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Expression and production of thermophilic alginate lyases in *Bacillus* and direct application of culture supernatant for seaweed saccharification

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ABSTRACT

Brown seaweeds are rich in carbohydrates and may be used as a source of fermentable sugars. Saccharification of the seaweed biomass can be carried out enzymatically by a combination of cellulases and alginate lyases. In this study, thermotolerant *exo*- and *endo* alginate lyases were cloned and expressed in *Bacillus subtilis*. The lyases were secreted to the culture supernatant and used directly together with a commercial cellulase preparation to saccharify *Saccharina latissima* biomass. The results showed that the strategy of using the culture supernatants directly as a source of alginate lyases worked very well, releasing glucose, mannitol, and uronic acids. The ratio between the *exo*- and *endo*-acting alginate lyases proved to be very important for saccharification yield, and under optimal reaction conditions the use of culture supernatants containing alginate lyases improved final glucose concentration by 73%, when compared to only applying cellulases. This direct use of culture supernatants as a source of alginate lyases shows that enzyme purification steps are not needed, saving seaweed processing costs and points to the possibility of a relatively simple on-site enzyme production for seaweed biorefining.

1. Introduction

Seaweeds (macroalgae) are marine plant-like multicellular organisms that predominantly grow in the ocean. They belong to three different groups, empirically distinguished by the color of their thallus: red (phylum: Rhodophyta), brown (phylum: Phaeophyta), and green (phylum: Chlorophyta) [1]. The chemical composition of seaweeds shows annual changes, varies considerably between species and growth sites, but generally seaweeds consist of varying amounts of different polysaccharides, minerals, proteins, and lipids [2–4]. Today seaweeds are mostly used for food and feed applications, e.g. as direct food consumption or to produce the gelling food-hydrocolloids carrageenan, agar, and alginate, but seaweeds are also used in e.g. cosmetics, in the pharmaceutical industry, and in biotechnological applications [5]. An emerging bio-economy has led to an increased interest in the polysaccharide-rich seaweeds as a sustainable resource for the production of fuels [6,7], bioactive compounds [8,9], feed [10] and food [11]. The use of seaweeds offers several advantages over terrestrial biomass as seaweeds have a high growth rate, can be cultivated directly in saltwater, and thus requires no arable land or industrial fertilization for growth. Compared to lignocellulosic biomass, seaweeds does not contain any lignin and does not require extensive pretreatments prior saccharification to fermentable sugars [12].

Brown seaweeds, such as *Saccharina latissima*, are found in high quantities along the Norwegian coast. Fucoidans and alginate are the main components of the brown seaweed cell walls, constituting up to 45% of dry weight, while cellulose, contrary to land plants, is only present in minor fractions (1–8% of dry weight) [13]. Laminarin and mannitol are storage carbohydrates that accumulate in the seaweed during the high-light season and depending on species and time of year these components can account for up to 25% and 30% of the seaweed dry weight, respectively [4]. Laminarin, a linear polysaccharide of β-1,3 bonded glucose moieties and with β-1,6 branches, and cellulose are easily hydrolyzed to glucose using available commercial enzyme cocktails of cellulolytic enzymes [14]. Mannitol, the sugar alcohol of mannose, can be easily extracted from seaweed and may be fermented to ethanol by some bacteria like *Zymobacter palmae* and the *Escherichia coli* KO11 [15,16]. Alginate, a heteropolymer composed of the two uronic acids β-D-mannuronic (M) and α-L guluronic (G) acid, can be enzymatically degraded by alginate lyases. It is well known that many

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microorganisms can grow aerobically on the alginate degradation product 4-deoxy-L-erythro-5-hexoseulose uronic acids (DEH), which can enter the Entner-Doudoroff pathway [17]. However, anaerobic fermentation of DEH to specific products like ethanol is rarely reported and may require genetically engineered microbes [7,18].

Efficient saccharification processes are needed for using seaweeds as a source of fermentable sugars. This can be done by acid hydrolysis [19], but enzymatic conversion is generally considered more sustainable and avoids the risks of unwanted sugar degradation. Enzymatic saccharification of brown seaweeds has been reported using cellulases, laminarases, and alginate lyases [14,20–25]. The use of alginate lyases in combination with cellulases has been shown to increase glucose yields and also to increase accessibility for enzymes by decreasing viscosity [14,20,24,26]. Alginate lyases catalyze the depolymerization of alginates and are divided into G-block specific and M-block specific enzymes depending on substrate preference, though several of the alginate lyases appear to have activity towards both G- and M-blocks in alginate. Identified alginate lyases belong to the polysaccharide lyase families PL5, PL6, PL7, PL14, PL15, PL17, and PL18, where most bacterial endolytic alginate lyases belong to family PL5 or PL7, while most exolytic alginate lyases are assigned to family PL15 or PL17 [27]. Alginate lyases, including commercially available once, normally have lower temperature optima than commercial cellulase mixtures, meaning that a two-step hydrolysis process at different temperatures may be needed to achieve high saccharification yields [14,20].

