enzymes were added in concentrations of 20 nM each, equalling a total of 0.071 mg/g DM.

3.4.1 Degradation of alginate to monomeric G and M

Standard sodium alginate (Sigma-Aldrich) was combined with dH_2O to a concentration of 2% (w/v) by weighing out 1.0 g and dissolving in 50 mL dH_2O in a 50 mL Nunc tube on a Vortex minishaker (Fisher Scientific). The sodium alginate was then allocated into 10 mL Nunc tubes, with the working stock stored at 4°C, and the remaining stock frozen at -20°C.

An enzyme loading of 25.4 nM AMOR_PL7A as suggested by previous research (10), and 1 µM Apl17-2 was added to 1% sodium alginate in 25 mM Sodium acetate, pH 5.6 and 300 mM NaCl for initial alginate degradation experiments. Reactions were run at 50°C in eppendorf tubes, and samples heated for 5 minutes in the thermoblock before enzyme addition. All reactions with sodium alginate were stopped by immediately storing the samples in the freezer at -20°C before analysis, or directly added to DNS reagent for analysis. The alginate degradation was followed by monitoring the increase in reducing ends using the DNS method (86) described in Section 3.5 with glucose or guluronic acid as standards.

Alginate lyase reactions on ground seaweed was prepared in the same manner with final concentrations of lyases and parameters described above. When stopping the seaweed reactions, all samples were centrifuged at 14000 rpm for 2

minutes, and supernatant collected into new eppendorf tubes before storing at -20°C or adding to DNS reagent. Seaweed samples were diluted 1:10 with dH_2O for DNS analysis. The samples were not analysed with HPLC when looking at alginate degradation, as there is currently no standard available to determine the monomeric product from these reactions.

Endolytic alginate lyases degrade alginate by cleaving the glycosidic bond by a β-elimination reaction, producing unsaturated oligomers. Alginate lyases with an exolytic mode of action further cleaves the oligomers from the end of the chain into monomeric G and M.

3.4.2 Enzymatic conversion of cellulose to glucose

The cellulosic part of the seaweed was converted to glucose via the action of GH48.3, a CBHI, and GH6.4, a CBHII, as well as the β-glucosidase therm3. GH48.3 and GH6.4 cleave the β-1,4-linkages in the cellulose chains and form small oligosaccharides. GH48.3 cleaves off two units from the reducing end of the chain, and GH6.4 from the non-reducing end. A β-glucosidase is then needed to cleave the resulting cellobiose into glucose. Therm3 was used to hydrolyse cellobiose, the end-product of cellulase action, to glucose. Enzymes were diluted to working stocks with 20 mM TrisHCl pH 8.0. Cellic® CTec2 was added to separate samples and used as a control.

Enzymes were added to a final concentration of 20 nM each, unless stated otherwise, to 500 µl reactions of 15% DM *S. latissima* (w/v) in 50 mM sodium acetate buffer pH 6.0. Reactions were run at 50°C in Eppendorf tubes, and all samples were heated for 5 minutes in the thermoblock before enzyme addition. When stopping the reaction, the tubes were first centrifuged at 14000 rpm for 2 minutes and the supernatant transferred to a new eppendorf tube, then stored directly in the freezer at -20°C. Samples were analysed with the DNS method (86) described in Section 3.5 with glucose standards, and with a Dionex™ UltiMate 3000 HPLC system (Thermo Fischer, U.S.A.) as described in Section 3.7.

3.4.3 Enzymatic conversion of laminarin to glucose

To hydrolyze the 1,3-β-D-glycosidic bonds of laminarin to glucose, endo- and exolytic GH16 and GH5, respectively, were first tested individually and combined on laminarin in concentrations of 10x, 100x and 1000x dilution from a stock of 1 µM. In saccharification experiments on *S. latissima*, the enzymes were added to the reaction mixture in equal amounts to a final concentration of 20 nM each unless stated otherwise. None of these enzymes have been observed to cleave the sidechains with 1,6-linkages.
GH16 is an endo-1,3-β-D-glucanase that catalyzes the hydrolysis of 1,3-inkages in β-D-glucans such as laminarin by cleaving the glucose chains at internal position. The exo-1,3-β-D-glucanase GH5 cleaves of β -glucose successively from the non-reducing end of β-1,3-oligomers produced by endolaminarinase.

For initial enzyme activity assays, a reaction mixture of 5 g/L laminarin with 50 mM NaOAc pH 6 was incubated in the thermoblock at 50°C with 20 nM each of the exo- and endolaminarinase, first for 60 minutes and then for 8 hours in a new, separate assay with the two enzymes combined in suitable concentrations. Results were measured as glucose yield, recorded in terms of reducing sugars with the DNS method (86) as described in Section 3.5.
Reactions were then carried out with brown seaweed *S. latissima* as substrate, then in combination with other enzymes, as described further in Section 3.4.4.

3.4.4 Time course analysis on ground *Saccharina latissima*

Whole *S. latissima* was harvested in the Trondheim fjord in July 2014 by Seaweed Energy Solutions. The seaweed was subsequently frozen in fresh state, transported and stored at -20°C at NMBU, Faculty of Chemistry, Biotechnology and Food Science (KBM), Ås. Samples were dried at 35-105°C to a dry matter content of 95%, milled and screened through a 1 mm sieve, then stored in room temperature. *S. latissima* used in this study contains 23.0% glucose (cellulose and laminarin), 20.0% uronic acids, 20.5% mannitol, 5.2% fucose (fucoidan), and 24.8% ash on a dry weight basis (9).

Prior to time course analysis, the seaweed was mixed with 50 mM sodium acetate buffer with pH 6.0 to a dry matter content of 15% (w/v). 1.5 grams dried

ground seaweed was weighed into a 15 ml Nunc tube using a scale of 0.1 mg accuracy (Sartorius, Germany), and 10 ml buffer was added to the tube before mixing well on a Vortex minishaker.
Individual triplicate samples of 500 µl per time point were allocated into eppendorf tubes by using a pipette with modified pipette tips (a scissor was used to make a broader tip opening to allow sampling of particles) for better homogeneity in the samples. The reaction tubes were mixed thoroughly between each sampling. All samples were preheated in the thermoblock to 50°C for 5 minutes before addition of enzymes.

Samples were incubated at 50°C in the thermoblock for 24 hours at 1000 rpm unless stated otherwise, and individual triplicates per timepoint by putting the samples directly into a -20°C freezer. Samples were analysed with the DNS method (86) described in Section 3.5 with glucose standards, and with a Dionex™ UltiMate 3000 HPLC system (Thermo Fischer, U.S.A.) as described in Section 3.7.

3.4.4.1 Drying temperatures
Seaweed samples used initially were dried at 35°C overnight before storing at room temperature. When testing the effect of drying temperature, these samples were compared to a separate batch which initially was dried at 105°C. Part of the 35°C-samples were then dried a second time at 105°C in a Heratherm OGS60 oven (Thermo Scientific), and the three samples compared in terms of glucose yield after enzymatic saccharification as described above. Glucose yield was measured with a Dionex™ UltiMate 3000 HPLC system (Thermo Fischer, U.S.A.) as described in Section 3.7.

3.5 DNS method for quantification of reducing ends
DNS stopping reagent was made by dissolving 16 g NaOH in approximately 800 mL water, which was heated to around 40°C on a magnetic stirrer, making sure the temperature stayed below 45°C. A mixture containing 300 g K-Na-tartrate (Potassium sodium tartrate tetrahydrate $C_4H_4KNaO_6 \bullet 4H_2O$) and 10 g 3,5-Dinitrosalicylic acid was added in small portions with continuous stirring.

Magnetic stirring continued until all crystals were dissolved, after which the reagent was cooled to room temperature and made up to 1000 mL with water in a volumetric flask. The reagent was stored in a dark tightly closed bottle at room temperature.

Product formation from reaction time series was measured with the DNS method (86). The method consists of recording the amount of reducing ends at absorption wavelength 540 nm, which is proportional to the color change in DNS reagent. Glucose standards were prepared by dissolving glucose in dH_2O in the range 0.156-2.500 g/L.

50 µl sample was added directly to a 96-well plate after a reaction and combined with DNS reagent in a ratio of 1:3. 24 hour samples were diluted 1:2 with dH_2O to stay within the detection range, and likewise all samples from seaweed saccharification were diluted 1:10. The plate was covered with plastic tape and incubated at 100°C for 15 minutes, then cooled down before reading the absorbance at 540 nm in a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (Biotek).

3.6 Determination of double bond formation by UV Spectrophotometer

Initial enzyme activity assays for AMOR_PL7A and Apl17-2 were conducted by measuring the change in absorbance at 235 nm. As both the unsaturated product of endolytic cleavage by AMOR_PL7A and the unsaturated monomer from Apl7-2 results in UV-active products due to a double bond formation between C4 and C5, the yield can be analysed as formation of double bonds shown as increase in absorbance.

Reactions containing 25.4 nM AMOR_PL7A and 1 µM Apl17-2 in 50 mM NaOAc pH 6 250 mM NaCl with a sodium alginate concentration of 0,5% w/v were set up in triplicates with AMOR_PL7A and Apl17-2 alone and combined. Control reactions containing everything except enzymes were also set up in triplicates. Product formation shown as increase in absorbance at 235 nm was recorded successively for 1 hour using a Synergy™ H4 Hybrid Multi-Mode Microplate Reader (Biotek). Absorbance was measured for all samples at 1 minute intervals.

The plate was incubated at 50°C throughout the reaction, and continuously stirred in between each absorbance measurement.

3.7 Determination of enzymatic saccharification yield with HPLC

Products of enzymatic saccharification experiments were determined with ion exhange chromatography (IEC) analysis. The principle behind IEC is to separate molecules based on the net charge of the surface. A Dionex™ UltiMate 3000 HPLC system (Thermo Fischer, U.S.A.) was used for sample analysis, and all samples were diluted 1:20 with dH_2O and filtered through a 0.45 µM filter in advance. A 300 x 7.8 mm Rezex ROA-Organic Acid H+ analytic column (Phenomenex) with a cation-H cartridge guard column and refractive index detector was used in the system, and the analysis was conducted at 65°C with 5 mM H_2SO_4 eluent at a flow rate of 0.6 mL/min.

Calibration standards of 0.5, 1, 2.5, 5 and 10 g/L combined glucose, xylose, mannitol and fucose were prepared and diluted from stocks dissolved in dH_2O. Results were viewed and analyzed with Chromeleon 7.0 software.

3.8 Monomeric product analysis

3.8.1 MALDI-TOF

Reaction products from alginate lyases used on alginate were analyzed with mass spectrometry using an ultrafleXtreme™ MS instrument. Matrix Assisted Laser Desorption/Ionization (MALDI) with Time of Flight (TOF) separation was used. The principle for MALDI-TOF is to mix the sample with matrix on a target plate, which is inserted into the MS instrument and irradiated with laser. This irradiation causes the sample with matrix to vaporize and generates charged ions. The ions are immediately detected according to the time it takes them to reach the detector. As ions with lower m/z (mass to charge) value will move faster than smaller ions before they are detected, the TOF method enables separation of ions based on their m/z ratio.

Reaction mixtures were prepared with 80 nM AMOR_PL7A and 0.8 µM Apl17-2 combined and separate on 1% sodium alginate in 50 mM NaOAc pH 7 250 mM

NaCl. Individual samples were incubated for 30 hours on 55°C and 1000 rpm mixing. Samples were taken out after 30 hours, put directly in the freezer until all samples were ready for MS analysis, and then thawed before application on the target plate. All samples were diluted 1:100 before application.
1 µl sample was mixed with 2 µl matrix consisting of a solution of 30% acetonitrile and 60 mM 2,5-dihydroxybenzoic acid by pipetting up and down on the plate. Additionally, a cellohexaose (Glc6) were added to calibrate the instrument. The samples were then dried on the target using hot air from a hairdryer, and analysed with MALDI-TOF.

3.8.2 NMR

Reaction products from alginate lyase hydrolysis of sodium alginate were analysed with ^{1}H NMR after incubating for 24 hours with 80 nM AMOR_PL7A and 800 nM AMOR_PL17A in 25 mM NaOAc pH 5.6, 300 mM NaCl on 2% sodium alginate in a Thermomixer at 55°C and 1000 rpm. The samples were split in two, one prepared for NMR immediately and another left in room temperature for 24 hours before analysis.
Samples were filtered using a Vivaspin ® filtration unit with 10,000 MWCO in a Mega Star 1.6R Centrifuge (VWR) at 4°C on 5000 xg for 30 minutes, until all of the enzymes was removed. The samples were then left in a -20°C freezer overnight, and freeze-dried before NMR analysis.
NMR spectra were recorded at 25 °C using a Bruker Ascend™ 400 (Bruker MA, USA) with a "PA BBO 400S1 BBF-H-D-05 Z SP" probe. D_2O was used as solvent, and the relaxation delay was 2.0 seconds with a pulse width of 10,000.
The frequency used for ^{1}H NMR was 400 MHz. Chemical shifts are reported in parts per million (δ) relative to the solvent. The spectra were processed using MestReNova version 9.1.0.14011 and Topspin version 3.7.

4. Results and discussion

4.1 Enzyme production

Alginate lyases AMOR_PL7A and AMOR_PL17A, β-glucosidase therm3 and endo-glucanase GH6.4 were all produced using existing *E. coli* glycerol stocks and purified with an ÄKTA™ protein purification system. SDS-PAGE analysis was carried out on the purified protein samples to verify production of the enzymes. Strong bands at the expected molecular weights appeared for each enzyme (Figure 8.). Alginate lyases had thick bands without visible contamination from other proteins at 82,3 kDa (AMOR_PL17A) and 28,5 kDa (AMOR_PL7A) (Figure 8A). Therm3 showed the expected band at 82,6 kDa (Figure 8B) and GH6.4 at 62,1 kDa (Figure 8C). The protein samples were upconcentrated and used further in enzymatic degradation experiments.

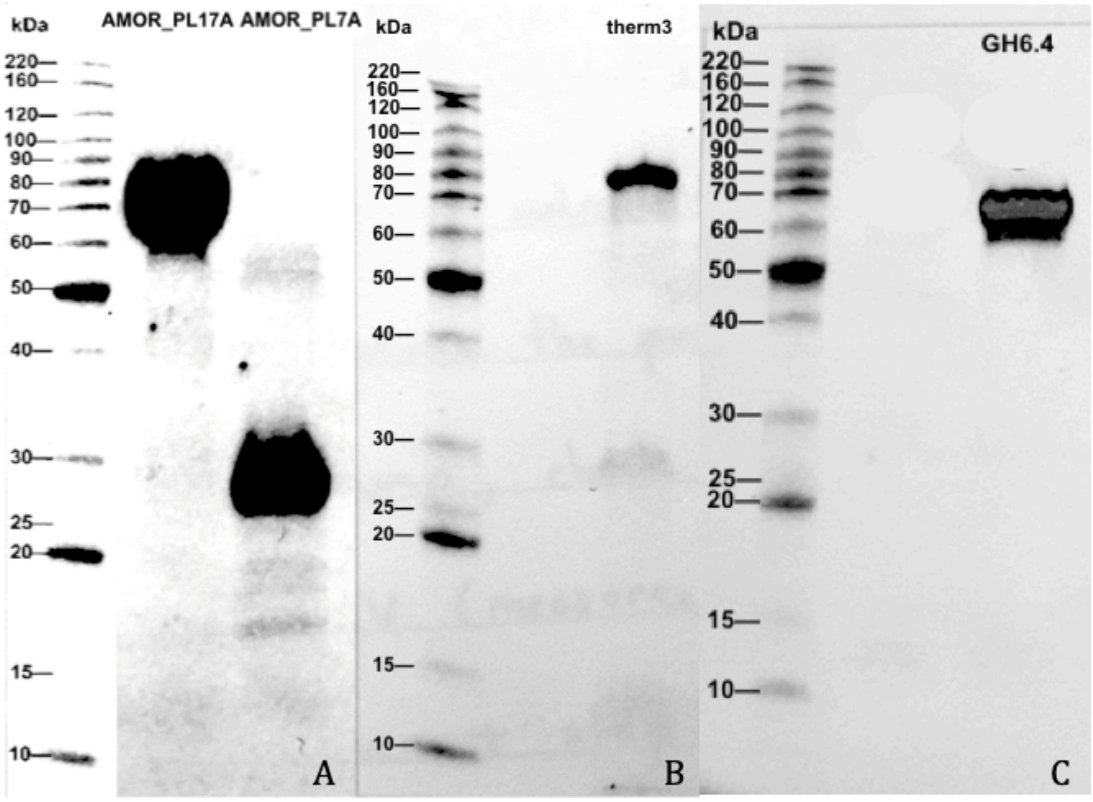

Figure 8. SDS-PAGE analysis after enzyme purification. The figure shows SDS-PAGE analysis of the four enzymes purified for this study. 10 µL protein sample and BenchMark™ ladder marking the molecular weight was applied to each gel plate. Panel A: the alginate lyases AMOR_PL17A and AMOR_PL7A, Panel B: the β-glucosidase therm3, and Panel C: the endo-glucanase GH6.4.

