Production, characterization and application of an alginate lyase, AMOR_PL7A, from hot vents in the Arctic Mid-Ocean Ridge

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Abstract

Enzymatic depolymerization of seaweed polysaccharides is gaining interest for the production of functional oligosaccharides and fermentable sugars. We describe a thermostable alginate lyase belonging to Polysaccharide Lyase family 7 (PL7), which can be used to degrade brown seaweed, *Saccharina latissima,* at conditions also suitable for a commercial cellulase cocktail (Cellic CTec2). This enzyme, AMOR_PL7A, is a β-D-mannuronate specific (EC 4.2.2.3) endo-acting alginate lyase, which degrades alginate and poly-mannuronate within a broad range of pH, temperature and salinity. At 65 ^{o}C and pH 6.0, its *K*m and k_{cat} values for sodium alginate are 0.51 +/- 0.09 mg/mL and 7.8 +/- 0.3 s^{-1} respectively. Degradation of seaweed with blends of Cellic CTec2 and AMOR_PL7A at 55 ^{o}C in seawater showed that the lyase efficiently reduces viscosity and increases glucose solublization. Thus, AMOR_PL7A may be useful in development of efficient protocols for enzymatic seaweed processing.

Keywords

Alginate lyase, Brown seaweed, *Saccharina latissima*, Biorefining, PolyM, Salt tolerance

Introduction

Saccharina latissima, better known by its common name sugar kelp, is a widely abundant brown seaweed (macroalgae) in Norwegian coastal areas. *S. latissima* contains up to 40 % alginate of its dry weight depending on season and depth [12]. In Norway, exploitation of brown seaweed has so far largely been based on harvesting of natural biomass and production of valuable compounds such as alginate [3]. In recent years, new possibilities have opened within industrialized multi-trophic aquaculture, where seaweeds are grown in close proximity to salmon farms [4]. In addition to alginate, *S. latissima* is rich in polysaccharides such as cellulose, laminarin, and fucoidan, and also contains considerable amounts of mannitol [5]. Because of this composition, brown seaweed may be utilized for biofuel production [6,7] or other fermentative production processes such as the production of single cell protein [8]. Considering the compositional complexity, complete saccharification of brown seaweed requires a multitude of enzymes. Key enzymes include cellulases and alginate lyases, because they

35 together release a considerable amount of the available sugar [9] and because the viscosity reducing effect of alginate lyase
36 has a general positive effect on enzymatic degradability [10,11].

37 Alginate lyases, characterized as either mannuronate (EC 4.2.2.3) or guluronate (EC 4.2.2.11) lyases, catalyze
38 depolymerization of alginate, a co-polymer consisting of the uronic acids ß-D-mannuronate (M) and its C5 epimer α-L-
39 guluronate (G). In alginates, these monomers occur as homopolymeric blocks of consecutive M-residues (polyM) or
40 consecutive G-residues (polyG), or in heteropolymeric blocks of alternating M and G-residues (polyMG). According to the
41 Carbohydrate-Active Enzymes database (CAZy), alginate lyases occur in several families of polysaccharide lyases (PL). To
42 date, alginate lyases are found in PL families 5, 6, 7, 14, 15, 17, and 18 and most of them work endolytically [12].
43 Polysaccharide lyases (EC 4.2.2.-) are active on uronic acid containing polysaccharides and cleave the substrate by a ß-
44 elimination reaction, which generates a new reducing end and an unsaturated uronic acid at the new non-reducing end
45 [13]. Alginate lyases acting mainly on the M-M bond or G-G bond are classified as poly-mannuronate (polyM) lyases, poly-
46 guluronate (polyG) lyases, respectively [12]. Due to the enzymatic formation of a double bond between C4 and C5, the
47 unsaturated residues originating from guluronic acid (G) or mannuronic acid (M) are identical. This urinate, 4-deoxy-L-
48 erythro-hex-4-enepyranosyluronate, is often shown as Δ in illustrations [12,14].

49 Alginate lyases, including commercially available ones, typically have a lower temperature optimum than commercial
50 cellulase cocktails. Hydrolysis of seaweed must therefore be done in two phases, at two different temperatures, requiring
51 long processing times [9], while the low temperature processing step with the alginate lyase increases the risk of bacterial
52 contamination. Here, we describe the cloning and characterization of a novel, thermostable alginate lyase (AMOR_PL7A)
53 whose gene was retrieved from a metagenomic dataset collected from the Arctic Mid-Ocean Ridge (AMOR). Furthermore,
54 we show that AMOR_PL7A promotes saccharification of seaweed by the commercial enzyme cocktail Cellic CTec2 in a
55 single step reaction.