Two thermophilic alginate lyases have recently been described; the endolytic AMOR-PL7 [24] and the exolytic AMOR-PL17 [28], where enzymatic brown seaweed saccharification studies with the AMOR-PL7 in combination with a commercial cellulase mixture have been shown to increase glucose yield, most likely due to a reduction in viscosity by degradation of alginate [24]. The majority of characterized alginate lyases, including AMOR-PL7 and AMOR-PL17, have been cloned into the Gram-negative *E. coli* [24,28–34]. *E. coli* is one of the preferred hosts for recombinant protein expression, as it offers a toolbox for genetic engineering, fast growth, and high cell density. However, intracellular protein expression, the lack of post-translational machinery, production of inactive proteins due to formation of inclusion bodies, and requirements for extended downstream protein purification processes are some disadvantages of this host [35]. *Bacillus subtilis*, a well-studied Gram-positive bacterium, has several advantages regarding protein production, one of them being a superior protein secretory capability. Downstream purification of secreted heterologous proteins is relatively easy because the proteins are harvested directly from the culture medium. Direct use of the culture supernatant as a source of enzymes is also possible, thus avoiding the time and equipment used for downstream processing.

In this study, two thermophilic alginate lyases, AMOR-PL7 and AMOR-PL17, were successfully expressed in the *B. subtilis* strain RIK1285. The secreted alginate lyases were used directly as culture supernatants in combination with the commercial cellulase mixture Cellic® CTec2 for enzymatic saccharification of the brown seaweed *S. latissima* at 55 °C. This proof-of-concept study demonstrates that alginate lyases can be successfully produced in a *Bacillus* expression system and used directly as culture supernatants for saccharification of brown seaweed biomass without the need of protein purification. This points to the possibility of relatively simple on-site production of alginate lyases for use in seaweed biorefining processes.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli TOP10 (Thermo Fisher Scientific Inc., Waltham, MA) was grown in brain heart infusion (BHI) broth (Oxoid Ltd., Basingstoke, UK) at 37 °C with shaking. *Bacillus subtilis* RIK1285 (Takara BIO INC. Mountain View, CA) was grown in lysogeny broth (LB) at 37 °C with shaking. Solid media were prepared by adding 1.5% (w/v) agar to the broth. When appropriate, antibiotics were added as follows: 100 μg/mL ampicillin and 50 μg/mL kanamycin for *E. coli* and 10 μg/mL kanamycin for *B. subtilis*.

2.2. Cloning strategy and plasmid construction

Both alginate lyases, AMOR-PL7 (GenBank accession number MH727998) and AMOR-PL17 (GenBank accession number MT444120) without signal peptides, were cloned into the pBE-S vector in *B. subtilis* secretory protein expression system (Takara). The N-terminal end of the alginate lyase sequences were fused in frame to the second amino acid downstream of the cleavage site of the aprE signal sequence in the pBE-S vector. The C-terminal end of the alginate lyase sequences were fused in-frame to the Histidine tag (His-tag) in the expression vector. All primers used in this study are listed in Table 1. The alginate lyase genes were inserted in the pBE-S vector using overlap extension cloning [36]. The AMOR-PL17 sequence was amplified using the primer pair Ap17-F1 and Ap17-R1, with pUC-ApL17-2 [28] as a template. The primers have extensions that are complementary to the aprE signal sequence or the sequence encoding the His-tag of the pBE-S plasmid. The PCR amplified 2.2 kb fragment was employed as mega-primers in the second PCR reaction with pBE-S as a template to insert the AMOR-PL17 sequence in the pBE-S vector, yielding pBE-AMOR-PL17. The PCR reaction was followed with *Dpn*I digestion to degrade the template plasmid prior to transformation of pBE-AMOR-PL17 to *E. coli*. The AMOR-PL7 was inserted in the pBE-S vector analogously to the AMOR-PL17 using the primer pair Ap7-F1 and Ap7-R1 with pNIC-CH_PL7A [24] as a template. The amplified 0.7 kb fragment was inserted in the backbone of pBE-S using the overlap extension method with subsequently *Dpn*I treatment, yielding pBE-AMOR-PL7. The plasmid constructions were first established in chemical competent *E. coli* TOP10 (Thermo Fisher Scientific Inc., Waltham, MA), following the manufacturer's procedure and subsequently transformed into *B. subtilis* according to the method described in Guan et al. [37].