4.2 Conditions for enzymatic reactions

4.2.1 Effect of seaweed drying temperature and storage

Enzymatic saccharification assays were initially conducted using *Saccharina latissima* samples dried at 35°C, (95% dry matter) which previously had be shown to give high glucose release during enzymatic saccharification (9). Samples with the cellulase preparation Cellic® CTec2 and blank samples containing only seaweed and 50 mM NaAc pH 6.5, were incubated for 48 hours at 50°C at a substrate concentration of 15% w/v, and analysed at timepoints 0, 24 and 48 hours with HPLC. Surprisingly, glucose release in seaweed blanks were only slightly lower after 48 hours as the samples that were treated with Cellic® CTec2 (Figure 9).

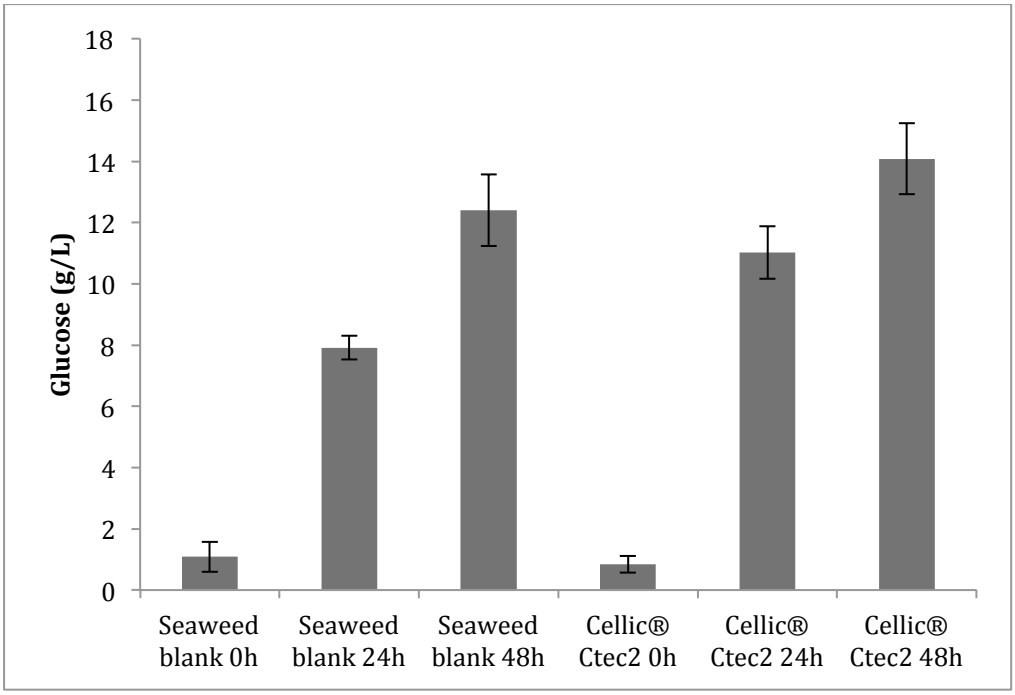

Figure 9. Glucose release from blanks and samples after enzymatic saccharification with Cellic® CTec2. Reactions were incubated at 50°C in 50 mM NaAc pH 6.5 and samples taken after 0, 24 and 48 hours. Each bar represents the average of independent triplicates ± standard deviation. The substrate concentration was 15% DM w/v with a final enzyme concentration of 0.045 mg/g DM Cellic® CTec2.

The assays detected no clear improvement of glucose release with treatment of cellulase, and it is possible that glucose had been leaking from the seaweed dried at 35°C because they had been stored in room temperature for 3 years. Drying temperature may affect the accessibility of enzymes to the substrate, and higher drying temperatures have been observed to yield lower glucose after treating *S. latissima* with blends of an alginate lyase (from Sigma) and Cellic® CTec2 (9). Furthermore, milling has been found to facilitate the release of free glucose monomers without enzymatic treatment, as the cell wall is deconstructed (87). This may also involve the release of endogenous enzymes that contribute to glucose production (87). The seaweed used in this study was harvested in 2014 and stored in room temperature after drying and milling. Seaweed dried at 35°C can be good to use for the highest glucose release from enzymatic saccharification (9), but after 5 years of storage, glucose was leaking out of the milled substrate without any enzyme treatment. This is indicated by the observed effect of cellulases on the seaweed dried at 105°C, which has no glucose leakage without enzymes (Figure 10).

Further experiments were done to investigate whether increased drying temperatures and/or the addition of alginate lyases would improve enzymatic saccharification assays. *Saccharina latissima* dried at 35°C was compared with a batch that had been dried at 105°C and stored for 3 years. A sample of the seaweed dried at 35°C was also subsequently dried at 105°C to investigate whether or not glucose leakage could be stopped with higher drying temperatures (e.g. by inactivating endogenous enzymes).
The alginate lyases AMOR_PL7A and AMOR_PL17A were included to investigate if lyase activity would facilitate a higher glucose yield.
Interestingly, drying the seaweed at 105°C before storage showed a severe decrease in glucose release of blank samples (Figure 10). Additionally, including alginate lyases showed a slight improvement in samples that had been dried at 105°C. As noted by Sharma & Horn (2016), high drying temperatures may form additional hydrogen bonds in cellulose and laminarin, the biopolymers that are the source of glucose, and make the substrate less accessible (9). In my study, blank seaweed samples dried at 105°C showed stable glucose values throughout

48 hours. Less glucose leakage due to added hydrogen bonds could be the reason why higher drying temperature stops further increase in free glucose monomers in the seaweed blank during a reaction. However, a more probable explanation is that high drying temperature reduced glucose release due to inactivation of endogenous enzymes in the seaweed, i.e. laminarinases and cellulases (Figure 10). There was a negligible effect of adding Cellic® CTec2, AMOR_PL7A and AMOR_PL17A to the seaweed dried at 35°C. This was probably because of glucose leakage, since high levels of glucose was released from the substrate in the blank reactions after incubation at 50°C.

When drying this seaweed again at 105°C, and when using the original batch dried at 105°C, the blank samples did not show any significant glucose release. However, adding alginate lyase had a clear effect on glucose release in these samples. This further supports that endogenous enzymes from the seaweed release glucose during incubation of the blank samples for seaweed dried at 35°C. Glucose release increased when the endo alginate lyase AMOR_PL7A was added to the seaweed. Thus, this enzyme probably opened up the seaweed matrix and allowed more degradation of laminarin and cellulose. However, when adding the exo-lyase AMOR_PL17A to the reaction, no significant effect was seen on glucose release. The data also shows that the seaweed dried at 35°C had a higher release of glucose than seaweed dried at 105°C, which is in accordance with a previous study (9).

Certainly, it is worth noting that increased drying temperatures could be beneficial to avoid glucose leakage directly from the milled seaweed, and that the presence of endogenous enzymes can be of great help in the production of fermentable sugars. Endogenous activity could be enhanced by milling the seaweed. If whole seaweed was used, as is the case on industrial scale, this activity could be lower and this should be tested in future studies.. However, for the purpose of studying the effect of added enzymes on seaweed degradation, it is better to limit the activity of endogenous enzymes. Storing the seaweed wet in the freezer and subsequently drying at 105°C and milling directly before use is potentially a better way to prepare the substrate to ensure maximum enzyme efficiency.

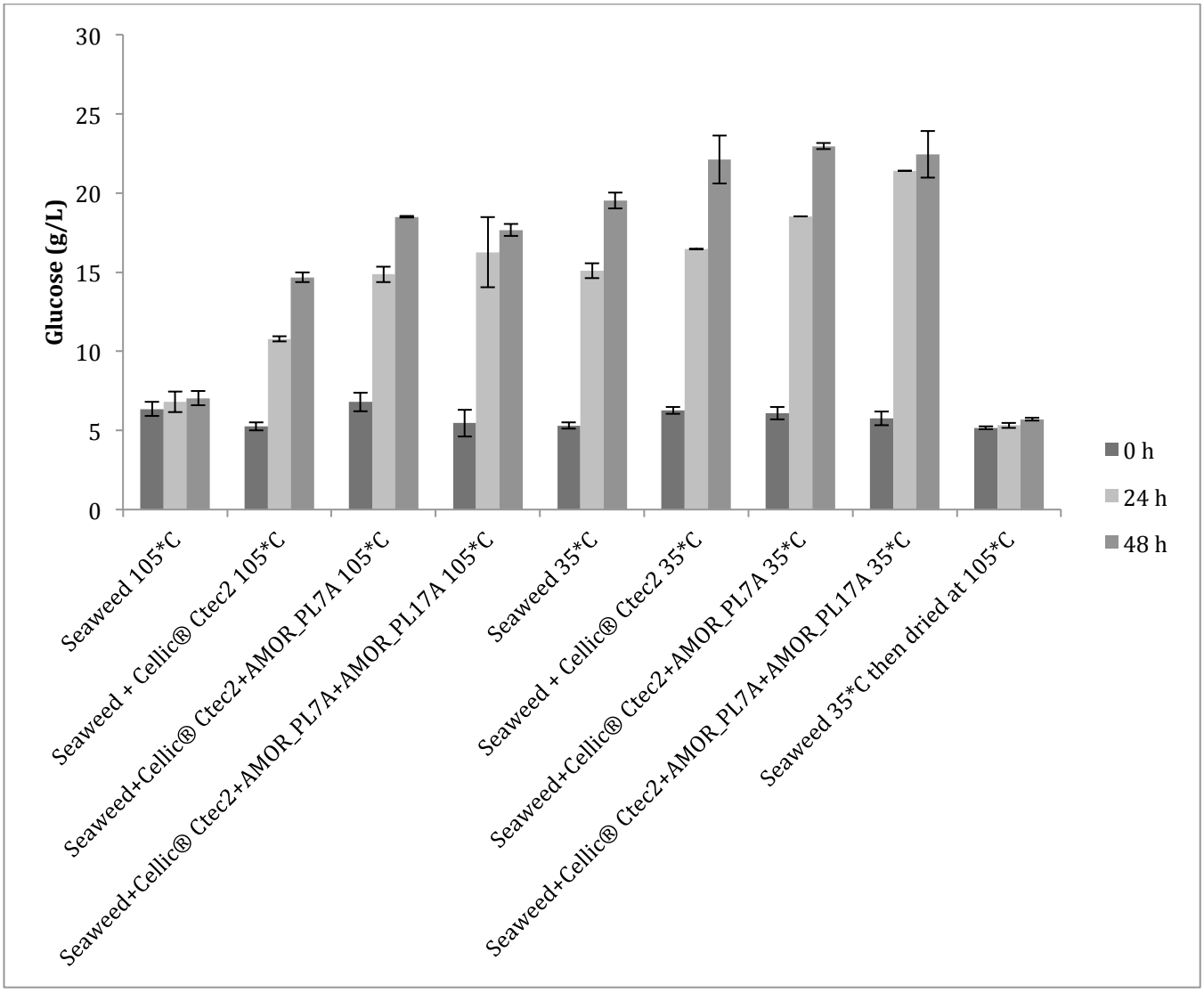

Figure 10. Glucose release from blanks and samples after enzymatic saccharification with Cellic® CTec2, AMOR_PL7A and AMOR_PL17A. Reactions were incubated at 50°C in 50 mM NaAc pH 6.5 and samples taken from 0-48 hours. Each data point represents the average of independent triplicates ± standard deviation. The substrate concentration was 15% DM w/v with a final enzyme concentration of 20 nM each of AMOR_PL7A and AMOR_PL17A, and 0.045 mg/g DM Cellic® CTec2.

4.2.2. Buffer

Seawater with a pH of 7.1 and 430 mM NaCl is optimal for AMOR_PL7A activity (10). When combining lyases with cellulases and laminarinases, the chosen reaction conditions cannot accommodate for each enzyme optimum. Optimum buffers were not obtainable for all individual enzymes. AMOR_ PL7A and

AMOR_PL17A work best at pH 7.1 and 5.6, respectively (10,88), but AMOR_PL17A has not been tested in seawater. GH6.1 is reported to work in phosphate-citrate buffer pH 5.0 and 6.0 depending on the substrate (89). To accommodate the need of the different enzymes, the performance of enzyme blends were tested with both seawater and 50 mM NaAc pH 6.5, 500 mM NaCl. All enzyme blends tested performed best in 50 mM NaAc pH 6.5, 500 mM NaCl (Figure 11), and therefore this buffer was kept as a standard buffer for further experiments. Also, the blend between the commercial Cellic® Ctec2 and AMOR_PL7A were tested against the in-house prepared blend of individual enzymes, and the former performed best.

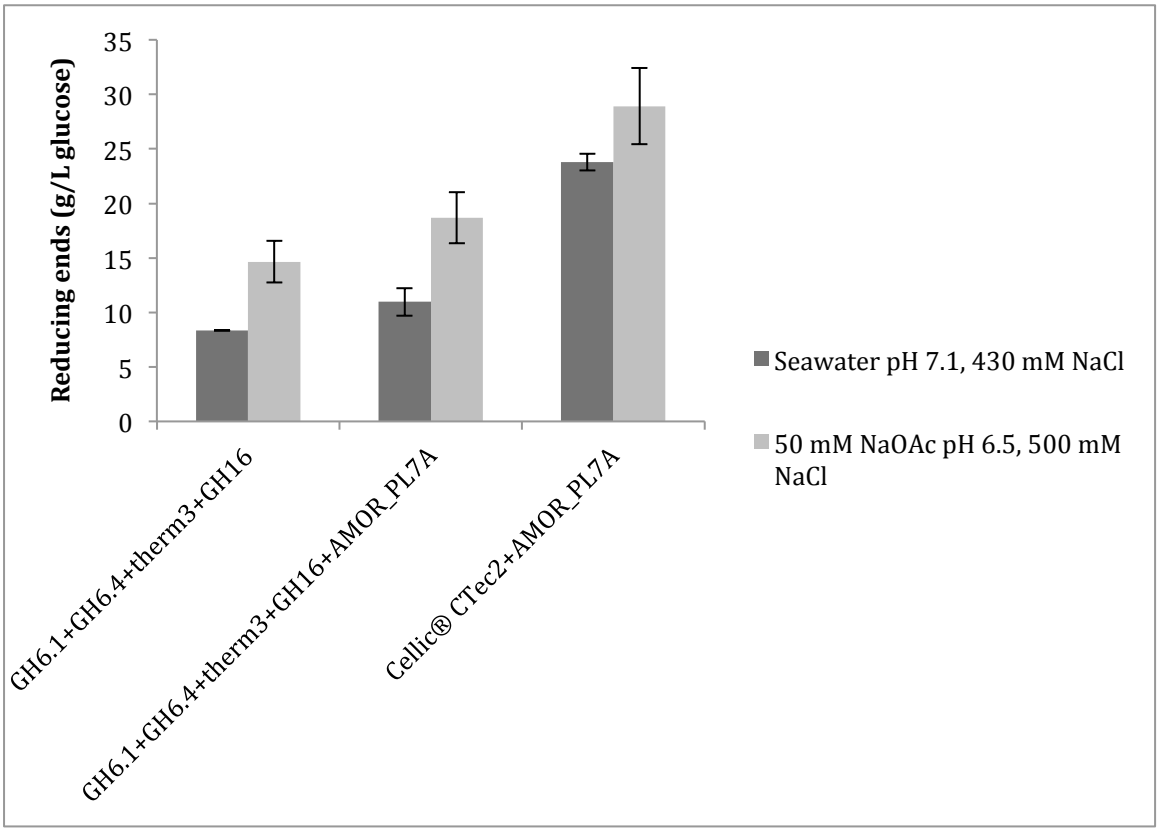

Figure 11. Performance of enzyme blends in different buffers. Reactions were run in seawater pH 7.1, 430 mM NaCl, or in 50 mM NaAc pH 6.5, 500 mM NaCl. Reactions were incubated at 50°C and samples taken after 24 hours. Each datapoint represents the average of individual triplicates ± standard deviation. Total enzyme loading was 6 mg/g DM for both in-house enzymes and Cellic® CTec2 with AMOR_PL7A, with 40% each of GH6.1+GH6.4, 15% therm3 and 5% GH16, or 3% GH16 with 2% AMOR_PL7A, and 98% Cellic® CTec2 + 2% AMOR_PL7A, to a substrate concentration of 15% DM (w/v).