Material and methods

Sampling, DNA extraction and sequencing

A sample of unbleached Norway spruce (*Picea abies*) that had been pretreated by sulfite-pulping using the BALI™ process [15,16] at Borregaard AS (Sarpsborg, Norway), was incubated for one year in ~70°C hot sediments at the Arctic Mid-Ocean Ridge (AMOR), 570 meters below sea level[17,18]. In short, one gram of spruce material was mixed with approximately 16 ml of sediment sampled at the site and placed in the middle chamber of a titanium incubator with three vertically aligned chambers of 2.5 cm in length, a volume of 16 ml and 1 mm pores. DNA was extracted from 4.6 grams of material and 1.8 µg of DNA was submitted for sequencing. Further details of the substrate and the sampling procedure, as well as the procedures used for DNA extraction and sequencing have been described elsewhere [19].

Filtering, assembly and ORF-prediction

Raw Illumina MiSeq 300 paired-end reads were filtered and assembled using the CLC genomics workbench (Qiagen, v.9.5.3), with CLC default parameters for filtering (quality 0.05, length min. 40 and max. 1000 nucleotides) and assembly (automatic k-mer size and bubble size). Before filtering, one nucleotide was removed from terminal read ends. The minimum contig length was set to 1000 bases, with scaffolding enabled. Open reading frames were predicted using the *-p meta* option in Prodigal v.2.6.3 [20,21] for metagenomics datasets. A full description of the resulting dataset will be published elsewhere.

The metagenomic data, which were generated for the discovery of cellulolytic enzymes, were also mined for putative endo-type lyases from polysaccharide lyase family 7 (PL7) using dbCAN (csbl.bmb.uga.edu/dbCAN)[22]. This analysis resulted in the identification of a 783 bp gene encoding a putative PL7, here named *amor_PL7A* (See Figure S1 for the protein sequence). The AMOR_PL7A amino acid sequence was Blasted against the PDB database (rcsb.org) and submitted to the Phyre2 server (www.sbg.bio.ic.ac.uk/phyre2;[23]) to investigate similarities to known alginate lyases and to check for occurrence of expected active site residues. LipoP analysis [24] indicated a signal peptidase II cleavage site between residues 24-25, suggesting that AMOR_PL7A is a lipoprotein anchored to the cell membrane via a cysteine at position 25. The sequence of AMOR_PL7A has been submitted to GenBank under accession number MH727998.

Sequence analysis

Sequence alignments were produced in MEGA v.7 [25] using the Muscle algorithm [26] and the aligned sequences were visualized using ESPript 3 [27]. To infer phylogenetic placement of AMOR_PL7, a pre-computed alignment of PL7 was downloaded from the dbCAN server and aligned with additional PL7 sequences as identified by using the EMBL-EBI HMMER biosequence analysis [28]. Only sequences sharing the conserved amino acids of the active site (Fig. S1) were considered and aligned using mafft-linsi [29,30]. In total, 161 sequences were used for construction of a phylogenetic tree using IQTREE [31].

Cloning, expression and purification of AMOR_PL7A

The *amor_PL7A* gene (codon-optimized for *Escherichia coli* expression) was synthesized by Genscript (Piscataway, NJ, USA) and a gene fragment comprising bp 76-783 (omitting the predicted 24 amino acid signal peptide and the cysteine residue at position 25) was amplified by PCR using the Q5 DNA polymerase (New England Biolabs, Ipswich, Massachusetts, USA) and forward and reverse primers 5'TTAAGAAGGAGATATACTATGAATAGCGACGACGGTCTGCT3' and 5'AATGGTGGTGATGATGGTGCGCCTCGTAATAATACTTCAGGCTCTTAAAT3' (Eurofins, Ebersberg, Germany), respectively. The resulting PCR product encodes for a protein with a C-terminal hexaHis-tag and was cloned into the pNIC-CH expression vector (AddGene, Cambridge, Massachusetts, USA) by Ligation-Independent cloning [32], as described previously [19]. Transformed OneShot *E. coli* TOP10 cells (Invitrogen, Carlsbad, California, USA) were propagated, plasmids were isolated and the sequence of *amor_PL7A* was confirmed by Sanger sequencing (GATC, Konstanz, Germany), after which a correct plasmid was transformed to OneShot BL-21 Star™ (DE3) *E. coli* cells for protein expression, all as described previously [19].