2.3. Expression and activity

Expression of AMOR-PL7 and AMOR-PL17 was performed in the *B. subtilis* strain RIK1285. Transformant colonies were picked from a LB kanamycin plate and inoculated in 5 mL LB kanamycin media (37 °C, 180 rpm). 3% v/v of starter culture was used for expression in Terrific Broth (TB) media. The optimal expression period was determined by monitoring cell density, protein concentration, and protein activity over time for a total of 105 h. Cell growth was monitored with OD_{600} (Ultraspec 10, Amersham Bioscience). Cell free supernatant (SN) fractions were obtained by centrifugation (4500 rpm, 20 min.). Protein concentrations in the SN were determined by Bradford [38] and further analyzed by SDS-PAGE and Western Blot. FOR SDS-PAGE analysis, proteins were separated on a 12% stain-free acrylamide gel (BioRad, Hercules, CA) before being transferred to a PVDF membrane for Western Blot analysis, using the eBlot™ L1 Fast Wet Transfer System (GenScript, Piscataway, NJ). The membrane was blocked with a Tris-buffered saline (TBS), pH 7.6, solution containing 3% BSA and 0.01% Tween-20 prior incubation with a 1:5000 dilution of monoclonal mouse anti-His antibody (Qiagen, Germany) and 1:10,000 horse radish peroxidase (HRP) conjugated anti-mouse IgG, respectively. Blocking, antibody incubations and washing steps were all performed on the SNAP i.d.® 2.0 Protein Detection System (Merck) following the manufacture's procedure. Antibody-protein complexes were detected using the SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and visualized using an Azure c400 system and AzureSpot Analysis Software (Azure Biosystems, Dublin, CA, USA). Preliminary activity tests of AMOR-PL7 and AMOR-PL17 were performed with the dinitrosalicylic acid (DNS) method [39]. 50 μL 1% w/v alginate (Sigma-Aldrich) was mixed with 400 μL culture supernatant and 200 μL sodium acetate buffer, pH 5.6, to reach a final concentration 25 mM NaOAc and 200 mM

Table 1
Primers used in this study.

Primer	Sequence[a]
Ap7-F1	GCGTTCAGCAACATGTCTGCGCAGGCTGCGGCCTGCAATAGCGACGACGGTCTGC
Ap7-R1	AACTACCGCATTAGTGGTGATGATGGTGATGCTCGTAATAATACTTCAGGC
Ap17-F1	GCGTTCAGCAACATGTCTGCGCAGGCTGCGGCCATGCTGCC GGCGGGTGAACATCCG
Ap17-R1	AACTACCGCATTAGTGGTGATGATGGTGATGGATTTTCTCCACCTTGTAATTGCC

[a] Underlining indicates extensions that are complementary to pBE-S plasmid.

NaCl. Enzymatic reactions were incubated for 24 h, 55 °C. Enzyme reactions were stopped by boiling and 50 μL of the reaction mixture was mixed with 100 μL DNS solution [39]. For color development, the solution was placed in a Thermomixer (ThermoMixer® C, Eppendorf) at 100 °C for 15 min. Solution was cooled, absorbance at 540 nm was read using a UV spectrophotometer (Synergy™ H4 Hybrid Reader, BioTek) and amount of reducing ends were calculated using glucose as a standard.

2.4. Enzymatic saccharification of S. latissima

Enzymatic saccharification of *Saccharina latissima* biomass was performed in reactions containing 10% w/v dry matter (DM) seaweed (grinded and then dried at 105 °C to remove endogenous enzyme activity) in 25 mM NaOAc buffer, pH 5.6. Reaction conditions were based on previously published results for the two alginate lyases, where it was shown that AMOR-PL7 has an optimum at 65 °C, pH 6, and 500 mM NaCl [24], while AMOR-PL17 has high activity above 50 °C, pH 5–6, and 200–300 mM NaCl [28]. The cellulase preparation Cellic® CTec2 has an optimal activity around 50 °C and a pH of 5–5.5. All reactions were performed at 55 °C, with a salt concentration of 200 mM NaCl. Enzymes, i.e. the commercial Cellic® CTec2 (Novozymes A/S, Denmark) mixture and the culture supernatants containing the AMOR-PL7 and AMOR-PL17 alginate lyases, were dosed according to protein concentrations determined by Bradford. Cellic® CTec2 was added at a concentration of 10 mg protein per g of dry seaweed, while AMOR-PL7 and AMOR-PL17 was each supplied as 0.025 mg lyase protein per g of dry seaweed. Concentrations of the two alginate lyases were estimated by subtracting the protein concentration in the pBE negative control from total protein concentrations in the alginate lyase culture supernatants. The three different enzyme preparations were used for brown seaweed saccharification alone, all of them together, or each lyase with the cellulase cocktail. Culture supernatants from *B. subtilis* containing an empty vector, pBE, were used as a control. Reactions were incubated at 55 °C, 400 rpm, for 24 h before enzymes were inactivated by boiling for 5 min. and subjected to chromatographic analysis of solubilized sugars.