4.2.3 Salinity

To investigate the best suited salt concentration for the enzyme cocktail, 50 mM NaAc pH 6.5 with NaCl concentration ranging from 0-500 mM was tested as buffers and compared with seawater on ground *S. latissima*. After 24 hours, the enzyme cocktail with cellulases GH6.4, GH6.1 and therm3, laminarinase GH16 and alginate lyase AMOR_PL7A performed best in 50 mM NaAc pH 6.5, 0 mM NaCl (Figure 12), however, with minor difference from the remaining buffers. The enzyme cocktail was compared to a combination of AMOR_PL7A and Cellic® CTec2, which performed better in seawater. The relatively low sugar values for in-house enzyme reactions is likely due to the lack of exolytic enzymes in this experiment. The cocktail lacks exolytic laminarinase, GH5, and exo-lyase AMOR_PL17A, which significantly add to the release of sugars.

Alginate lyases AMOR_PL7A and AMOR_PL17A perform well in seawater, a natural buffer for brown seaweed experiments, which in this study contained 430 mM NaCl. The lyases are both thermostable, thus ideal for enzymatic saccharification at higher temperatures, with AMOR_PL7A performing best at 65°C (10) and AMOR_PL17A at 90°C (88). This may have implications for combining these enzymes with cellulases and laminarinases at lower temperatures and with less salt. 50 mM NaAc buffer pH 6.5 with no salt added was a slightly better buffer condition for the in-house enzyme combination, but there was no large significant difference between NaCl concentrations ranging from 0-500 mM. Although not tested, AMOR_PL17A would probably be more sensitive to salinity concentrations. This enzyme requires salt to be active (88), whereas AMOR_PL7A also works without any salt (10).

Exo-lyase AMOR_PL17A was expected to show an increase in sugar release due to more of the alginate network being broken down by lyases. Surprisingly, early reactions that explored the difference in enzymatic saccharification from seaweed dried at different temperatures had no effect of adding AMOR_PL17A (Figure 10). The buffer was subsequently changed to 50 mM SoAc pH 6.5 including 300 mM NaCl, because salt proved to be essential for AMOR_PL17A activity, while not severely inhibiting the action of cellulases and laminarinases.

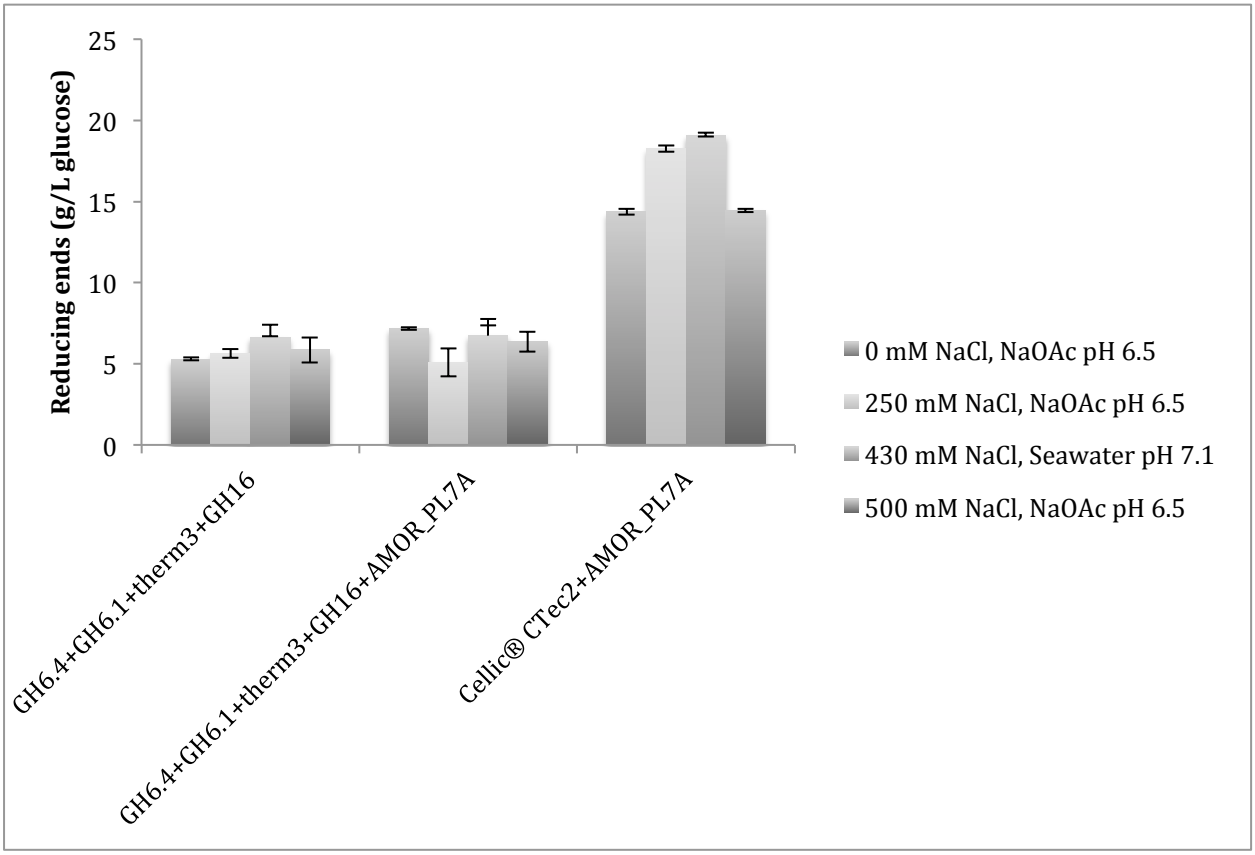

Figure 12. Performance of enzyme blends in different salinity concentration. Reactions were run in real seawater pH 7.1, 430 mM NaCl and 50 mM NaAc pH 6.5, 0-500 mM NaCl. Each datapoint represents the average of independent triplicates ± standard deviation. Total enzyme loading was 6 mg/g DM with 40% each of GH6.4+GH48.3, 15% therm3 and 5% GH16, or 3% GH16 with 2% AMOR_PL7A, and 98% Cellic® CTec2 + 2% AMOR_PL7A to a substrate concentration of 15% DM.

4.3 Enzyme activity assays

Enzymes were initially tested independently and combined in endo- and exolytic groups according to the target substrate. Cellulases were tested directly on ground *S. latissima* for their effect on glucose release, whereas alginate lyases were tested on sodium alginate and laminarinases on laminarin. The initial aim was to verify enzyme activity and find a suitable loading to degrade cellulose and laminarin down to glucose and sodium alginate to uronic acids. These findings could be used as guidelines for creating a combination on brown seaweed.

4.3.1 Alginate lyases

Saccharification of alginate was tested in a 24 hour reaction on 0.5% sodium alginate at 55°C in 25 mM NaAc pH 5.6, 300 mM NaCl. A final concentration of 25.4 nM AMOR_PL7A and 1 μM AMOR_PL17A were added, and samples taken from 0-24 hours. Alginate degradation was measured with DNS for reducing ends with guluronic acid (GA) standards. The reaction containing both AMOR_PL7A and AMOR_PL17A performed best, albeit only slightly better than AMOR_PL7A alone (Figure 13). The highest sugar concentration was measured at 8 hours with 1.2 g/L reducing ends. In comparison, the highest yield for AMOR_PL7A was measured after 24 hours with 1.00 g/L reducing ends and for AMOR_PL17A with 0.72 g/L (Figure 13). However, the data in figure 13 is not conclusive, as more data points could be taken between 8 and 24 hours. Earlier work on endolytic AMOR_PL7A and exolytic AMOR_PL17A were used as a reference for final enzyme concentrations (10,88). However, no previous work has been done with these enzymes combined for seaweed saccharification.

AMOR_PL17A has activity on both poly-G and poly-M, but as AMOR_PL7A is an M-specific endo-lyase, its efficiency on alginate may vary according to the distribution of G- and M-blocks in the substrate. A higher monomeric sugar yield might be obtainable if the alginate lyase has a very high content of M-blocks, which would be better in terms of substrate specificity. If a larger part of the alginate is hydrolyzed and the exo-lyase can subsequently work from the newly formed ends, the degradation rate of seaweed could be improved overall. Alginate would be more completely hydrolyzed and other enzymes would more quickly get access to laminarin and cellulose.

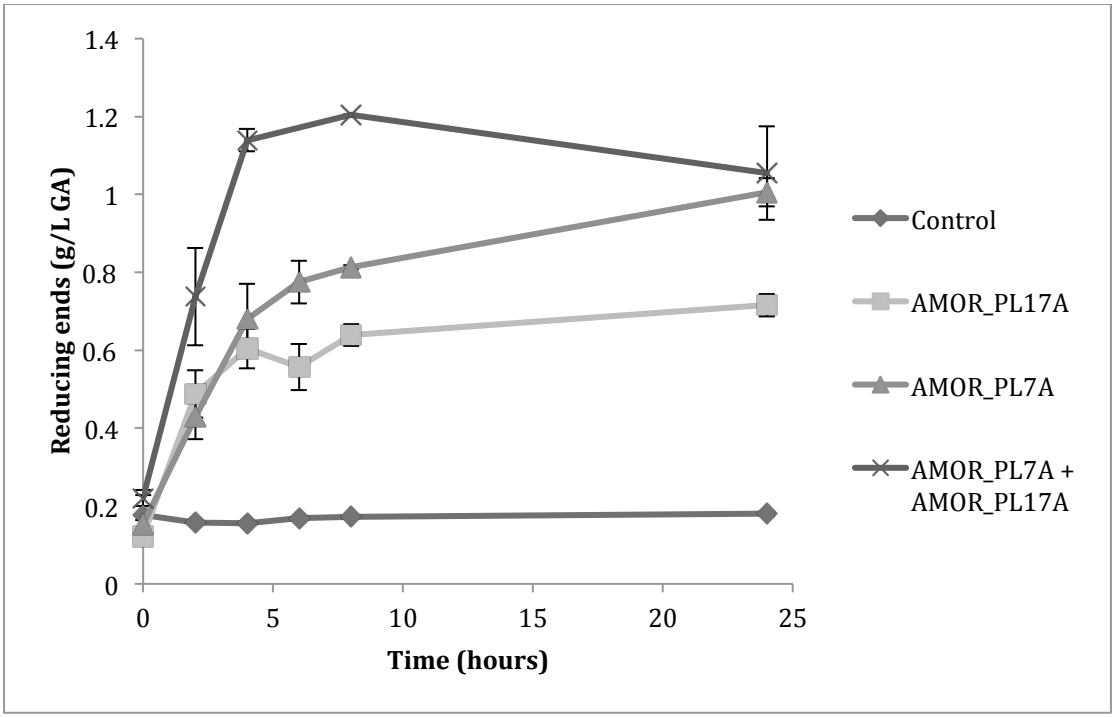

Figure 13. Saccharification of alginate. The degradation of alginate over 24 hours at 55°C was measured as reducing ends (g/L guluronic acid (GA)) with the DNS assay. Each data point represents the average of independent triplicates ± standard deviation. Reactions were done on 0.5 % w/v sodium alginate with 25.4 nM AMOR_PL7A and 1 µM AMOR_PL17A in 25 mM NaAc pH 5.6, 300 mM NaCl.

4.3.2 Laminarinases

The activities of endo-laminarinase GH16 and exo-laminarinase GH5 were tested individually and combined on 0.5 g/L laminarin. Both enzymes were added from a stock of 1 µM in 10x, 100x and 1000x dilutions and incubated for 60 minutes at 50°C in 50 mM NaAc pH 6.5.

The endo-laminarinase GH16 was most active at 0.1 µM concentration, which released a total of 0.21 g/L sugars from laminarin after 60 minutes (Figure 14). Exo-laminarinase GH5 was most active at 0.1 µM, with a total of 0.40 g/L sugars measured after 60 minutes (Figure 15). However, as this enzyme concentration resulted in an initial (time 0 h) large sugar concentration, the lower concentration of 0.01 µM was chosen for saccharification experiments on seaweed. This reaction, too, had a satisfactory yield of 0.25 g/L reducing ends after 60 minutes, compared to 0.07 g/L with 0.001 µM GH5 (Figure 22). GH16

was kept at 0.1 µM final concentration, as lower loadings than this gave a very low yield (Figure 14).

A longer time series reaction of 8 hours was completed to compare how GH16 and GH5 would perform combined with the same concentration of both enzymes. A final concentration of 20 nM was tested and samples taken from 0-8 hours. After 8 hours, 3.60 g/L glucose was released from laminarin as measured with HPLC using glucose standards (Figure 16). This corresponds to 72% out of the maximum yield. This reaction was carried out as a single reaction. Ideally, this reaction should be done with triplicates to improve reliability.

GH16, an endo-1,3(4)-β-glucanase, have activity on substrates with β-1,3-glycosidic linkages (data not shown) and laminarin. It is uncertain to what extent GH16 can hydrolyze the β-1,6-linkages present in laminarin. According to Figures 15 an 16, it cleaves laminarin relatively efficiently with dosages as low as 100 nM and releases glucose in combination with exolytic GH5. Both enzymes are active on 50°C, which similar to temperature optima of commercial cellulases.

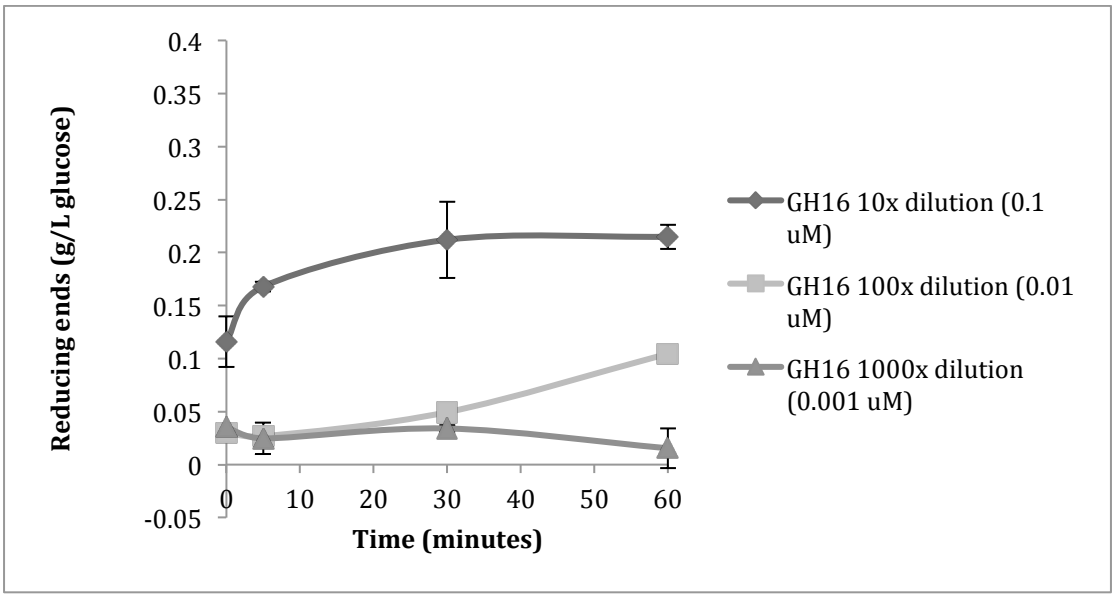

Figure 14. Enzyme activity of GH16. Glucose release with 1-100 nM GH16 on 0.5 g/L laminarin during 60 minutes incubation at 50°C in 50 mM NaAc pH 6.5. Each data point

represents the average of independent triplicates ± standard deviation as measured by the DNS assay with reducing ends, using glucose standards.