For expression, cells were grown in Terrific Broth (TB) supplemented with 50 µg/mL kanamycin at room temperature, overnight, using a Harbinger system (Harbinger Biotechnology & Engineering, Markham, Canada). Protein expression was then induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM, followed by further incubation at room temperature for 24 h. Cell pellets were collected by centrifugation at 5,000 *g*, T = 4 °C, for 15 minutes using a Beckman Coulter Avanti J-26S XP centrifuge (Brea, California, USA). The cell pellet was placed at –80 °C for 1 hour to promote cell lysis. After thawing, the cells were resuspended in 50 mM Tris-HCl (pH 8.0) containing 500 mM NaCl and 5 mM imidazole, and sonicated on ice using a Vibracell sonicator (Sonics & Materials Inc., Newtown, Connecticut, USA)

with 5 seconds on/off pulses for 3 minutes at 30% amplitude. After removal of cell debris by centrifugation at 15,000 *g* for 15 minutes, the supernatant was filtered using a 0.45 µm syringe filter (Sarstedt, Nümbrecht, Germany). The resulting cell-free protein extract was then used for purification of AMOR_PL7A by immobilized metal affinity chromatography (IMAC) using an Äkta pure chromatography system and a Ni^{2+} affinity HisTrap™ HP 5 mL column (GE HealthCare, Chicago, USA). Elution was achieved by applying a linear gradient of 5-500 mM imidazole in 50 mM Tris-HCl (pH 8.0), 500 mM NaCl. After analysis of protein-containing fractions by SDS-PAGE (Bio-Rad, Hercules, California, USA) (Figure S2), fractions containing AMOR_PL7A were combined and the resulting solution was concentrated using a 3,000 MWCO Vivaspin ultrafiltration tube (Sartorius, Göttingen, Germany), with concomitant buffer exchange to 20 mM sodium acetate, pH 6.0, 300 mM NaCl. For determination of the protein concentrations, the absorbance at 280 nm was recorded with a Biophotometer (Eppendorf, Hamburg, Germany) and converted to a concentration using the theoretical extinction coefficient (web.expasy.org/protparam) of AMOR_PL7A. Solutions with purified protein were stored at 4°C.

Activity assays

The activity of AMOR_PL7A was quantified either by determining production of reducing sugar equivalents using the 3,5-dinitrosalicylic acid (DNS) reagent [33] or by following double bond formation through monitoring absorbance at 235 nm. Absorbance at 235 nm was converted to product concentration using an extinction coefficient of 6150 $M^{-1}cm^{-1}$ [34] and a path length of 0.56 cm (200 µL reaction volume in microtiter plates). Enzyme reactions were carried out in triplicates and the values presented below represent the mean ± standard deviation. All reported buffer pHs were measured at 65 °C. Note that while the pH of sodium acetate buffer is almost independent of temperature, the pH of Tris-HCl buffer with a pH of 7.0 at room temperature is approximately 6.0 at 65 °C.

Dinitrosalicylic acid (DNS) assay for determination of reducing sugars

The optimal temperature for the enzyme was determined using the DNS method for product quantitation [33]. Reaction mixtures contained 25.4 nM AMOR_PL7A in 50 mM sodium acetate (NaAc) buffer containing 500 mM NaCl (pH 6.0) and 1% (w/v) standard sodium alginate from Sigma Aldrich (F_G = 0.44, Mw = 107.9±2.7 kDa) and the reaction mixtures were incubated at different temperatures (37 °C to 100 °C) for up to 50 min. Samples were taken at regular intervals and subjected to boiling for 5 minutes. Samples were then mixed with two volumes of DNS reagent, followed by boiling for 15

minutes. Subsequently, the absorbance was measured at 540 nm using a Synergy H4 Microplate reader (Biotek Instruments Inc, Winooski, USA). Standard curves were made using guluronic acid at concentrations ranging from 0.09 to 1.80 mg/mL. The linear regions of the obtained progress curves were used to determine initial velocities.