The optimal ratio of the *endo*- and *exo*-acting alginate lyases (PL7/PL17 ratio) during seaweed saccharification was determined by saccharification studies using 10% w/w DM seaweed with a constant concentration of 10 mg Cellic® CTec2 per g of dry seaweed and varying ratios of the two alginate lyses (0.05 mg alginate lyases in total per g of dry seaweed). Reaction conditions were as described above.

2.5. Monosaccharide analysis

Glucose and mannitol concentrations were determined by high performance anion exchange chromatography (HPAEC) using an ICS6000 system (Dionex) equipped with a pulsed anomeric detector (PAD). Samples were passed through a 0.22 μm filter and mannitol and glucose were separated with a CarboPac™ PA210 guard (4 × 30 mm) – and an analytical column (4 × 150 mm). Elution was done isocratic with 1 mM KOH for 13 min. Quantification was carried out using Chromeleon software with mannitol (Sigma-Aldrich, St. Louis, MO) and D-glucose (VWR, Darmstadt, Germany) as standards. The amounts of uronic acids were also determined by HPAEC-PAD, using the ICS5000 system (Dionex) on a CarboPac™ PA1 column. Elution was done with 15 mM NaOH and a linear NaOAc gradient from 0 to 150 mM. Quantification was carried out using Chromeleon software with D-mannuronic acid sodium salt (Sigma-Aldrich, St. Louis, MO) and L-guluronic acid sodium salt (CarboSynth Ltd. UK) as standards. The composition of the *S. latissima* biomass was determined in the same way following a two-step acid hydrolysis. 100 mg/mL dry *S. latissima* was mixed with 72% w/w H_2SO_4 and left in a water bath for 1 h at 30 °C. Milli-Q water was added to reach a final concentration of 4% w/w H_2SO_4 and hydrolysis was continued for 40 min at 120 °C in an autoclave [19]. Acid hydrolysate and seaweed residuals were separated by centrifugation and the hydrolysates were passed through a 0.22 μm filter prior to chromatographic analysis, as described above. Recovery values for the monosaccharides were estimated from parallel acid hydrolysis runs of the monosaccharaide standards.

2.6. Statistics

All experiments were performed in triplicates and data are presented as means ± standard deviations. Analyses of variances (ANOVA) were used to determine significant differences using the Tukey-Kramer test from pooled standard deviations (JMP Pro 15, SAS). Values of $P < 0.05$ are considered statistically significant.

3. Results and discussion

3.1. Growth and expression in Bacillus subtilis

B. subtilis harboring AMOR-PL7, AMOR-PL17 or the empty plasmid pBE were cultivated on TB medium in shake flaks. Cell density, protein concentration, and lyase activity were monitored over time and showed that the *B. subtilis* was able to grow with the transformed vectors. As expected, the control strain harboring the empty vector pBE showed lowest extracellular protein concentration and no lyase activity (Fig. 1B and Fig. 1C). In contrast, Fig. 1B shows increasing protein concentrations up to 50 h incubation for the strains harboring pBE-AMOR-PL7 and pBE-AMOR-PL17. This can be ascribed to secreted alginate lyases, as evidenced by the presence of alginate lyase activity (Fig. 1C). Fig. 1B shows that extracellular protein concentration was highest after 50 and 70 h for AMOR-PL7 and AMOR-PL17, respectively, while the highest alginate lyase activity was observed after 50 h of incubation for both enzymes (Fig. 1C). Expression and secretion of the two alginate lyases in *B. subtilis* were further confirmed by Western Blot analysis of the SN fraction (Fig. 2), where the protein band at expected size of 30 kDa shows the AMOR-PL7 and the one around 80 kDa band shows the AMOR-PL17, respectively.

For further analyses, the pBE-AMOR-PL7 and pBE-AMOR-PL17 transformants were cultivated for 50 h, and the supernatants were harvested and used as sources of alginate lyases. Protein concentration of the two alginate lyases was estimated based on the difference in protein levels between the pBE control strain and the two strains harboring the plasmids pBE-AMOR-PL7 and pBE-AMOR-PL17.

Previous work on alginate lyase expression in *Bacillus* expression systems is very limited. The alginate lyase A1-III derived from *Flavobacterium* sp. has been recombinantly expressed in a different *Bacillus* expression system (*B. subtilis* ANA-1) [40], otherwise most reported recombinant alginate lyases have been expressed in *E. coli* [29,31,34,41]