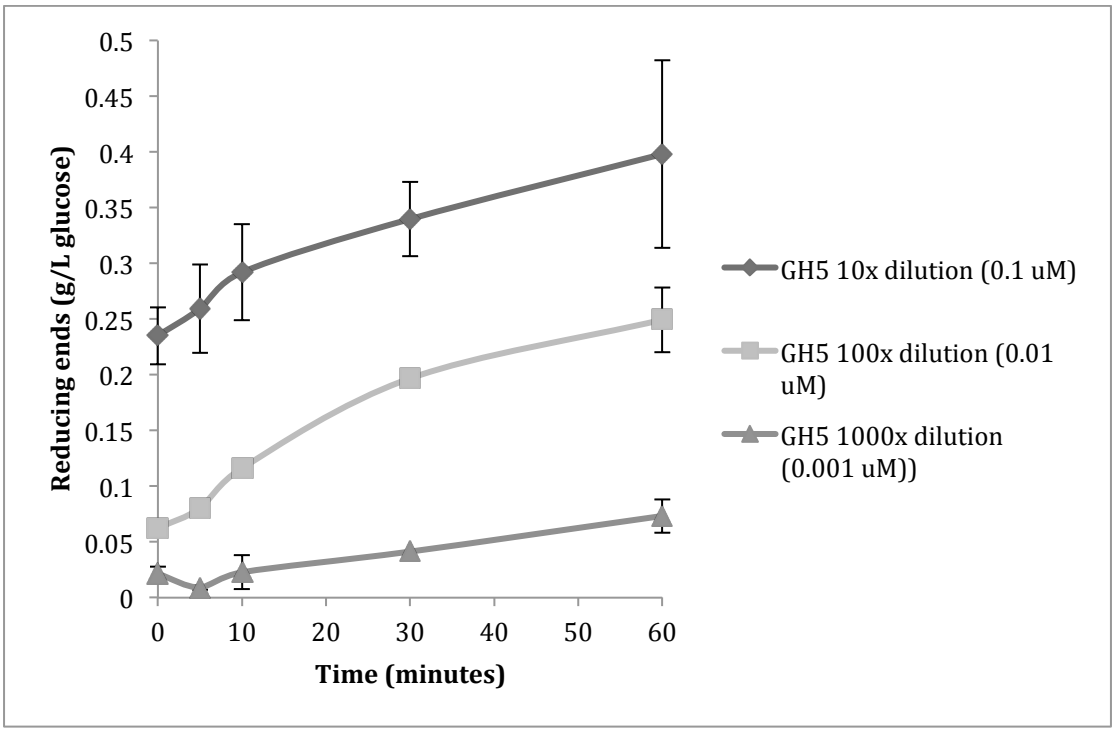

Figure 15. Enzyme activity of GH5. Glucose release with 1-100 nM GH5 on 0.5 g/L laminarin during 60 minutes incubation at 50°C in 50 mM NaAc pH 6.5. Each data point represents the average of independent triplicates ± standard deviation as measured by the DNS assay with reducing ends, using glucose standards.

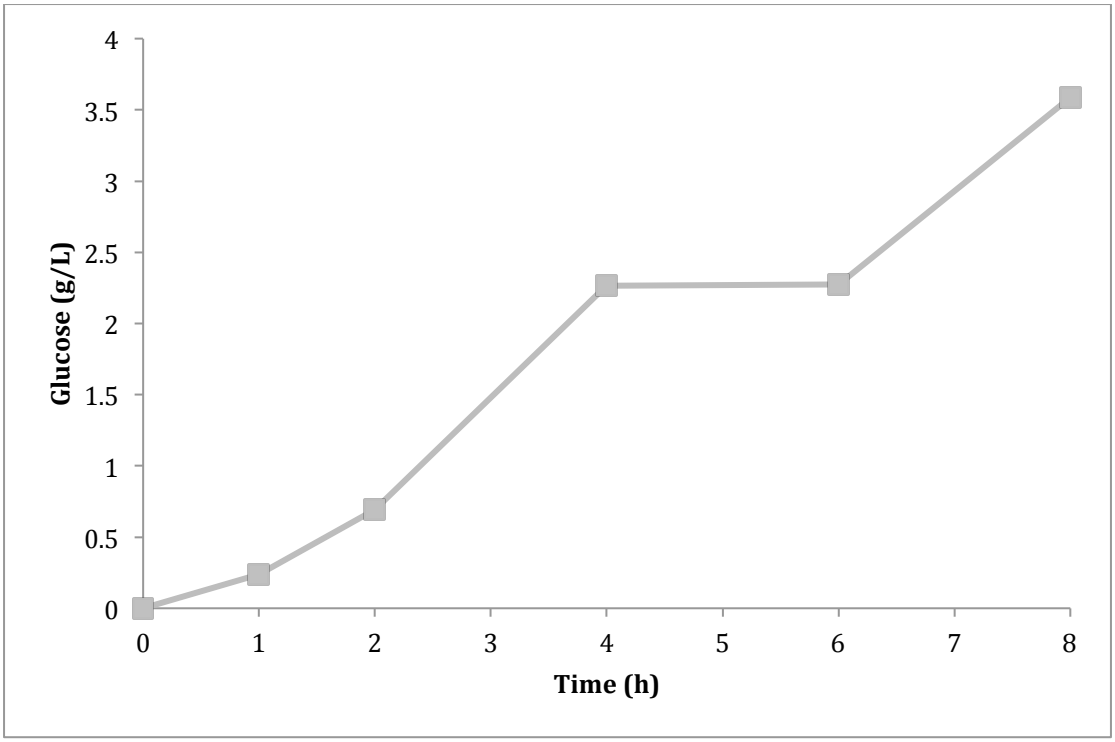

Figure 16. Enzyme activity of GH16 and GH5 combined over 8 hours. Glucose release with 20 nM GH5 + GH16 on 5 g/L laminarin during incubation at 50°C in 50 mM SoAc pH 6.5. Each data point represents the individual glucose value as measured by HPLC with glucose standards.

4.3.3 Cellulases

An initial selection of three endoglucanases, GH6.1, GH6.4 and GH48.3, were tested on 15% DM (w/v) *S. latissima* in 50 mM NaAc pH 6.5 at 50°C for 45 hours. The enzymes were tested alone and combined, with a final enzyme concentration of 20 nM. GH6.4 and GH48.3 were tested in combination as these both are cellobiohydrolases (CBHs) that cleave from the non-reducing and reducing end of the cellulose chain, respectively.

Both GH6.4 and GH6.1 efficiently hydrolyzed seaweed into reducing sugars, also proving that the seaweed contains cellulose. GH6.4 performed best with a concentration of 20.31 g/L reducing ends as measured by DNS assay after 45 hours, and GH6.1 released17.83 g/L sugars (Figure 17). GH48.3 was not as efficient in releasing sugar alone, with only 11.75 g/L reducing sugars after 45 hours, but performed better in combination with GH6.4 (Figure 17). Surprisingly, combining GH48.3 (CBHI) and GH6.4 (CBHII) was not the most effective

treatment on ground seaweed, with 15.91 g/L sugars. As GH6.4 is highly efficient alone, the lower sugar release might be due to microbial contamination of the GH48.3 enzyme stock. These two enzymes were initially combined for saccharification experiments on seaweed, due to their CBH activity on both ends of the chain, but only GH6.4 was used in later synergy experiments.

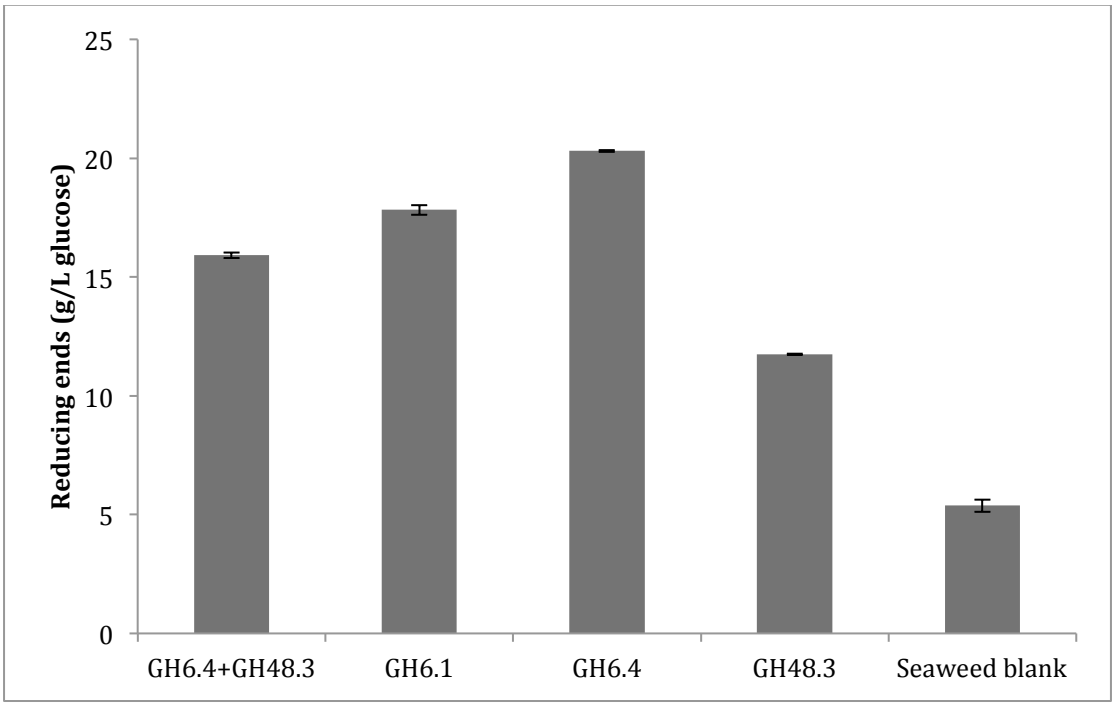

Figure 17. Glucose release from cellulose in *S. latissima*. The graph shows glucose release measured as reducing sugars in g/L by DNS assay after treatment with different cellulases for 45 hours at 50°C. GH6.1, GH6.4 and GH48.3 were tested at a final concentration of 20 nM each in 50 mM NaAc pH 6.5 alone and combined for their endoglucanase activity. Each datapoint represents the average of independent duplicates ± standard deviation.

4.3.4 Enzyme cocktail on *S. latissima*

The commercial cellulase preparation Cellic® CTec2 was used as a positive control to compare with the enzyme cocktails developed in this study. Initial reactions included a total enzyme load of 6 mg/g DM with endoglucanases GH6.4 and GH48.3, which were believed to be the best combination before all cellulases were compared, β-glucosidase therm3, endo-laminarinase GH16 and endo-lyase AMOR_PL7A (Figure 18). GH48.3 was omitted later when performing synergy experiments, as it proved to be less efficient than GH6.1 and GH6.4 (Section

4.3.3.). The cocktail as mentioned above was compared to reactions including Cellic® CTec2 and AMOR_PL7A, alone and combined.

Combined Cellic® CTec2 and AMOR_PL7A produced almost three times as much glucose equivalents (26.23 g/L), as the in-house designed cocktail with (9.91 g/L; Figure 18). AMOR_PL7A appears to facilitate an increase in glucose release, both in combination with Cellic® CTec2 and the in-house cocktail. The yield increased from 23.04 g/L to 26.23 g/L in the former, and from 7.10 g/L to 9.91 g/L when adding AMOR_PL7A to the latter (Figure 18). Alginate degradation by AMOR_PL7A probably makes the cellulose and laminarin more accessible for the other enzymes.

Furthermore, a time series of the enzyme combinations were conducted over 48 hours (Figure 19). The enzyme blends were supplemented with exo-laminarinase GH5 and exo-lyase AMOR_PL17A for more complete degradation of laminarin and alginate to monomeric sugars. Cellulases were added from the start and after 24 hours. The buffer used was 50 mM NaAc pH 6, and all enzymes were added in final individual concentrations of 20 nM (0.071 mg/g DM), apart from Cellic® CTec2 which was added to a concentration of 0.045 mg protein per g DM. As this commercial enzyme cocktail cannot be converted to molar concentration due to unknown molar weight of the contents, the loading of Cellic® CTec2 compared to the in-house cocktail will be different. All concentrations should ideally have been measured as mg/g DM from the start when comparing to Cellic® CTec2.
The highest sugar release for the in-house cocktail was 11.29 g/L reducing sugars after 48 h (Figure 19). Evidently, adding either the in-house cellulases (GH6.4, GH48.3 and therm3) or the commercial cellulase cocktail Cellic® CTec2 from the start resulted in a slightly higher release of monomeric sugars than when adding them after 24 hours (Figure 19).
Only laminarinases (GH16+GH5) and alginate lyases (AMOR_PL7A+AMOR_PL17A) released 10.38 g/L reducing ends as measured by DNS after 48 hours, which was better than Cellic® CTec2 alone (which released 8.57 g/L after 48 hours).

Reactions containing Cellic® CTec2 instead of in-house cellulases still performed better, with the highest yield of 13.22 g/L after 48 consecutive hours and 11.98 g/L when adding Cellic® CTec2 after 24 hours, but the performance of the in-house cocktail was significantly higher than in the previous experiment (Figure 18). The effect of exo-enzymes is clearly shown, as exo-lyase AMOR_PL17A and exo-laminarinase GH5 were not included in the 24 hour-experiment (Figure 18) but added in the time series.

As the seaweed used in this study contains 23.0% monomeric glucose (from laminarin and cellulose) (9), the maximum theoretical sugar release with a dry matter of 15% w/v is 34.5 g/L glucose if everything is hydrolyzed.

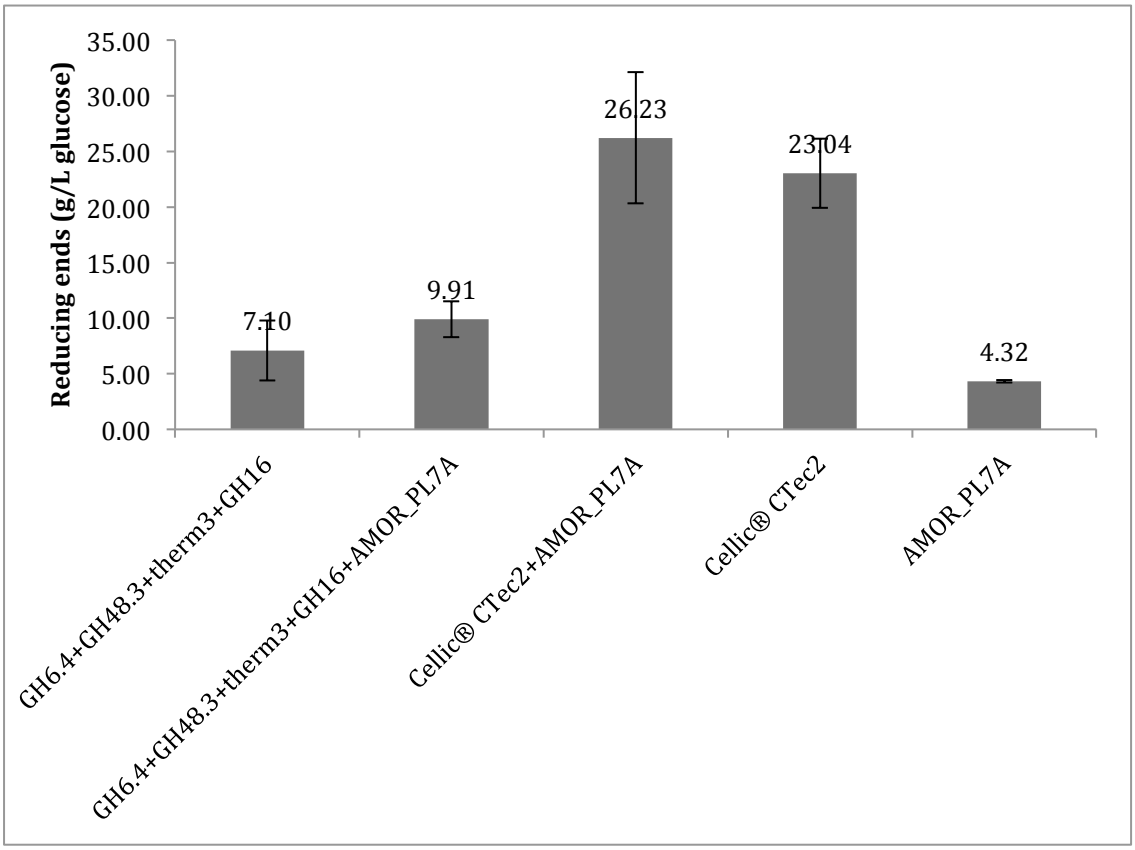

Figure 18. Enzymatic saccharification of seaweed. Combinations of in-house enzymes GH6.4, GH 48.3, therm3, GH16 and AMOR_PL7A compared with conventional cellulase cocktail Cellic® CTec2 on 15% DM (w/v) *S. latissima*. Reactions were incubated at 50°C for 24 hours in seawater pH 7.1, 430 mM NaCl, and endpoints analysed for monomeric sugars with a reducing end assay (DNS). Each datapoint represents the average of independent triplicates ± standard deviation.