Monitoring the formation of double bonds

For determination of the pH optimum, kinetic measurements and assessment of thermostability, enzyme activity was assessed by recording the change in absorbance at 235 nm [35]. Standard reactions contained 12.7 nM AMOR_PL7A in 50 mM Tris-HCl, pH 6.0, containing 500 mM NaCl and 0.5 % (w/v) sodium alginate. Reactions were set up in triplicates and product formation was monitored in real time using a Synergy H4 Microplate reader (Biotek). Samples were incubated for up to 60 minutes at 65°C with continuous stirring in between absorbance measurements at 235 nm with one-minute intervals. The linear regions of the progress curves were used to determine initial velocities.

pH optimum and salt tolerance

The pH optimum of AMOR_PL7A was investigated using the conditions described above for determination of absorbance at 235 nm, with varying buffers covering a pH range of 4.2 to 9.3, either 50 mM NaAc (pH 4.2-5.8), 50 mM Tris-HCl (pH 6.0-6.9) or 50 mM Glycine-NaOH (pH 7.9-9.3) (Nb. pH measured at 65 ^{o}C). For testing the effects of salinity, the standard 50 mM Tris-HCl buffer, pH 6.0, was supplied with 0 to 2 M NaCl. Seawater (pH 6.8 at 65 ^{o}C) was obtained from Norsk Institutt for Vannforskining (NIVA), Drøbak, Norway, and was collected from 60 m depth in the Oslo Fjord and a temperature of 7.6°C. Seawater salinity was measured with a Sal-Bta salinity probe (Vernier, Beaverton, USA) and corresponded to approximately 430 mM NaCl.

Determination of Michaelis-Menten enzyme kinetics

Steady-state kinetic constants, K_M and V_{max}, at 65°C, were calculated by direct fitting of experimental data to the Michaelis–Menten equation. Data points were collected from reactions with sodium alginate at concentrations ranging from 0.1 to 9 mg/mL, containing 12.7 nM enzyme in 50 mM Tris-HCl, pH 6.0, and 500 mM NaCl. Enzyme activity was measured by recording the change in absorbance at 235 nm and all progress curves used for rate determination were linear. Enzyme

153 rates were calculated by dividing the linear increase in the concentration of saturated ends, calculated as described above,
154 by time.

155 ## Thermostability

156 Thermostability was determined by pre-incubating the enzyme (0.1 μM) in 50 mM Tris-HCl, pH 6.0, and 500 mM NaCl
157 without the substrate at 65°C for 0 to 24 hours before running an activity assay using standard conditions (50 Tris HCl, pH
158 6.0, 500 mM NaCl, 65°C), with product monitoring at 235 nm.

159 For determination of the apparent protein melting temperatures, we used a Nano-Differential Scanning Calorimeter III
160 (Calorimetry Sciences Corporation, Lindon, USA). Protein samples were dialyzed over night at 4°C against 50 mM sodium
161 acetate pH 6.0 containing either 50 or 500 mM NaCl, or against pure unbuffered seawater. The dialyzed protein samples
162 (final concentration adjusted to 1.3 mg/mL) and samples of dialysis buffer, used for recording baselines, were degassed
163 prior to the DSC experiments. The scan rate was 1°C/minute and the temperature range was 20-100°C. The data were
164 analyzed using the NanoAnalyze software (tainstruments.com).

165 ## Substrate specificity

166 The substrate specificity of AMOR_PL7A was investigated using polyM (M_w = 275 kDa, F_G = 0.0, [36]), polyG (DPn =20, F_G =
167 0.93, [1]) or polyMG (M_w = 275 kDa, F_G = 0.46, F_{GG} = 0.0 [37]) as substrate. The reactions were performed in 50 mM Tris-HCl,
168 pH 6.0, containing 250 mM NaCl, 12.7 nM enzyme and 0.5% (w/v) substrate. Products were analyzed by High Performance
169 Anion Exchange Chromatography (HPAEC) for qualitative analysis and with the DNS method for quantification. Preparation
170 of alginate oligomers for product identification, by fractionation of alginate hydrolysates on SEC columns, has been
171 described previously by Aarstad et al. [14] .