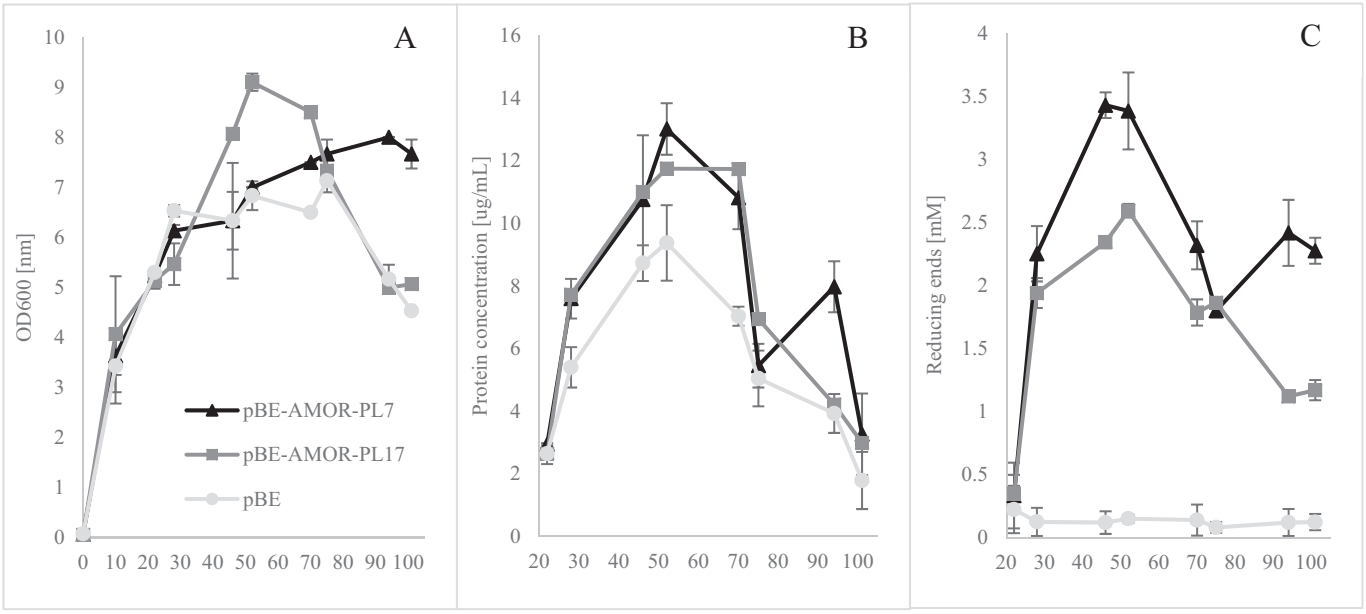

Fig. 1. Growth curves measured as OD_{600nm} (A), total extracellular protein concentrations (B), and alginate lyase activity (C) for *B. subtilis* harboring the plasmids; pBE-AMOR-PL7, pBE-AMOR-PL17, or pBE (negative control).

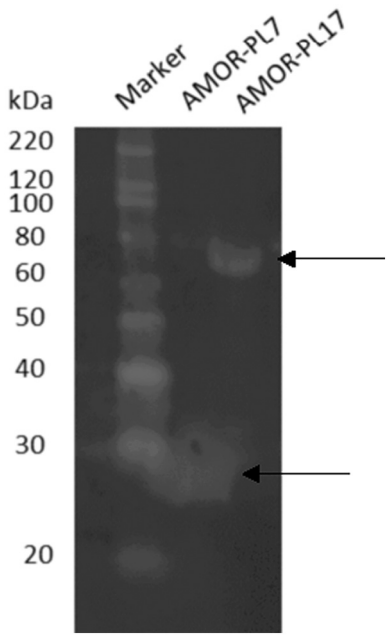

Fig. 2. Western Blot of protein marker and concentrated culture supernatants of AMOR-PL7 and AMOR-PL17 harvested after 50 h. of cultivation.

and *Pichia* [42–44]. Additionally, there are a few studies on heterologous expression of β-agarases in *Bacillus*, enzymes responsible for the degradation of agar derived from red seaweeds [45–47].

3.2. Enzymatic saccharification of S. latissima

To investigate if supernatants from *Bacillus* cultivation broths could be applied directly as an enzyme source, degradation experiments of seaweed biomass were carried out. It is known from literature that blends of cellulases and alginate lyases can efficiently saccharify brown seaweeds [14,20,24,26]. Thus, cultivation broths containing alginate lyases AMOR-PL7 and AMOR-PL17 were added to seaweed substrate in the form of *S. latissima* together with the commercial cellulase preparation Cellic® CTec2. On a dry matter basis, the seaweed was composed of 26 ± 1.5% (w/w) glucose (dehydrated), 16 ± 0.4% (w/w) mannitol, 13 ± 1.1% (w/w) mannuronic acid (dehydrated), and 9 ± 2.1% (w/w) guluronic acid (dehydrated; Chromatograms in Fig. S1).

In the absence of alginate lyases, Cellic® CTec2 released approx. 137 g glucose from 1 kg DM seaweed (Fig. 3), corresponding to a glucose solubilization yield of 54%. Glucose yields were significantly increased when saccharification reactions were supplemented with supernatants containing alginate lyases, where combined addition of the *endo*-acting AMOR-PL7 and the *exo*-acting AMOR-PL17 resulted in the highest glucose yield of 203 g glucose per kg DM, accounting for 79% of the total glucose present in the seaweed biomass (Fig. 3). Compared to the cellulases alone, the addition of alginate lyases under these reaction conditions improved the glucose release by 48%. The lyases themselves do not release glucose through enzymatic reactions, but rather ease the accessibility of the cellulases to laminarin and cellulose by degrading surrounding alginates, opening the seaweed biomass structure and reducing viscosity. This synergy effect of lyases in combination with cellulase fits the model of the brown seaweed cell wall, where the matrix polysaccharides alginate and glucans are tightly associated [13].