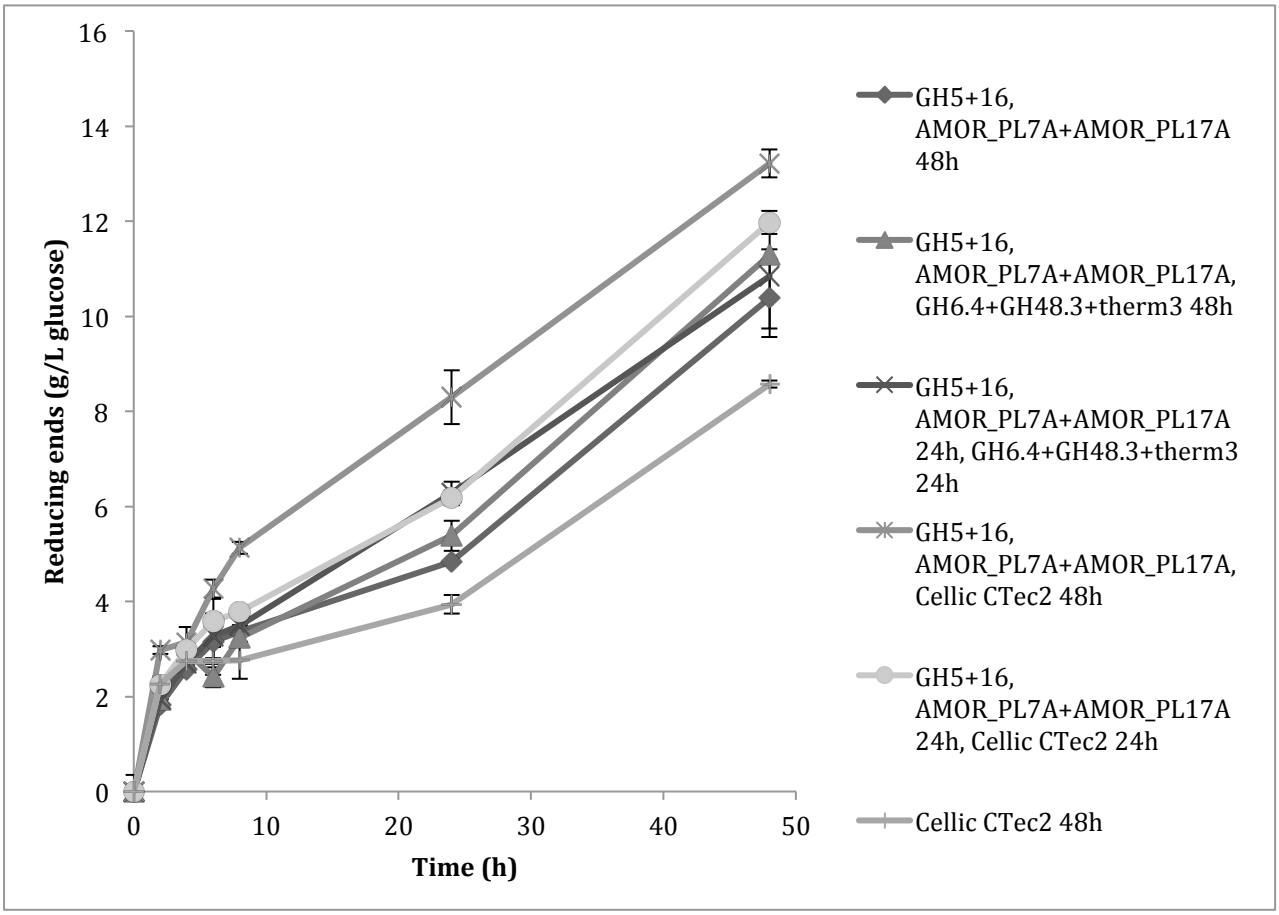

Figure 19. Enzymatic saccharification of seaweed over 48 hours. Combinations of in-house enzymes GH6.4, GH 48.3, therm3, GH16, GH5, AMOR_PL7A and AMOR_PL17A compared with conventional cellulase cocktail Cellic® CTec2 on 15% DM (w/v) *S. latissima*. Reactions were incubated at 50°C in 50 mM NaAc pH 6, and samples analysed from 0-48 hours with DNS for reducing sugar yield. Each datapoint represents the average of independent triplicates ± standard deviation.

Enzyme loading in the initial cocktail was set to 6 mg/g DM, modelled after an earlier experiment which found that a concentration of 7 mg protein per gram of dry matter *S. latissima* was best (9). According to this study, glucose release was rather similar with enzyme loadings in the range 6-10 mg/g DM, which is why the loadings in this study were started in the lower range, but the in-house cocktail varied a lot from the conventional alginate lyase (Sigma) and Cellic® CTec2 used before. The closest comparison to the current enzyme cocktail is a study with AMOR_PL7A tested in combination with Cellic® CTec2 on ground

seaweed. Here, the alginate lyase activity was still high when lowering the loading from 0.7 to 0.0175 mg/g DM (10).
With previous studies in mind, final enzyme concentrations of 25.4 nM AMOR_PL7A and 1 µM AMOR_PL17A were used in reactions on sodium alginate. The concentration was adjusted to 20 nM when including all enzymes, which should still be sufficient for AMOR_PL7A but might be a too small loading for AMOR_PL17A. Regarding cellulases and laminarinases, the enzymes in this study have never been tried in combination on seaweed before. The enzyme loadings were mostly modelled after initial reactions with laminarinases and cellulases on laminarin and ground seaweed, respectively. Also, to spot differences between enzyme blends the enzyme concentrations should not be too high as this may mask differences. Seeing that the sugar yield, although notable, was far from maximum yield, enzyme loadings were increased to 200 nM each in the following synergy experiment (see Section 4.3.5). Further studies should focus on optimizing the enzyme load especially with cellulases and laminarinases for complete saccharification.

Cellulases were tested directly on seaweed to check their activity and get an idea of how to best combine them. This was also done to verify that seaweed contains considerable amounts of cellulose. In order to reduce the amount of enzymes used in the cocktail and considering the lower yield by GH6.4 and GH48.3 combined, only one endoglucanase (GH6.4) was chosen towards the later experiments and paired with β-glucosidase therm3. The activity of cellulases on seaweed is enhanced in combination with alginate lyase, which has been shown in previous experiments with combined lyase and Cellic® CTec2 (10,85). The lyase reduces viscosity by breaking down the alginate network, making the cellulosic part more accessible for cellulolytic enzymes.
Early combinations of enzymes with AMOR_PL7A as the sole alginate lyase suggest that the lyase facilitates glucose release which most likely comes from the cellulosic part (Section 4.3.4). Cellic® CTec2 or the corresponding in-house enzyme cocktail releases glucose that probably originates from both cellulose and laminarin hydrolysis.

Adding all enzymes at the start of the reaction appeared to be beneficial for the sugar release, as opposed to letting alginate lyases and laminarinases break down the alginate and laminarin parts of seaweed for the first 24 hours. However, the difference was not profound, and the results are therefore not conclusive in this regard.

4.3.5 Synergy experiments

For a minimal in-house enzyme cocktail, it is desirable with only a few, essential enzymes due to high costs of enzyme production. Therefore, an enzyme combination experiment was conducted with seven different reactions, A-G, to investigate if any of the enzymes could be omitted from the cocktail and if synergistic effects could be detected.
Reaction A contained all the essential groups of enzymes: endo-lyase AMOR_PL7A, exo-lyase AMOR_PL17A, endo-laminarinase GH16, exo-laminarinase GH5, β-glucosidase therm3 and endoglucanase GH6.4, added to a final concentration of 200 nM each to 15% DM (w/v) ground *S. latissima*. In reactions B-G, each enzyme was systematically eliminated (Table 12).

Table 12. Setup of enzyme blend reactions for enzymatic saccharification. Seven different reactions A-G were set up in parallel to investigate the effect of removing individual enzymes. All reactions were performed with 15% DM (w/v) ground seaweed for 48 hours at 50°C in 50 mM NaAc pH 6, 100 mM NaCl, with a final enzyme concentration of 200 nM for each enzyme.

Enzyme mixture	**A**	**B**	**C**	**D**	**E**	**F**	**G**
AMOR_PL7A endo-lyase	X		X	X	X	X	X
AMOR_PL17A exo-lyase	X	X		X	X	X	X
GH16 endo-laminarinase	X	X	X		X	X	X
GH5 exo-laminarinase	X	X	X	X		X	X
therm3 beta-glucosidase	X	X	X	X	X		X
GH6.4 endo-glucanase	X	X	X	X	X	X	

Samples were taken at timepoints 0, 24 and 48 hours and analyzed with HPLC using a glucose standard, as well as with DNS using both glucose and guluronic acid standards for comparison. After 48 hours, reactions B and C had the highest glucose release, with 9.91 and 10.15 g/L, respectively, as measured by HPLC (Figure 20). Reaction A, containing a complete set of enzymes, released 8.18 g/L glucose. Notably, reactions E, F and G had significantly lower glucose yield with only 4.78 g/L, 4.63 and 6.77 g/L glucose, respectively. E and F were the reactions without exolytic laminarinase and β-glucosidase, respectively, for cleaving laminarin and cellulose down to glucose. Thus, these reactions probably accumulate oligosaccharides, and the exolytic laminarinase and β-glucosidase are essential for the very fast initial release (time 0) of glucose seen in most reactions. Reaction G lacked the endo-cellulase, showing that this enzyme also is important to yield high glucose release. An interesting observation is that little glucose is released between 0 h and 24 h in most reactions, while considerable amounts are released between 24 h and 48 h. This could indicate that some time is needed for degradation of alginate before more glucose can be released from initially inaccessible cellulose and laminarin. Testing the release after 72 hours would also be of interest.

DNS results showed considerably higher sugar concentrations than measured glucose concentrations, suggesting that there are other reducing sugars than glucose present, presumably from alginate degradation (Figures 21 and 22). Different oligosaccharides will also contribute to the DNS signal. When guluronic acid (GA) was used for calibration of the DNS data lower concentrations were measured (Figure 21 and 22). In terms of reducing ends measurement, reaction C again had the highest final concentration of sugars with 21.06 g/L glucose equivalents, but not visibly different from reaction A and G with 20.65 g/L and 20.41 g/L, respectively (Figure 21). The relatively low levels of reducing ends in reaction B (15.19 g/L) (which had a high glucose release; Figure 20) indicates lower degradation of alginate in this reaction. This can explained with the lack of the endo alginate lyase (Table 4.3.1). This is in contrast to reactions E and F. These probably contained a lot of glucose-containing oligosaccharides, as

glucose concentrations were low while reducing end concentrations were 15.24 g/L and 17.69 g/L, respectively.

When using guluronic acid as a standard, the highest reducing ends concentrations were measured in reaction A and C with 17.89 g/L and 17.83 g/L, respectively (Figure 22). These were the reactions containing all enzymes (A) or where the exo-alginate lyase was lacking (C). This shows the importance of combined endolytic and exolytic lyase activity in degrading alginate down to uronic acids, as reaction B without endolyase omitted had a about 3 g/L lower sugar release.

Using DNS as an analytical method for seaweed is inaccurate due to it recording only the reducing ends being formed during degradation, instead of the exact glucose and uronic acid concentration. Comparing DNS with glucose and GA standards in this experiment shows that the signal is slightly different and it needs to be taken into consideration when recording reducing ends from monomeric M and G and glucose.

As monomeric G and M in alginate is difficult to measure with the current HPLC method, partly because these monomers are unstable, uronic acid yield from alginate lyases is most easily detected as reducing ends with DNS. When all enzymes are combined on seaweed, instead of only alginate lyases on sodium alginate, reducing ends signal from the entire substrate will be present, as Figure 22 illustrates.

In this study, HPLC was used as the preferable analytical method for measuring glucose yield as it is accurate. However, the DNS results are also useful as they show all reducing sugars (Figure 21). This may indicate alginate degradation and the amount of substrate that remains as oligosaccharides. In contrast to glucose release, which was low between 0 and 24 h (Figure 20), the release of reducing ends in this period was much higher (except reaction B lacking endolytic alginate lyase). This probably reflects alginate degradation in this period.

The main focus of this study was to combine enzymes that would degrade all the carbohydrate components of brown seaweed. With this in mind, the effect of the combined enzymes proved to be efficient for hydrolysis of both alginate, laminarin and cellulose. The degradation by the in-house cocktail was at best

only 29.42% of maximum expected sugar yield, as measured by HPLC with 10.15 g/L glucose after 48 hours (Figure 20). None of the reactions were incubated for more than 48 hours, but with the current composition of the enzyme cocktail, it could be valuable to test the glucose release after a longer incubation.
In this study, relative low enzyme doses were used to better visualize difference between the enzyme blends. A previous study done with a combination of alginate lyase (Sigma) and Cellic® CTec2 on *S. latissima* tested enzyme loadings from 1-10 mg of protein per g of DM, where the effect of the enzymes decreased below 6 mg protein, but stays almost the same from 6-10 mg (9). This stands in contrast to the findings of this study, where the concentration was 100x lower (0.071 mg/g DM) than what was used in the aforementioned study (7 mg/g DM). This shows that the in-house enzymes are efficient in very small concentrations and removing a single one can have a great effect on glucose release.

Perez et al. (2018) investigated the effect of cellulases and laminarinases on brown seaweed saccharification and found that combining the enzymes had a possible synergistic effect with higher sugar release (90). This is useful to note when constructing a complete minimal enzyme cocktail, as combining the two enzyme groups may lead to more efficient enzymatic saccharification, thus limiting the enzyme production cost and treatment steps.
As expected, the exolaminarinase GH5 and β-glucosidase therm3 are important for release of glucose from oligosaccharides released seaweed (Figure 20). However, the addition of an endoglucanase and an endo-laminarase are important for the degradation of the polysaccharides cellulose and laminarin, respectively. This is in accordance with the previous study where such enzymes were combined on brown seaweed (90).

Interestingly, the release of total sugars (Figures 21 and 22) compared to glucose release (Figure 20) show that the reaction with all enzymes present has one of the highest sugar, but not glucose, yields after 48 h. This is surprising, as one would expect the synergistic reaction of both lyases to best facilitate glucose release by laminarinases and cellulases. Evidently, AMOR_PL7A is important for alginate degradation as the exclusion of this enzyme in reaction B has one of the

lowest sugar yields, which is not as significant when excluding AMOR_PL17A in reaction C. AMOR_PL7A is clearly efficient in alginate degradation and high release of reducing ends. Thus, this enzyme is important for maximizing the production of fermentable sugars from seaweed

This study shows that the cellulase, β-glucosidase, therm3 and exo-laminarinase GH5 have key roles in glucose release. Alginate lyases did not significantly impact final glucose yield, although they might affect the kinetics of the reaction. More studies looking at what happens between 24 and 48 hours are needed to conclude on this.