Chromatographic product analysis

Samples were passed through a 0.22 µm filter prior to chromatographic analysis. Analysis of monosaccharides and mannitol was done using a Dionex Ultimate 3000 (Sunnyvale, California, USA) HPLC system equipped with a refractive index detector and a 300 × 7.8 mm Rezex ROA-Organic Acid H+ analytical column fitted with a cation-H cartridge guard column, operated at 65 °C with 5 mM H_2SO_4 as the mobile phase, with a flow rate of 0.6 mL/min. Glucose and mannitol at concentrations ranging from 0.50 to 10 g/L were used as calibration standards. Analysis of oligosaccharides by high-performance anion exchange chromatography (HPAEC) was done using an ICS3000 system from Dionex (Sunnyvale, California, USA) equipped with a pulsed amperiometric detector (PAD) with a disposable electrochemical gold electrode. Separation was achieved using a 4 × 250 mm IonPac AS4A column (Dionex) connected to IonPac AG4A (4x50) guard column, operated at 30 °C. Samples were analyzed essentially as previously described [14]. In brief, the mobile phases were 0.1 M sodium hydroxide (A) and 1 M sodium acetate in 0.1 M sodium hydroxide (B) and a linear gradient was developed from 1% B to 88.5% B over 100 minutes, i.e. 8.75 mM sodium acetate/min, at a flow rate of 1 mL/min. The PAD detector was set to use an AAA waveform for optimal signal-to-noise detection. Data acquisition and analysis were done using Chromeleon 7.2 (Thermo Scientific).

Enzymatic saccharification of *S. latissima*

Enzymatic saccharification of *S. latissima* was performed in reactions containing 15 % dry matter (DM) seaweed (grinded and then dried at 50 °C; see Sharma and Horn [2016] for details) in seawater, which were incubated at 55°C for 24 hours. The commercial enzyme preparations were Cellic CTec2 (Novozymes A/S, Denmark) and alginate lyase A1603 from Sigma Aldrich, Germany. AMOR_PL7A was dosed according to its protein content and the amounts of protein dosed were similar to appropriate amounts of powdered commercial lyase that had previously been determined by [9]. Cellic CTec2 was dosed according to its protein content determined by the Bradford method [38] at 6.3 mg/g DM. The hydrolysates were subjected to rheological measurements without further processing.

Rheological measurements

The viscosity measurements were done with continuous rotation using an MCR301 rheometer from Anton Paar fitted with a PP50/P2 measuring system and a Peltier element ensuring a temperature of 20 ^{o}C. The shear rate was ramped during

the analysis from 10 s^{-1} to 200 s^{-1}. Amplitude sweep measurements were done using the same rheometer at a frequency of 1 Hz, changing the amplitude from 0.01% to 100% strain (i.e. relative change in length). The limit of the linear viscoelastic range was defined as the point where the storage modulus, G', was reduced by 3%.

Results and discussion

Sequence analysis

The deduced amino acid sequence was subjected to dbCAN analysis [22], leading to the identification of a putative PL7 domain; the protein was therefore named AMOR_PL7A (Figure S1). The presence of an N-terminal lipoanchor predicted by the LipoP server indicates that AMOR_PL7A is most likely secreted and anchored to the cell membrane. Alignment of AMOR_PL7A with available protein sequences from GenBank and the PDB showed that AMOR_PL7A has the highest sequence identity (46%) to two putative endo alginate lyases from *Rhodopirellula sp.* SWK7 and *Vibrio hyugaensis*, both of which belong to species commonly found in salt water [39, 40]. The most similar protein with a known structure is an alginate lyase called A1-II' (PDB id: 2CWS) from *Sphingomonas sp. A1* [41], with 29 % identity (Figure S1) within 83 % of the sequence. Another related enzyme with known structure is PA1167 from *Pseudomonas aeruginosa* [42](30% identity within 62 % of the sequence; Figure S1). Candidate key catalytic residues in AMOR_PL7A are His163 and Tyr256, which correspond to His191 and Tyr284 in A1-II' [43]. Additional active site residues previously shown to be crucial for A1-II' activity [41], are also present in AMOR_PL7A and include Arg118, Glu120, Arg122, Gln161 and Lys253 (Figure S1). Phylogenetic analysis (Fig. S3) showed that AMOR_PL7A does not belong to one of the five best known subfamilies of PL7s that were described by Lombard et al [44]. The protein groups with PL7s from a variety of other, mostly marine, bacteria, including thermophiles.