All reactions containing Cellic® CTec2 gave a relatively high release of mannitol (from 115 to 131 g/kg), with highest mannitol yield (79%) for the blend of Cellic® CTec2 and AMOR-PL7 (Fig. 3). Blends of Cellic® CTec2 and an endo alginate lyase from *Flavobacterium multivorum* have previously been described to efficiently release mannitol from seaweeds [14]. Alginate lyases have a positive effect on mannitol release, but the presence of cellulases is clearly more important.

The presence of alginate lyases seemed to improve the yield of glucose released by the cellulases. However, the opposite was not the case; the alginate lyases released similar levels of uronic acids irrespective of the presence of cellulases in the reaction (Fig. 3).

Alginate lyases alone only released small amounts of glucose from the seaweed biomass (Fig. 3). The lyases degrade alginate to a blend of oligosaccharides with an unsaturated moiety at the non-reducing end and the monomer 4-deoxy-Lerythro-4-hexenopyranouronate which spontaneously is hydrated (via ring-opening) to 4-deoxy-L-erythro-5-hexulosuronate (DEH). Interestingly it was recently shown that DEH is spontaneously transformed to two cyclic hemiketals (4-deoxy-D-manno-(5*S*)-hexulofuranosidonate hydrate and 4-deoxy-D-manno-(5*R*)-

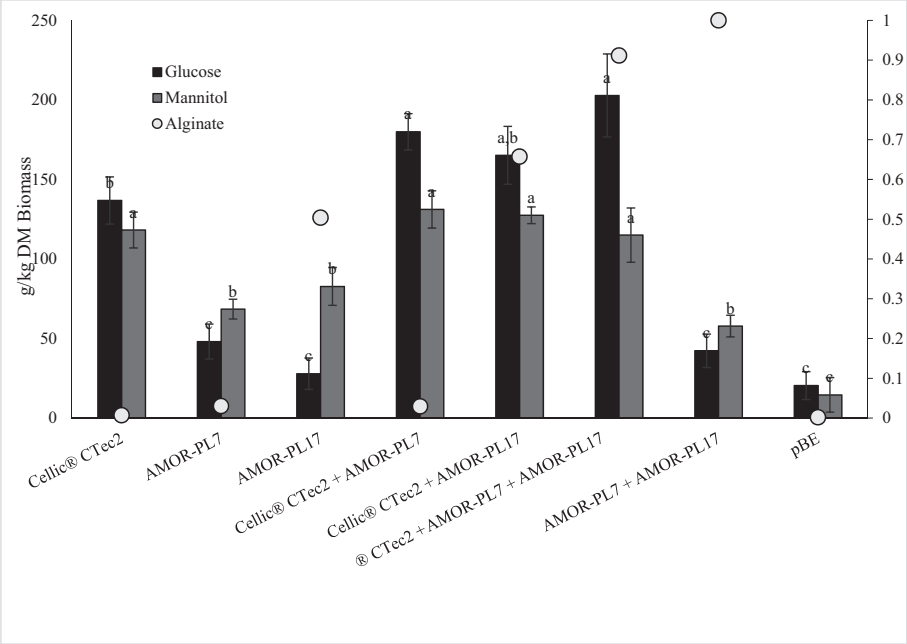

Fig. 3. Enzymatic release (g/kg DM of *S. latissima*) of glucose, mannitol, and uronic acids as function of applied enzymes (Cellic® CTec2, AMOR-PL7 and AMOR-PL17). Each data point represents the mean value of independent triplicates ± SD. Different letters indicate significant differences ($P < 0.05$) by one-way ANOVA. Relative levels of alginate solubilization were estimated by summarizing HPLC peak areas for mannuronic acid and guluronic acid and normalizing this to the highest release level. pBE indicates negative control without alginate lyase.