The activity of β-glucosidases is essential for avoiding product inhibition of endoglucanases and cellobiohydrolases by cellobiose, as it immediately hydrolyses this to glucose (91). A study by Driskill et al. (1999) looked at the synergy between two endoglucanases, degrading 1,3- and 1,4-glycosidic linkages respectively, and one β-glucosidase. They found that hydrolysis was most efficient when combining all three enzymes, as the β-glucosidase stopped the product inhibition that occurred with only endoglucanases together (92). Similar results are observed in this study, where the presence of only endoglucanase GH6.4 for cellulose hydrolysis (Reaction F) is not efficient for glucose release, compared to when endoglucanase, β-glucosidase and exolytic 1,3-glucanase (GH5) are included (Figure 20). Some product inhibition in reaction E and F of the β-1,3- and 1,4- endoglucanases (GH16 and GH6.4) could therefore be possible. To the author's knowledge, a combination of the current types of enzymes have not been tested on brown seaweed before, and further research is needed to support the findings of this study.

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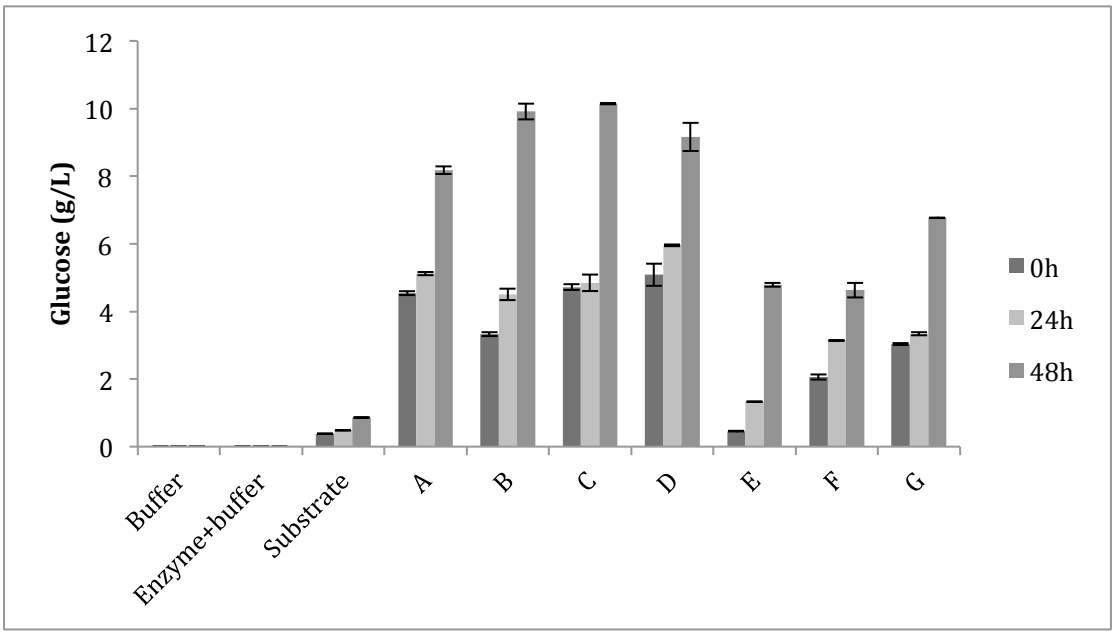

Figure 20. Effect of varying enzyme blends on seaweed saccharification. The graph shows the release of glucose measured by HPLC of reactions A-G, as well as blanks for buffer, enzyme and buffer, and substrate, from 0-48 hours. All reactions were performed at 50°C in 50 mM NaAc pH 6, 100 mM NaCl on 15 % DM (w/v) in a Thermomixer (Eppendorf) at 1000 rpm, with final enzyme concentrations of 200 nM for each enzyme. Each datapoint represents the average of individual replicates ± standard deviation.

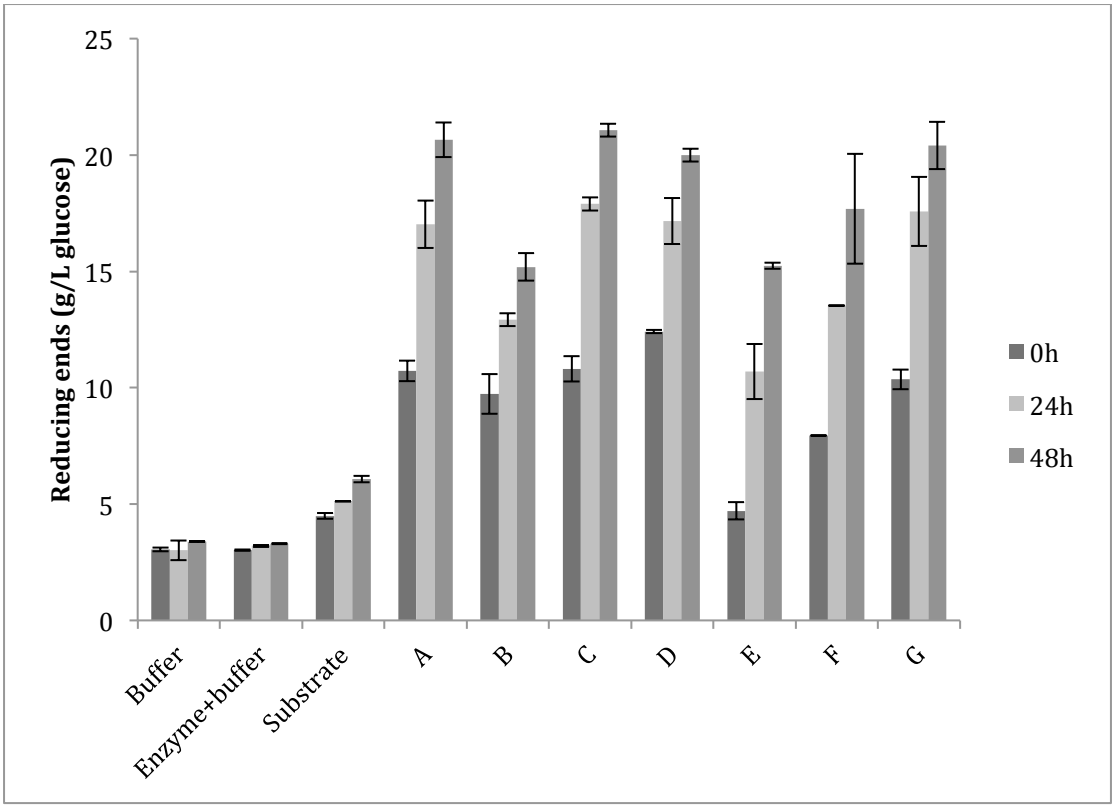

Figure 21. Effect of varying enzyme blends on seaweed saccharification. The graph shows the release of glucose measured as reducing ends using DNS with glucose standards of reactions A-G, as well as blanks for buffer, enzyme and buffer, and substrate, from 0-48 hours. All reactions were performed at 50°C in 50 mM NaAc pH 6, 100 mM NaCl on 15 % DM (w/v) in a Thermomixer (Eppendorf) at 1000 rpm, with final enzyme concentrations of 200 nM for each enzyme. Each datapoint represents the average of individual replicates ± standard deviation.

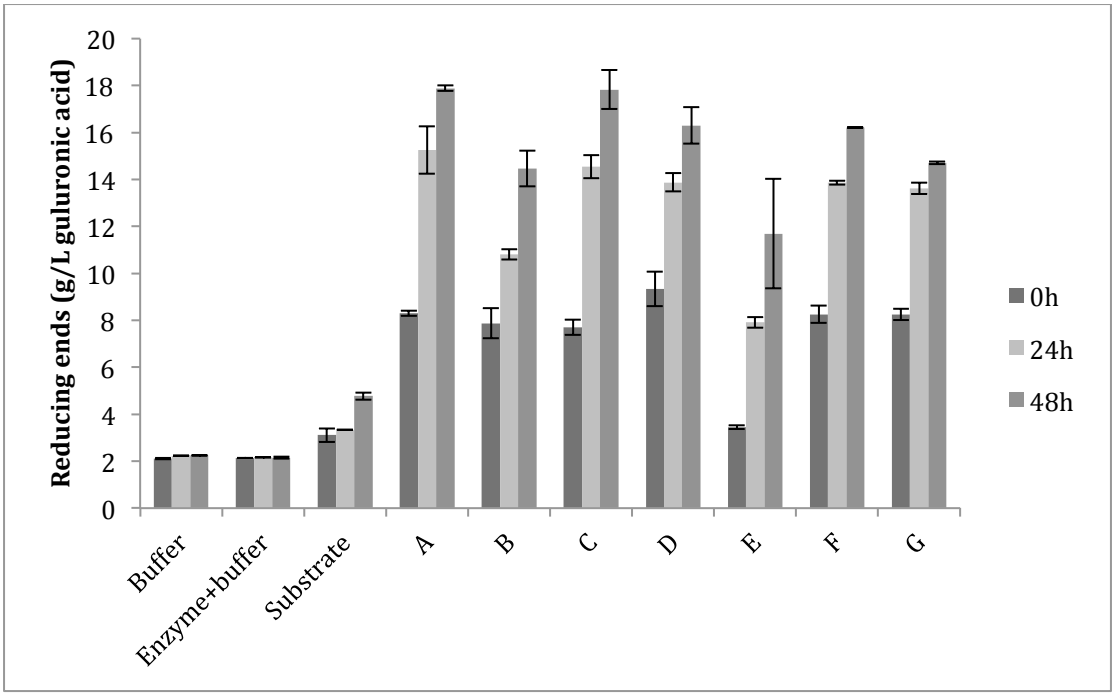

Figure 22. Effect of varying enzyme blends on seaweed saccharification. The graph shows the release of glucose measured by reducing ends using DNS with guluronic acid standards of reactions A-G, as well as blanks for buffer, enzyme and buffer, and substrate, from 0-48 hours. All reactions were performed at 50°C in 50 mM NaAc pH 6, 100 mM NaCl on 15 % DM (w/v) in a Thermomixer (Eppendorf) at 1000 rpm, with final enzyme concentrations of 200 nM for each enzyme. Each datapoint represents the average of individual replicates ± standard deviation.

4.4 The nature of the alginate monomer

Endpoint samples of a 30 hours long reaction with AMOR_PL7A and AMOR_PL17A on sodium alginate were analysed with mass spectrometry using negative mode MALDI-TOF/TOF. The reaction was incubated at 50°C with 80 nM AMOR_PL7A and 800 nM AMOR_PL17A with 1% sodium alginate in 50 mM NaAc pH 7 250 mM NaCl. All samples were stopped by immediately freezing the tubes and defrost them later when performing the MALDI analysis.

Product analysis showed a clear peak at m/z = 175 and m/z = 193 (Figure 23 (A)). Peak 175 indicates the presence of 4-deoxy-L-erythro-hexoseulose uronic acid, DEHU (M-H), whereas peak 193 can indicate the hydrated form of DEHU with mass 175 +18, which corresponds to H_2O. Oligomers of DP2 and DP3

appear to still be present in the reaction mixture after 30 hours, as peak m/z = 351 corresponds to ΔM2, a dimer, and peak m/z = 527 corresponds to ΔM3, a trimer (Figure 23 (B)). AMOR_PL7A is β-mannuronate specific, thus the products are M-chains and not G, It appears that most of the substrate has been hydrolysed by combining endo- and exo-lyase, with only a portion of the shorter oligomers remaining.

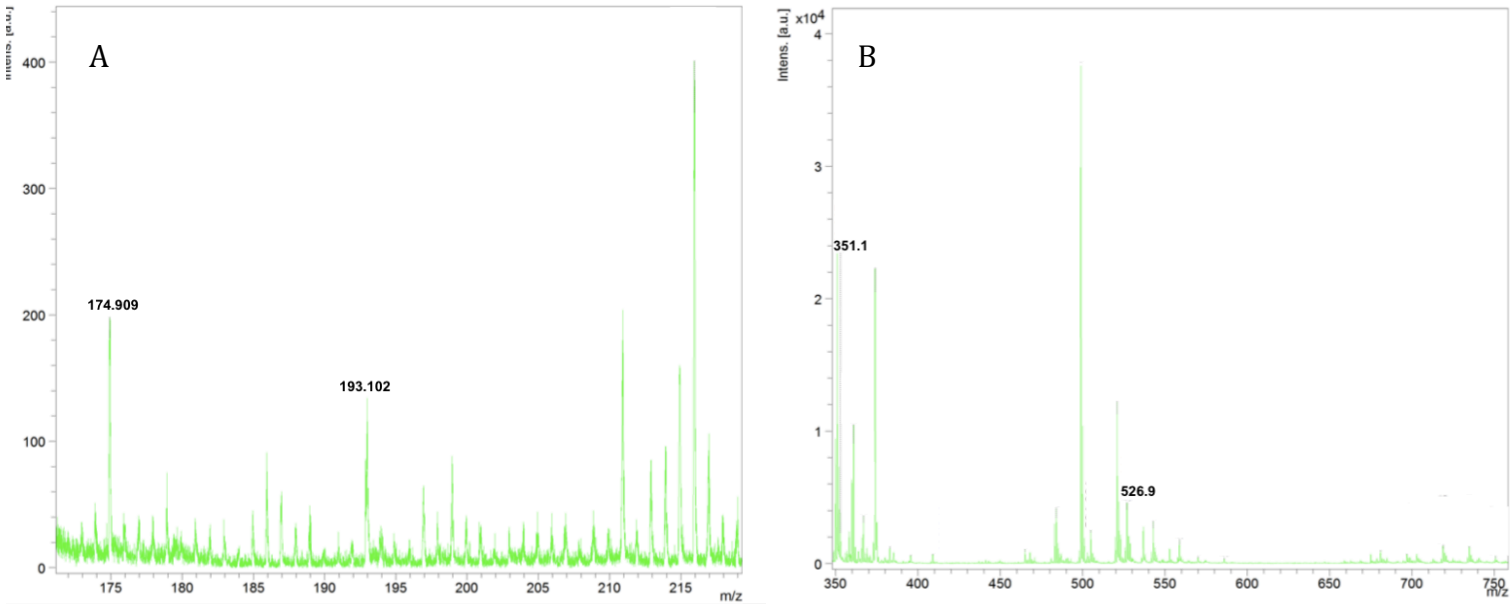

Figure 23. MALDI-TOF/TOF analysis with AMOR_PL7A and AMOR_PL17A on 1% sodium alginate. The figure shows product analysis done by mass spectrometry of reaction results after 30 hours incubation on 50°C. Monomer masses of alginate hydrolysis are shown in (A), whereas oligomer masses of ΔM2 and ΔM3 are shown in (B).

4.5 Monomer analysis with NMR

^{1}H NMR was performed on samples incubated at 55°C for 24 hours with 1 µM AMOR_PL17A and 80 nM AMOR_PL7A on 1% sodium alginate in 25 mM NaAc pH 5.6, 300 mM NaCl. One sample was analyzed immediately after the reaction finished, and the second after leaving it in room temperature for another 24 hours.

NMR spectra of the first sample showed alkene signals around 5.1 ppm (Figure 24 A), which partly disappear after 24 hours (Figure 24 B). This indicates the formation of 4-deoxy-L-*erythro*-hex-4-enopyranuronate (Δ) as the first monomeric product of alginate lyase hydrolysis. A previous study published NMR spectra of Δ (93) also show this signal and further supports the formation of Δ as the initial monomer form.