AMOR_PL7A was produced without its signal peptide and the cysteine putatively used for lipo-anchoring, and including a C-terminal His-tag (AHHHHHH). Typical yields of purified concentrated protein (Figure S2) were approximately 60 mg per 500 mL culture broth.

Characterization of AMOR_PL7A

The activity of AMOR_PL7A was characterized using commercially available sodium alginate (Sigma Aldrich) and purified polyM, polyG and polyMG (see materials and methods for details). Initial incubations of alginate with AMOR_PL7A resulted in increased absorbance at 235 nm, a clear indication of production of unsaturated oligosaccharides and thus alginate lyase activity [45]. Figures 1 and 2 show that AMOR_PL7A has a broad activity range in terms of temperature, pH and salinity. At pH 6.0, the enzyme has highest activity at 65°C, and more than 60 % of this activity is maintained in the temperature range of 60 °C to 80 °C (Figure 1A). At 65 °C, AMOR_PL7A has highest activity at pH 6.0, which is typical for alginate lyases of bacterial origin [46], and 70 % of maximal activity is maintained in a wide pH range spanning from pH 4.8 to 7.8 (Figure 1B). Figure 2 shows that AMOR_PL7A is active in a broad salinity range from 0 to 2M of NaCl, and has highest activity in seawater. The high tolerance to salts and broad pH range are useful in seaweed processing since these properties allow direct treatment, without applying pre-processing steps such as removal of salts [47] or buffering [48].

In the absence of substrate, the half-life of AMOR_PL7A at 65°C, pH 6.0, was approximately three hours (Figure 3). The high stability of AMOR_PL7A was confirmed by differential scanning calorimetry experiments which yielded apparent melting temperatures of approximately 71 °C both in buffer (pH 6.0) containing 500 mM NaCl and in pure seawater, whereas stability at lower salt concentrations (50 mM) was slightly reduced, but still high ($T_{m,app}$ = 64.1 °C) (Figure 4). To the best of our knowledge, AMOR_PL7A is one of the most thermoactive and thermostable PL7s known to date. Only one PL7, the M-type alginate lyase from *Sphingomonas* sp., seems to have similar thermophilic properties, having a reported temperature optimum of 70 °C [49], whereas other thermostable alginate lyases seem to operate optimally at lower temperatures [11,50–52]. The alginate lyase A1-II' shown in the alignment of Figure S1 lost the majority of its activity already after 10 minutes of incubation at 50°C [49]. The thermal tolerance of AMOR_PL7A is a highly desired property when used in combination with other enzymes whose optimum temperatures are high, such as the cellulolytic enzyme cocktail Cellic CTec 2, which works most efficiently at around 50 °C [53]. Thus, the use of AMOR_PL7A likely allows one-pot combined lyase-cellulase processing of seaweed biomass at high temperatures, as is shown below.

Using optimal conditions (pH 6.0, 500 mM NaCl, 65°C) steady state kinetic analysis of AMOR_PL7A activity on sodium alginate (Figure S4) yielded a K_M of 0.51 +/- 0.09 mg/mL and a k_{cat} of 7.8 +/- 0.3 s^{-1}. These values are in the same order of

magnitude as for other kinetically characterized alginate lyases (e.g. [34,54]). At 0.5% (w/v) substrate concentration, AMOR_PL7A showed similar reducing-end liberating activities for sodium alginate and PolyM, whereas activity towards PolyG and PolyMG was very low (Figure 5). The progress curves in Figure 5 indicate specific activity on alginate in the order of 3.8 μmol/mg.min, whereas the initial specific activity towards PolyM may be up to three times higher. Clearly, AMOR_PL7A is an M-specific alginate lyase.

Figure 6A shows that AMOR_PL7A generates oligosaccharides from polyM with an unsaturated hexenuronic acid residue, 4-deoxy-L-erythro-hex-4-enepyranosyluronate (Δ), at the non-reducing end and hence is a true alginate lyase. After one hour of degradation, oligomers of DP3-40 were observed in low amounts, whereas after 24 hours, there were only trace amounts left of oligomers with DP >5. Addition of fresh AMOR_PL7A after 24 hours followed by incubation for another 24 hours led to only minor additional degradation, reflected in a small reduction of ΔM5. Using M12 and M24 as substrates, we observed a mixture of saturated and unsaturated products after 24h incubation (Figure 6B), confirming that the enzyme is endo-acting.