hexulofuranosidonate hydrate) as the final product [28]. Due to lack of standards, we could not quantify the amount of hemiketals produced in the enzyme reactions. However, we detected mannuronic acid and guluronic acid in the hydrolysates (Fig. S2) that contained alginate lyases. These uronic acids must originate from the original non-reducing ends of alginate since all alginate lyase activity creates unsaturated sugars. We also propose that some of the detected uronic acids may originate from dimers with and unsaturated moiety at the non-reducing end which was degraded on column due to instability at the high pH conditions. Such on column degradation of relatively unstable non-reducing end sugar moieties have been observed previously for other types of dimers and oligosaccharides [48]. Thus, we cannot quantify the extent of alginate saccharification, but we have estimated the relative extent by using the peak area of the detected uronic acids. Fig. 3 shows that uronic acids were readily detected in all in reactions containing the exolytic AMOR-PL17. The amount of uronic acids detected were significantly increased when the two alginate lyases where combined, with highest amount for the reaction containing only these lyases (Fig. 3). This increase in alginate degradation was probably due to synergy between the enzymes since one of them is an *endo*-acting enzyme while the other is an *exo*-acting [24,28]. Generally, *endo*-acting lyases degrade alginate substrates into a range of oligomers while *exo*-acting lyases produce monomers from alginate oligomers. More mannuronic than guluronic acid was detected (Fig. S2), supporting the fact that both AMOR-PL7 and AMOR-PL17 have previously been described to have preference for poly-mannuronic acid sequences, with less activity on poly-guluronic acid sequences [24,28].

To investigate how different ratios of *exo* and *endo* alginate lyases affect the degradation process, seaweed saccharification was performed with a constant concentration of Cellic® CTec2 and with varying ratios of the two alginate lyases (see Fig. 4).

At these reaction conditions, the PL7 alginate lyase alone with

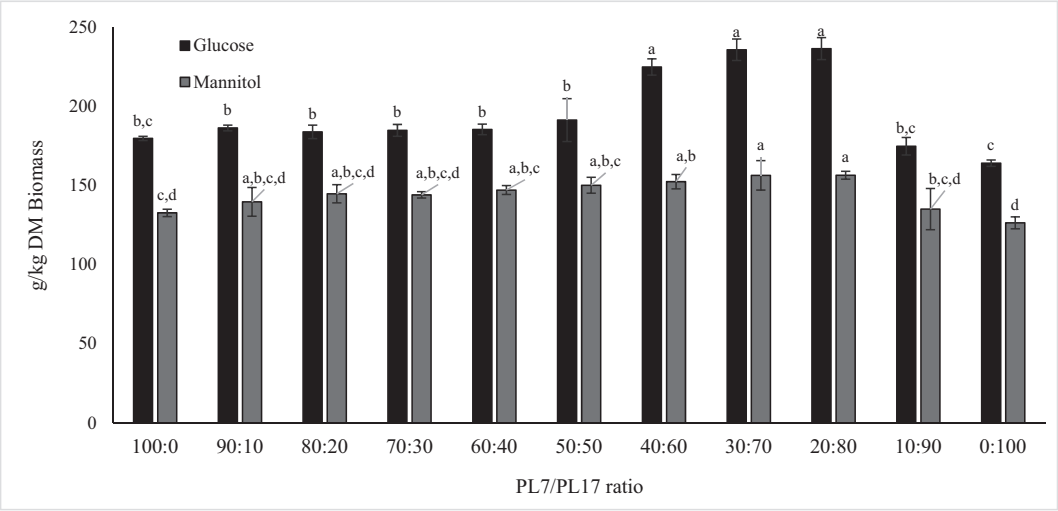

Fig. 4. Enzymatic release (g/kg DM of *S. latissima*) of glucose and mannitol as function of applied AMOR-PL7/PL17 ratio. Cellic® CTec2 was applied at 10 mg/g DM and the alginate lyases at a total protein concentration at 0.05 mg protein/g DM with varying ratios of the two enzymes indicated on the x-axis. Each data point represents the mean value of independent triplicates ± SD. Different letters indicate significant differences ($P < 0.05$) by one-way ANOVA.

Cellic® CTec2 was shown to release 180 g glucose from 1 kg DM seaweed, whereas the PL17 alginate lyase together with Cellic® CTec2 released 164 g glucose (Fig. 4). The highest sugar release, of 236 g glucose and 156 g mannitol per kg seaweed, was observed at the PL7/PL17 ratios of 20:80 and 30:80, highlighting the importance of high levels of exo-acting alginate lyases in the enzyme blend. These saccharification yields accounted for approx. 94% of the total glucose and 93% of the total mannitol present in the seaweed biomass. Compared to using only cellulases, the addition of PL7 and PL17 in the ratios 20:80 and 30:70 improved the final sugar release by 73% and 32% for glucose and mannitol, respectively.

Synergy between exo- and endo-acting alginate lyases have been described before using alginate as a substrate, but not on seaweed biomass. On an alginate substrate the highest degrees of synergy between an exo- and endo-acting alginate lyase was also shown to take place at a ratio of 30% endo-acting alginate lyase and 70% exo-acting lyase [42]. These optimal conditions may vary depending on reaction conditions, composition of substrates and substrate preference, i.e. M or G-specificity of the specific enzymes.