After 24 hours, the proton spectra showed weak chemical shifts characteristic for aldehydes in the area 8.4-8.6 ppm (Figure 24 B), which could correspond to the presence of 4-deoxy-L-erythro-hexoseulose uronic acid (DEHU). The aldehyde signal is weak and therefore not entirely conclusive, but the observations are supported by MALDI data and the disappearing UV-signal over time for AMOR_PL17A reactions (88). UV-analysis of AMOR_PL7A and AMOR_PL17A was conducted in preliminary work for this study, but not included in this study as DNS was the preferred method of measuring hydrolysis yield. It has been documented that the Δ form is UV-active at λ_{max} = 235 nm (88). This and the fact that this signal disappears considerably over 24 hours, further confirms the likely rearrangement of Δ into DEHU. However, the signals alone are not definite and only suggest that the main product may be Δ and that it spontaneously could be rearranged to DEHU over 24 hours, as illustrated in Figure 25.

The chemical shifts appearing after 24 hours around 1.7-2.3 ppm indicate H-4 equatorial and H-4 axial (Figure 24 B). These products may correspond to cyclic hydrated variants of DEHU, 4-deoxy-5-hydroxy-α-D-glucopyranuronate and 4-deoxy-5-hydroxy-α-D-idopyranuronate. The observations are supported by previous studies, which show that DEHU is predominantly hydrated to form two cyclic hemiacetal stereoisomers (94,95). The signal is in accordance with said earlier studies, but not conclusive based on this NMR data alone.

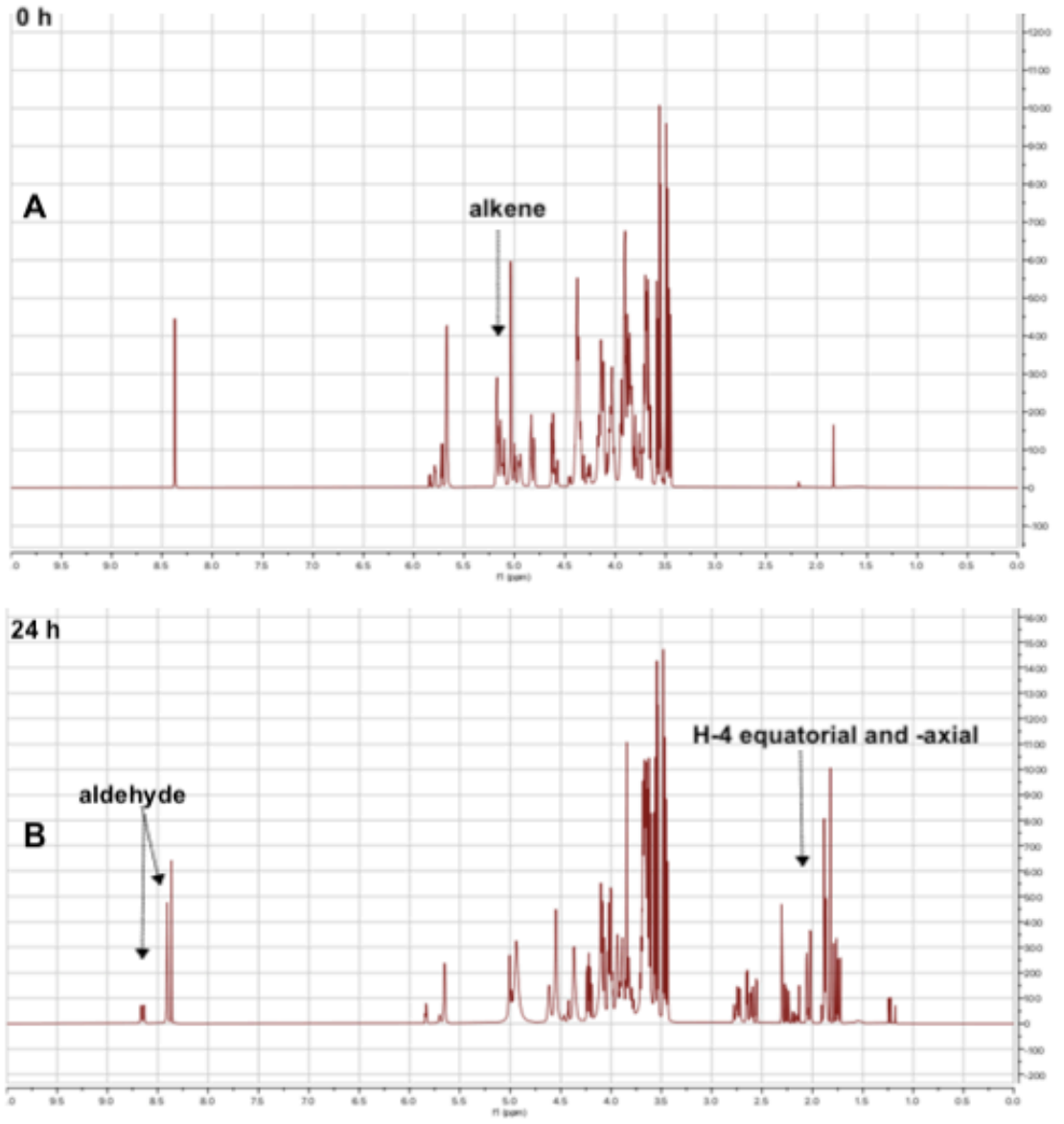

Figure 24. Product analysis of alginate lyase reaction samples with ^{1}H NMR. The figure shows ^{1}H NMR spectra of samples incubated with 1 µM AMOR_PL17A and 80 nM AMOR_PL7A for 24 hours at 55°C immediately after the reaction was stopped with 10% NaOH (final concentration) (A) and after leaving the sample in room temperature for 24 hours (B). Chemical shifts characteristic for alkene, aldehyde and H-4 equatorial and –axial are indicated with arrows.

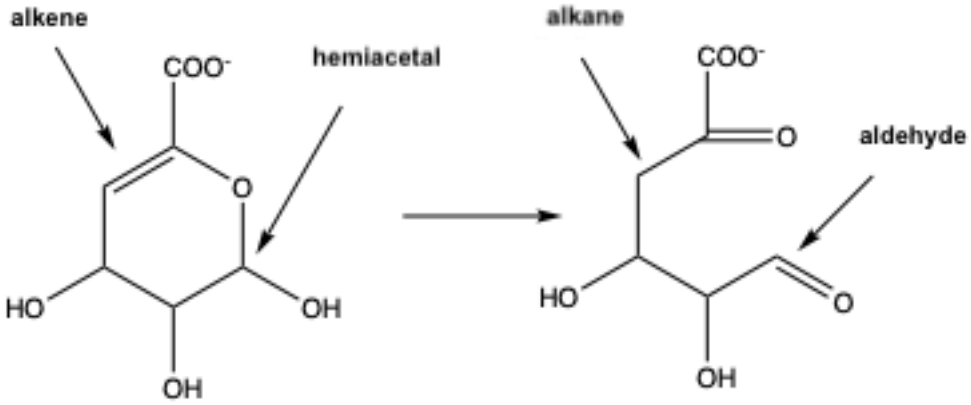

Figure 25. Suggestive rearrangement of Δ to DEHU. The figure shows the possible reaction that could take place after stopping the alginate lyase reaction with NaOH. At the 0h timepoint, NMR signals appear in the alkene area, which suggest the presence of Δ. The unsaturated Δ then rearranges to DEHU with ring opening, forming the aldehyde group which shows a weak NMR signal after 24 hours.

According to MALDI and NMR data, the substrate is degraded to uronic acid monomers, but even with the high enzyme concentrations in reactions for NMR, the substrate is only partly degraded. A recent study by Gimpel et al. (2018) shows that efficient degradation of alginate to monomers can be largely impacted by temperature, enzyme load ratio and the substrate bias towards M-, G- or MG-rich alginate (96). AMOR_PL7A is β-mannuronate specific and does not show significant activity on α-guluronate, but has activity on sodium alginate which contains alternating MG-blocks (10). AMOR_PL17A is active on both M and G, while its highest activity is on sodium alginate (88), which should indicate that the substrate biases would match well together on alginate from this perspective.

M and G content of *S. latissima* in this study, however, is unknown, and might have an impact on the synergy of these two enzymes combined. Further optimization of the lyase combination could include new tests with different enzyme ratios, as these have not been explored in full, and the monomer yield from lyases on ground seaweed should be investigated further.

4.6 Conclusion and future perspective

The seaweed industry in Norway is growing and the biomass harvest is expected to increase over the next years. Brown seaweeds are rich in polysaccharides, and one possible route for valorization is the production of monomeric sugars by saccharification. The sugars may be used in various fermentation processes.

The main aim of this study was to successfully degrade the carbohydrate components of the Norwegian brown seaweed *Saccharina latissima* by constructing a minimal enzyme blend, and to obtain monomeric sugars. These can later be used as substrate for fermentation. Furthermore, another aim was to identify the nature of the alginate monomer. To achieve this, an enzyme cocktail containing six enzymes, five of which were in-house produced, was developed. In addition, the alginate lyases and cellulases used are thermostable enzymes, which is very beneficial on an industrial scale.

There are currently no commercial enzyme cocktails available for complete hydrolysis of seaweed. This study, has contributed to creating such a seaweed degrading enzyme cocktail, which constitutes an alternative to blends of cellulase cocktails such as Cellic® CTec2 and alginate lyases from Sigma that has been used earlier.

This study has shown that this in-house cocktail can degrade seaweed to free monomeric sugars, but a high yield has not been demonstrated yet, partly because relatively low enzyme doses were used. The enzyme cocktail has several potential points of improvement, with the main aim of enhancing the glucose and DEHU yield, both of which are valuable for further work in fermentation studies. The reaction conditions and enzyme loadings for efficient hydrolysis must be optimized, as the experiments done in this study are in their preliminary stage.

This study shows that degradation of alginate is not a key factor for cellulose and laminarin degradation when considering final yield, as glucose is released also without the lyases. However, alginate lyases reduce the viscosity of the reactions and thus probably affects the kinetics of the reactions, and this should be tested in future work. Alginate lyases are essential for the degradation of alginate to

fermentable sugars, and the total sugar yield, as illustrated by DNS data. It is important to note that AMOR_PL7A and AMOR_PL17A can efficiently degrade alginate to monomers, and they are probably positive for the reaction kinetics when releasing glucose as well. This study also verified the nature of the monomer to most likely be 4-deoxy-L-*erythro*-hex-4-enopyranuronate (Δ), which is unstable and transforms spontaneously into DEHU by ring opening over time. Considering the potential for fermentation with DEHU as a substrate, which has been demonstrated previously by Enquist-Newman et al. (2014) (94), it is of interest to improve the cocktail with regards to complete hydrolysis of alginate. Mannitol is also a fermentable sugar found in brown seaweed. Since it can be easily extracted from brown seaweed without the help of enzymes it has not been in focus in this work (9). Finding and using fermentation strains that can ferment all monomeric sugars (glucose, mannitol and uronic acids) is desirable for future work, as it would utilize all of the degraded seaweed carbohydrates. This should include aerobic fermentation processes for e.g. production of microbial biomass (11).

Further work should focus on a more detailed investigation of enzyme synergy, and statistically designed experiments to identify optimal enzyme ratios. In this study, only a few selected in-house enzymes were chosen to target each carbohydrate component in brown seaweed, but especially with regards to cellulases and laminarinases, optimal enzyme choice could be investigated further. Thus, this study can be considered as a proof of concept study demonstrating that a relatively simple enzyme cocktail composed of only 6 enzymes can be used to hydrolyze the polysaccharides in *S. latissima*. Testing the enzyme cocktail on other species of brown seaweed is also needed to see if the blend can be used as a general enzyme cocktail for saccharification of brown seaweeds.

Further work could also focus on the use of crude enzyme preparation, where several enzymes can be produced simultaneously in the same bacteria, and preferable be used directly on the seaweed without elaborate enzyme purification steps. This would simplify enzyme production and save time and costs.

5. References

1. Visbeck M. Ocean science research is key for a sustainable future. Nat Commun. 2018;9(1):1–4.
2. Stévant P, Rebours C, Chapman A. Seaweed aquaculture in Norway: recent industrial developments and future perspectives. Aquac Int. 2017;27:1373–90.
3. Wang X, Olsen LM, Reitan KI, Olsen Y. Discharge of nutrient wastes from salmon farms: environmental effects, and potential for integrated multi-trophic aquaculture. Aquac Environ Interact. 2012;2:267–83.
4. FAO. FAO yearbook. Fisheries and Aquaculture Statistics 2016 [Internet]. Fao. 2016. pp. 104. Available from: http://www.fao.org/3/a-i5716t.pdf
5. Mac Monagail M, Cornish L, Morrison L, Araújo R, Critchley AT. Sustainable harvesting of wild seaweed resources. Eur J Phycol. 2017;52(4):371–90.
6. Horn SJ. Seaweed Biofuels. 1. Saarbrücken: VDM Verlag Dr. Müller Aktiengesellschaft & Co. KG; 2009. 102 p.
7. Kılınç B, Cirik S, Turan G, Tekogul H, Koru E. Seaweeds for Food and Industrial Applications. In: Food Industry. InTech; 2013. p. 735–48.
8. Kraan S. Mass-cultivation of carbohydrate rich macroalgae, a possible solution for sustainable biofuel production. Mitig Adapt Strateg Glob Chang. 2013;18:27–46.
9. Sharma S, Horn SJ. Enzymatic saccharification of brown seaweed for production of fermentable sugars. Bioresour Technol. 2016;213:155–61.
10. Vuoristo KS, Fredriksen L, Oftebro M, Arntzen MØ, Aarstad OA, Stokke R, et al. Production, Characterization, and Application of an Alginate Lyase, AMOR_PL7A, from Hot Vents in the Arctic Mid-Ocean Ridge. J Agric Food Chem. 2019;67(10):2936–45.
11. Sharma S, Hansen LD, Hansen JO, Mydland LT, Horn SJ, Øverland M, et al. Microbial Protein Produced from Brown Seaweed and Spruce Wood as a Feed Ingredient. J Agric Food Chem. 2018;66(31):8328–35.
12. Lobban CS, Wynne MJ. The Biology of Seaweeds. Lobban CS, Wynne MJ, editors. Los Angeles: University of California Press; 1981. 728 p.
13. Schiener P, Black KD, Stanley MS, Green DH. The seasonal variation in the

chemical composition of the kelp species Laminaria digitata, Laminaria hyperborea, Saccharina latissima and Alaria esculenta. J Appl Phycol. 2014;27(1):363–73.

14. Sharma S, Neves L, Funderud J, Mydland LT, Øverland M, Horn SJ. Seasonal and depth variations in the chemical composition of cultivated Saccharina latissima. Algal Res. 2018;32:107–12.
15. Vilg JV, Nylund GM, Werner T, Qvirist L, Mayers JJ, Pavia H, et al. Seasonal and spatial variation in biochemical composition of Saccharina latissima during a potential harvesting season for Western Sweden. Bot Mar. 2015;58(6):435–47.
16. Rioux L, Turgeon SL, Beaulieu M. Characterization of polysaccharides extracted from brown seaweeds. Carbohydr Polym. 2007;69:530–7.
17. Filomena M, Raposo DJ, Maria A, Bernardo M, Manuel R, Costa S. Emergent Sources of Prebiotics : Seaweeds and Microalgae. Mar Drugs. 2016;14:1–27.
18. Lahaye M. Marine algae as sources of fibres: Determination of soluble and insoluble dietary fibre contents in some 'sea vegetables.' J Sci Food Agric. 1991;54:587–94.
19. Deniaud-Bouët E, Kervarec N, Michel G, Tonon T, Kloareg B, Hervé C. Chemical and enzymatic fractionation of cell walls from Fucales: Insights into the structure of the extracellular matrix of brown algae. Ann Bot. 2014;114(6):1203–16.
20. Milledge JJ, Harvey PJ. Potential process ' hurdles ' in the use of macroalgae as feedstock for biofuel production in the British Isles. J Chem Technol Biotechnol. 2016;91(April):2221–34.
21. Milledge JJ, Harvey PJ. Ensilage and anaerobic digestion of Sargassum muticum. J Appl Phycol. 2016;28(January):3021–30.
22. Michel G, Tonon T, Scornet D, Cock JM, Kloareg B. The cell wall polysaccharide metabolism of the brown alga Ectocarpus siliculosus. Insights into the evolution of extracellular matrix polysaccharides in Eukaryotes. New Phytol. 2010;188(1):82–97.
23. Horn SJ, Moen E, Østgaard K. Direct determination of alginate content in brown algae by near infra-red (NIR) spectroscopy. J Appl Phycol.