Saccharification of brown seaweed (*Saccharina latissima*) at 55°C

The industrial applicability of AMOR_PL7A was assessed in degradation of milled and dried *S. latissima* with the commercial cellulase cocktail Cellic Ctec2 at 55°C for 24 h in seawater (pH 6.8; no buffer added). Both AMOR_PL7A and Cellic Ctec2 were expected to work at those conditions, whereas commercial alginate lyase from Sigma Aldrich has an optimal activity at lower temperature (37°C, according to the supplier's data sheet). Various enzyme combinations were compared based on their effectiveness in reducing viscosity and releasing glucose in reactions at an industrially relevant high-density solid loading (15 % DM). Inclusion of endo-type alginate lyases as such is not expected to directly improve glucose yield, but rather to make the reaction mixtures less viscous, thus likely increasing the efficiency of the cellulase cocktail. Figure 7A shows that inclusion of an alginate lyase leads to faster glucose release and about 25 % higher glucose yield at the final sampling point (24 h). Figure 7A also shows that AMOR_PL7A is more efficient than the commercial alginate lyase and this difference became more pronounced in experiments with a 40 times lower enzyme dosage (0.0135 mg/g rather than 0.7 mg/g lyase; Figure 7B). It is worth noting that, when using AMOR_PL7A, the enzyme dose could be lowered by 40-times (to 0.0135 mg/g DM) without altering the final glucose yield (compare Figures 7A and 7B).

Characterization of rheological properties are important to understand the behavior of polysaccharides such as alginate, which even at low concentrations significantly increases the viscosity of a solution. A typical measurement from an industrial perspective is viscosity (Pas). To further assess the impact of AMOR_PL7A on seaweed processing, we measured the viscosity of seaweed hydrolysates after 24 hours incubation at 55 °C in the absence and presence of an alginate lyase and Cellic Ctec2. Based on the rheological measurements, addition of an alginate lyase caused a large decrease in viscosity and AMOR_PL7A worked better than the commercial enzyme (Figure 8). Treatment with Cellic Ctec2 did not reduce the viscosity relative to a reaction without any added enzyme. Although direct comparison of the two enzyme samples is complicated by lack of information for the commercial enzyme, these results show that AMOR_PL7A is highly efficient for seaweed processing at higher temperatures. The AMOR_PL7A + Cellic Ctec2 hydrolysate was found to be much softer (lower G'; Figure S5) and weaker (lower shear stress at limit of LVR; Figure S6), than the other hydrolysates.

In conclusion, AMOR_PL7A, derived from the AMOR metagenomic dataset, is an M-specific alginate lyase (EC 4.2.2.3) that works optimally at 65 °C, pH 6.0, and has a broad tolerance to different NaCl concentrations. AMOR_PL7A is one of the most thermostable alginate lyases known to date (e.g.[11,50–52]). The enzyme is easy to produce and more efficient than an available commercial alginate lyase at reaction temperatures that are typical for other enzymes used in biomass processing, such as the Cellic CTec2 cocktail. Indeed, addition of small amounts of AMOR_PL7A improved glucose release from seaweed by Cellic CTec2 in terms of both speed and yield. This improvement was accompanied by a marked reduction in the viscosity of the reaction mixture. The present study adds to (a limited number of) previous studies on the effects of alginate lyases on saccharification by cellulase cocktails ([9–11]) by showing that AMOR_PL7A allows running simultaneous cellulase-lyase reactions at temperatures and dry matter concentrations that are higher than those used previously. AMOR_PL7A has the potential to increase the efficiency of enzymatic seaweed processing at large scale, not only because of the benefits of a fast reduction in viscosity (i.e. lower energy requirements for mixing and higher efficiency of other enzymes), but also because the enzyme works well in pure seawater, alleviating the need for additional chemicals or buffers.

It should be noted that the Cellic Ctec2 cocktail, used here as proof-of-principle, has been developed for conversion of lignocellulosic biomass. There are currently no commercial enzyme cocktails available for complete conversion of seaweed

to fermentable sugars. Since the market for seaweed-derived products and technologies for large-scale seaweed cultivations are expected to grow, there is a growing need for new enzyme cocktails designed specifically for seaweed processing. AMOR_PL7A may become part of such cocktails.