Enzymatic saccharification of brown seaweeds using alginate lyases and cellulases has been reported several times and it is well established that combination of the two enzymes can increase final glucose yield [14,20,24,26]. All reported studies have been using purified alginate lyases. Manns et al. (2016) expressed two bacterial PL7 alginate lyases from *Sphingomonas* sp. and *Flavobacterium* sp. in *E. coli*, and they were shown to be able to release all potential glucose from a *Laminaria digitata* sample when applied with 1% enzyme/substrate (E/S) level (% weight) together with 10% E/S (v/w) of Cellic® CTec2 [20]. The effects of different dosages of alginate lyases and cellulases have been investigated by Sharma & Horn, 2016. They showed that the cellulase mixture Cellic® CTec2 with 10% incorporation of alginate lyase gave the highest amount of glucose release [14]. However, lower levels of alginate lyase inclusion were not tested. The endo AMOR-PL7 alginate lyase used in the current study has also previously been tested for its ability to saccharify brown seaweeds in combination with Cellic® CTec2. Enzymatic saccharification of *S. latissima* with the *E. coli* recombinantly expressed AMOR-PL7 and Cellic® CTec2 resulted in 25% higher glucose yield than only using Cellic® CTec2, at protein concentrations of 0.7 mg/g and 6.3 mg/g DM, respectively. Interestingly, it was shown that the AMOR-PL7 concentration could be lowered 40 times without affecting the final glucose yield [24].

Using culture supernatants directly as protein solutions for enzymatic reactions, without purifications – and/or concentration steps, will naturally result in lower protein concentrations. In the current study, the applied alginate lyase dosage was relatively low. The protein concentration may be increased by downstream processes, but this will result in higher process costs, use of extra time, and potential activity loss. Several other factors affect the protein concentration, such as expression host, secretion efficiency, expression vector (i.e. which promoter) and can be optimized to increase protein expression. Nevertheless, the reported results in this study show that the strategy of using the culture supernatants directly as a source of alginate lyases worked very well. Only low inclusions levels of alginate lyases were needed to improve glucose yields up to 73% compared to only applying cellulases. Additionally, due to the inclusion of an *exo*-acting alginate lyase, alginate was degraded to monomeric sugars, demonstrating the possibility to produce high amounts of fermentable sugars (blends of glucose, mannitol and uronic acids/DEH) from brown seaweed. This production of alginate monomers can probably be improved by inclusion of lyases with G-block preference. Previous studies have shown that mannitol and alginate hydrolysates can be used as carbon sources in fermentation processes, e.g. studies by Horn et al. (2000) that produced ethanol from mannitol by *Zymobacter palmae* and *Pichia angophorae* [15] and Wargacki et al. (2012) that engineered an *E. coli* strain for ethanol production from alginate monomers [7]. It should be noted that seaweed hydrolysates also provide other useful microbial growth medium ingredients such as N, P and minerals [10].

Generally, alginate lyases, including commercially available enzymes, have lower temperature optima than commercial cellulase mixtures. However, the two alginate lyases used in the current study are thermotolerant lyases, making them appropriate candidates to combine with fungal cellulases. By using thermostable lyases, we achieved efficient enzymatic saccharification without the need for a two-step reaction adapted to different temperature optima [14].

Directly using the supernatant of fermentation broths as a source of enzymes without purifications is an effective way to decrease costs for enzymatic biomass saccharification and several studies report “on-site” production of cellulolytic enzymes for different biomass refineries [49–53]. Gool et al. (2012) reported the production of hemicellulolytic enzymes for the hydrolysis of wheat straw and corn fiber fractions using a wide range of mesophilic lignocellulolytic fungi grown on wheat straw as a carbon source [49]. Similarly, different *Trichoderma* species have been used for production of cellulases for hydrolysis of spruce [53], corn cob residues [51], and soybean and sugar cane [52]. Direct use of fermentation broths for seaweed biorefining is rare, but recently Li et al. (2019) have assessed the potential of an extracellular expressed β-agarase, the AgaM1, to be used directly for degradation of the red seaweed *Gracilaria lemaneiformis*. The *Bacillus* culture supernatants containing the recombinant AgaM1 agarase was shown to be able to produce agar-oligomers from a red seaweed sample pretreated by autoclavation [46].

Overall, this study has shown that culture supernatants of *Bacillus* expressing alginate lyases can be used as a source of enzymes, and together with cellulases these culture supernatants can be used to efficiently release glucose from seaweed biomass. Additionally, the combination of *exo* and *endo* alginate lyases resulted in release of alginate monomers, increasing the yield of fermentable sugars from the biomass. This opens the possibility for a cheap and simple on-site production of enzymes that can be applied directly for seaweed biorefining.

CRediT authorship contribution statement

NRK: Methodology, Investigation, Formal analysis, Writing original draft. **CG**: Methodology, Investigation, Formal analysis, Writing- review and editing. **GM**: Supervision, Methodology, Writing- review and editing. **SJH**: Conceptualization, Methodology, Supervision, Writing- review and editing, Funding acquisition, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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