1999;11(1):9–13.
24. Gacesa P. Enzymic degradation of alginates. Int J Biochem. 1992;24(4):545–52.
25. Rees DA. Shapely Polysaccharides. Biochem J. 1972;126(2):257–73.
26. Gacesa P. Alginates. Carbohydr Polym. 1988;8:161–82.
27. Festucci-Buselli RA, Otoni WC, Joshi CP. Structure, organization, and functions of cellulose synthase complexes in higher plants. Brazilian J Plant Physiol. 2007;19(1):1–13.
28. Baptista A, Ferreira I, Borges J. Cellulose-Based Bioelectronic Devices. In: Ven T v. d., Godbout L, editors. Cellulose - Medical, Pharmaceutical and Electronic Application s. Rijeka: InTech; 2013. p. 67–82.
29. Delmer DP, Amor Y. Cellulose Biosynthesis. Plant Cell. 1995;7(7):987–1000.
30. Atalla RH, Vanderhart DL. Studies on the structure of cellulose using Raman spectroscopy and solid state 13C NMR. Schuerch C, editor. Cellulose and Wood: Chemistry and Technology. New York: Wiley Interscience; 1989. 169–188 p.
31. Chen P, Ogawa Y, Nishiyama Y, Ismail AE, Mazeau K. Iα to Iβ mechano-conversion and amorphization in native cellulose simulated by crystal bending. Cellulose. 2018;25(8):4345–55.
32. Šturcova A, His I, Apperley DC, Sugiyama J, Jarvis MC. Structural details of crystalline cellulose from higher plants. Biomacromolecules. 2004;5(4):1333–9.
33. Brown RM, Saxena IMJ, Kudlicka K. Cellulose biosynthesis in higher plants. Trends Plant Sci. 1996;1(5):149–56.
34. Groisillier A, Shao Z, Michel G, Goulitquer S, Bonin P, Krahulec S, et al. Mannitol metabolism in brown algae involves a new phosphatase family. J Exp Bot. 2014;65(2):559–70.
35. Vas'kovskii VE, Isai S V. Determination of the amount of mannitol in brown seaweeds. Chem Nat Compd. 1972;8(5):566–9.
36. Tenny S, Patel R, Thorell W. Mannitol. In: StatPearls. Treasure Island (FL): StatPearls Publishing; 2020.
37. Iwamoto K, Shiraiwa Y. Salt-regulated mannitol metabolism in algae. Mar

Biotechnol. 2005;7(5):407–15.

38. Rioux LE, Turgeon SL, Beaulieu M. Structural characterization of laminaran and galactofucan extracted from the brown seaweed Saccharina longicruris. Phytochemistry. 2010;71(13):1586–95.
39. Read SM, Currie G, Bacic A. Analysis of the structuralheterogeneity of laminarin by electrospray-ionisation-mass spectrometry. Carbohydr Res. 1996;281(2):187–201.
40. Nelson TE, Lewis BA. Separation and characterization of the soluble and insoluble components of insoluble laminaran. Carbohydr Res. 1974;33(1):63–74.
41. Fedorov SN, Ermakova SP, Zvyagintseva TN, Stonik VA. Anticancer and cancer preventive properties of marine polysaccharides: Some results and prospects. Mar Drugs. 2013;11:4876–901.
42. Graiff A, Ruth W, Kragl U, Karsten U. Chemical characterization and quantification of the brown algal storage compound laminarin — A new methodological approach. J Appl Phycol. 2016;28(1):533–43.
43. Rioux LE, Turgeon SL, Beaulieu M. Effect of season on the composition of bioactive polysaccharides from the brown seaweed Saccharina longicruris. Phytochemistry. 2009;70(8):1069–75.
44. Garcia-Vaquero M, Rajauria G, O'Doherty J V., Sweeney T. Polysaccharides from macroalgae: Recent advances, innovative technologies and challenges in extraction and purification. Food Res Int. 2017;99:1011–20.
45. Hemmingson JA, Falshaw R, Furneaux RH, Thompson K. Structure and antiviral activity of the galactofucan sulfates extracted from Undaria pinnatifida (Phaeophyta). J Appl Phycol. 2006;18(2):185–93.
46. Rocha HAO, Moraes FA, Trindade ES, Franco CRC, Torquato RJS, Veiga SS, et al. Structural and hemostatic activities of a sulfated galactofucan from the brown alga Spagtoglossum schroederi: An ideal antithrombotic agent? J Biol Chem. 2005;280(50):41278–88.
47. Kusaykin M, Bakunina I, Sovo V, Ermakova S, Kuznetsova T, Besednova N, et al. Structure, biological activity, and enzymatic transformation of fucoidans from the brown seaweeds. Biotechnol J. 2008;3(7):904–15.
48. Khalafu SHS, Wan Aida WM, Lim SJ, Maskat MY. Effects of deodorisation

methods on volatile compounds, chemical properties and antioxidant activities of fucoidan isolated from brown seaweed (Sargassum sp.). Algal Res. 2017;25(June):507–15.

49. Senthilkumar K, Manivasagan P, Venkatesan J, Kim SK. Brown seaweed fucoidan: Biological activity and apoptosis, growth signaling mechanism in cancer. Int J Biol Macromol. 2013;60:366–74.
50. Cumashi A, Ushakova NA, Preobrazhenskaya ME, D'Incecco A, Piccoli A, Totani L, et al. A comparative study of the anti-inflammatory, anticoagulant, antiangiogenic, and antiadhesive activities of nine different fucoidans from brown seaweeds. Glycobiology. 2007;17(5):541–52.
51. Adamo R, Tontini M, Brogioni G, Romano MR, Costantini G, Danieli E, et al. Synthesis of laminarin fragments and evaluation of a β-(1,3) glucan hexasaccaride-CRM 197 conjugate as vaccine candidate against Candida albicans. J Carbohydr Chem. 2011;30(4–6):249–80.
52. Taylor G. Biofuels and the biorefinery concept. Energy Policy. 2008;36(12):4406–9.
53. Abraham RE, Su P, Puri M, Raston CL, Zhang W. Optimisation of biorefinery production of alginate, fucoidan and laminarin from brown seaweed Durvillaea potatorum. Algal Res. 2019;38.
54. Philippsen A, Wild P, Rowe A. Energy input, carbon intensity and cost for ethanol produced from farmed seaweed. Renew Sustain Energy Rev [Internet]. 2014;38:609–23. Available from: http://dx.doi.org/10.1016/j.rser.2014.06.010
55. Skjermo J, Aasen IM, Arff J, Broch OJ, Carvajal A, Christie H, et al. A new Norwegian bioeconomy based on cultivation and processing of seaweeds: Opportunities and R&D needs. Trondheim; 2014.
56. Cabrita ARJ, Maia MRG, Sousa-pinto I, Fonseca AJM. Ensilage of seaweeds from an integrated multi-trophic aquaculture system. Algal Res. 2017;24(May):290–8.
57. Ravanal MC, Sharma S, Gimpel J, Reveco-Urzua FE, Øverland M, Horn SJ, et al. The role of alginate lyases in the enzymatic saccharification of brown macroalgae, Macrocystis pyrifera and Saccharina latissima. Algal Res. 2017 Sep 1;26:287–93.

58. Lee JY, Kim YS, Um BH, Oh KK. Pretreatment of Laminaria japonica for bioethanol production with extremely low acid concentration. Renew Energy. 2013;54:196–200.
59. Maneein S, Milledge JJ, Nielsen B V, Harvey PJ. A Review of Seaweed Pre-Treatment Methods for Enhanced Biofuel Production by Anaerobic Digestion or Fermentation. Fermentation. 2018;4(100):1–31.
60. Schultz-Jensen N, Thygesen A, Leipold F, Tjalfe S, Roslander C, Lilholt H, et al. Pretreatment of the macroalgae Chaetomorpha linum for the production of bioethanol – Comparison of five pretreatment technologies. Bioresour Technol. 2013;140:36–42.
61. Adams JMM, Schmidt A, Gallagher JA. The impact of sample preparation of the macroalgae Laminaria digitata on the production of the biofuels bioethanol and biomethane. J Appl Phycol. 2015;27:985–91.
62. Vanegas C, Hernon A, Bartlett J. Influence of Chemical, Mechanical, and Thermal Pretreatment on the Release of Macromolecules from Two Irish Seaweed Species. Sep Sci Technol. 2014;49:30–8.
63. Karray R, Hamza M, Sayadi S. Evaluation of ultrasonic, acid , thermo-alkaline and enzymatic pre-treatments on anaerobic digestion of Ulva rigida for biogas production. Bioresour Technol. 2015;187:205–13.
64. Schiener P, Stanley MS, Black KD, Green DH. Assessment of saccharification and fermentation of brown seaweeds to identify the seasonal effect on bioethanol production. J Appl Phycol. 2016;28:3009–20.
65. Zhu B, Yin H. Alginate lyase: Review of major sources and classification, properties, structure-function analysis and applications. Bioengineered. 2015;6(3):125–31.
66. Kobayashi T, Uchimura K, Miyazaki M, Nogi Y, Horikoshi K. A new high-alkaline alginate lyase from a deep-sea bacterium Agarivorans sp . Extremophiles. 2009;13:121–9.
67. Ertesvåg H. Alginate-modifying enzymes : biological roles and biotechnological uses. Front Microbiol. 2015;6(May):1–10.
68. Kim HS, Lee CG, Lee EY. Alginate lyase: Structure, property, and application. Biotechnol Bioprocess Eng. 2011;16:843–51.
69. Gacesa P. Alginate-modifying enzymes: A proposed unified mechanism of

action for the lyases and epimerases. FEBS Lett. 1987;212(2):199–202.
70. Payne CM, Knott BC, Mayes HB, Hansson H, Himmel ME, Sandgren M, et al. Fungal Cellulases. Chem Rev. 2015;115:1308–448.
71. Wilson DB. Three Microbial Strategies for Plant Cell Wall. Ann N Y Acad Sci. 2008;1125:289–97.
72. Wilson DB. Microbial diversity of cellulose hydrolysis. Curr Opin Microbiol. 2011;14:1–5.
73. Watanabe H, Tokuda G. Animal cellulases. Cell Mol Life Sci. 2001;58:1167–78.
74. Vaaje-Kolstad G, Westereng B, Horn SJ, Liu Z, Zhai H. An Oxidative Enzyme Boosting the Enzymatic Conversion of Recalcitrant Polysaccharides. Science (80-). 2010;330(October):219–23.
75. Phillips CM, Beeson WT, Cate JH, Marletta MA. Cellobiose Dehydrogenase and a Copper-Dependent Polysaccharide Monooxygenase Potentiate Cellulose Degradation by Neurospora crassa. ACS Chem Biol. 2011;6:1399–406.
76. Bertini L, Lambrughi M, Fantucci P, Gioia L De, Borsari M, Sola M, et al. Catalytic Mechanism of Fungal Lytic Polysaccharide Monooxygenases Investigated by First-Principles Calculations. Inorg Chem. 2018;57:86–97.
77. Andlar M, Rezic T, Mardetko N, Kracher D, Ludwig R, Santek B. Lignocellulose degradation : An overview of fungi and fungal enzymes involved in lignocellulose degradation. Eng Life Sci. 2018;0(June):1–11.
78. AMFB - CNRS - Université d'Aix. Glycoside Hydrolase family classification [Internet]. Carbohydrate-active enzymes. 2019 [cited 2019 Mar 27]. Available from: http://www.cazy.org/Glycoside-Hydrolases.html
79. Teresa M, Bara F, Lima AL, Ulhoa CJ. Purification and characterization of an exo-β-1,3-glucanase produced by Trichoderma asperellum. FEMS Microbiol Lett. 2003;219:81–5.
80. Connell EO, Piggott C, Tuohy M. Purification of exo-1,3-beta-glucanase, a new extracellular glucanolytic enzyme from Talaromyces emersonii. Appl Microbiol Biotechnol. 2011;89:685–96.
81. Gaiser OJ, Piotukh K, Ponnuswamy MN, Planas A, Borriss R, Heinemann U. Structural Basis for the Substrate Specificity of a Bacillus 1,3-1,4-beta-

Glucanase. J Mol Biol. 2006;357(4):1211–25.

82. Reese ET, Parrish FW, Mandels M. β-d-1,6-glucanases in fungi. Can J Microbiol. 1962;8(3):327–34.

83. Lafond M, Navarro D, Haon M, Couturier M, Berrin J. Characterization of a Broad-Specificity -Glucanase Acting on β-(1,3)-, β-(1,4)-, and β-(1,6)-Glucans That Defines a New Glycoside Hydrolase Family. Appl Environ Microbiol. 2012;78(24):8540–6.

84. Fukuda K, Hiraga M, Asakuma S, Arai I, Urashima T. Purification and Characterization of a Novel Exo- β -1 , 3-1 , 6-glucanase from the Fruiting Body of the Edible Mushroom Enoki (Flammulina velutipes). Biosci Biotechnol Biochem. 2008;72(12):3107–13.

85. Manns D, Nyffenegger C, Saake B, Meyer AS. Impact of different alginate lyases on combined cellulase-lyase saccharification of brown seaweed. RSC Adv. 2016;6:45392–401.

86. Miller GL. Use of DinitrosaIicyIic Acid Reagent for Determination of Reducing Sugar. Anal Chem. 1959;31(3):426–8.

87. Manns D, Andersen SK, Saake B, Meyer AS. Brown seaweed processing: enzymatic saccharification of Laminaria digitata requires no pre-treatment. J Appl Phycol. 2016;28:1287–94.

88. Pedersen B. Characterization of a thermostable exolytic alginate lyase from hot vents in the Arctic Mid-Ocean Ridge. Norwegian University of Life Sciences; 2019.

89. Jensen MS, Fredriksen L, MacKenzie AK, Pope PB, Leiros I, Chylenski P, et al. Discovery and characterization of a thermostable two-domain GH6 endoglucanase from a compost metagenome. Berrin J-G, editor. PLoS One. 2018 May 24;13(5):e0197862.

90. Perez CMT, Pajares IG, Alcantara VA, Simbahan JF. Bacterial laminarinase for application in ethanol production from brown algae Sargassum sp. using halotolerant yeast. Biofuel Res J. 2018;792–7.

91. Geronimo I, Ntarima P, Piens K, Gudmundsson M, Hansson H, Sandgren M, et al. Kinetic and molecular dynamics study of inhibition and transglycosylation in Hypocrea jecorina family 3 -glucosidases. J Biol Chem. 2019;294(9):3169–80.

92. Driskill LE, Bauer MW, Kelly RM. Synergistic interactions among β-laminarinase, β-1,4-glucanase, and β-glucosidase from the hyperthermophilic archaeon Pyrococcus furiosus during hydrolysis of β-1,4-, β-1,3-, and mixed-linked polysaccharides. Biotechnol Bioeng. 1999;66(1):51–60.
93. Leone S, Molinaro A, Alfieri F, Cafaro V, Lanzetta R, Donato A Di, et al. The biofilm matrix of Pseudomonas sp. OX1 grown on phenol is mainly constituted by alginate oligosaccharides. Carbohydr Res. 2006;341:2456–61.
94. Enquist-newman M, Faust AME, Bravo DD, Santos CNS, Raisner RM, Hanel A, et al. Efficient ethanol production from brown macroalgae sugars by a synthetic yeast platform. Nature. 2014;505(7482):239–43.
95. Hobbs JK, Lee SM, Robb M, Hof F, Barr C, Abe KT, et al. KdgF, the missing link in the microbial metabolism of uronate sugars from pectin and alginate. Proc Natl Acad Sci U S A. 2016;113(22):6188–93.
96. Gimpel JA, Ravanal MC, Salazar O, Lienqueo ME. Saccharification of brown macroalgae using an arsenal of recombinant alginate lyases: Potential application in the biorefinery process. J Microbiol Biotechnol. 2018;28(10):1671–82.

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