Supporting information

Amino acid sequence alignment of the catalytic domain of AMOR_PL7A with related PL7 alginate lyase domains with a known structure; SDS-PAGE analysis of purified AMOR_PL7A; phylogenetic analysis of AMOR_PL7A; steady state kinetics of AMOR_PL7A; oscillatory measurements of seaweed hydrolysates treated with different enzymes.

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Figure legends

Figure 1. Effect of temperature and pH on the activity of AMOR_PL7A. Experiments were conducted in triplicate and activity was determined by recording initial catalytic velocity. The reactions were incubated up to 50 minutes. Reactions giving non-linear progress curves, which implies that only a few early sampling points could be used to estimate activity, are indicated by an asterisk. Activity was normalized to 100% for the most active sample. The temperature optimum was determined using 50 mM sodium acetate buffer, pH 6.0; the pH optimum was determined at 65 ^{o}C.

Figure 2. Salt tolerance of AMOR_PL7A at 65°C, pH 6.0. A reaction in seawater (no buffer or added salts) was also performed. The displayed activities reflect initial velocities, derived from a linear increase in absorbance at 235 nm during a 50-minute incubation at 65°C.

Figure 3. Stability of AMOR_PL7A at 65 °C. AMOR_PL7 (0.1 μM) was pre-incubated at 65 °C in 50 mM Tris-HCl, pH 6.0, with 500 mM NaCl without substrate for 0 to 24 hours, followed by assessment of remaining enzyme activity (initial velocity) at 65 °C. All reactions were carried out in triplicate and activity was normalized to 100% for the most active sample. Note that the presence of substrate stabilizes the enzyme as shown by the linearity of progress curves obtained under standard assay conditions, at 65 ^{o}C.

Figure 4. Stability of AMOR_PL7A assessed by Differential Scanning Calorimetry (DSC). The graph shows DSC thermograms for AMOR-PL7 (1.3 mg/mL) in 50 mM NaOAc, pH 6.0, 50 mM NaCl (dashed line, apparent T_m, $T_{m,app}$ = 64.1°C), 50 mM NaOAc, pH 6.0, 500 mM NaCl (dotted line, $T_{m,app}$ = 71.5°C) NaCl, and unbuffered seawater (solid line, $T_{m,app}$ = 70.5°C). The protein samples were heated at a rate of 1°C/min and protein unfolding was irreversible.

Figure 5. Reducing end formation (mM/mL) from sodium alginate, polyM, polyG and polyMG. The reaction mixtures contained 12.7 nM of AMOR_PL7A and 5 mg/mL of substrate in Tris HCl, pH 6.0, containing 250 mM NaCl and were incubated at 65 ^{o}C. The reactions were carried out in triplicates and the values presented represent the mean ± standard deviation.

Figure 6. Degradation of polyM by AMOR_PL7A. Panel A shows products generated from polyM after 0h, 1h, 4h, 24h incubation in seawater, at 65°C. "24 + 24h" represents a sample where fresh enzyme was added after 24 hours, followed by another 24 h incubation. The major peaks correspond to M-chains of varying DP with an unsaturated non-reducing end, i.e. a 4-deoxy-L-erythro-hex-4-enepyranosyluronate, denoted Δ. Panel B shows products obtained upon 1) acid hydrolysis of PolyM, 2) degradation of PolyM using a previously characterized M-lyase from *Haliotis tuberculata*[14], and degradation of 3) M12 and 4) M24 by AMOR_PL7 in seawater at 65°C for 24h. Reactions 3 and 4 show mixtures of saturated and unsaturated products.

Figure 7. Enzymatic release of glucose. The graphs show release of glucose from *S. latissima* at 15 % solid loading, in seawater, at 55 °C. The enzyme doses were 6.3 mg for Cellic Ctec2 and 0.7 mg (A) or 0.0175 mg (B) of AMOR_PL7A or the commercial alginate lyase (Sigma) per g DM. The reactions were carried out in triplicates and the values presented represent the mean ± standard deviation.

Figure 8. Viscosity (Pas) of hydrolyzed seaweed at different shear rates (1/s). Viscosity was measured after 24 hours incubation of *S. latissima* (15 % DM) in seawater at 55°C with no added enzymes (Control), and with 6.3 mg per g DM Cellic Ctec2 in the absence of an alginate lyase or in the presence of 0.7 mg per g DM Sigma Aly or AMOR_PL7A.

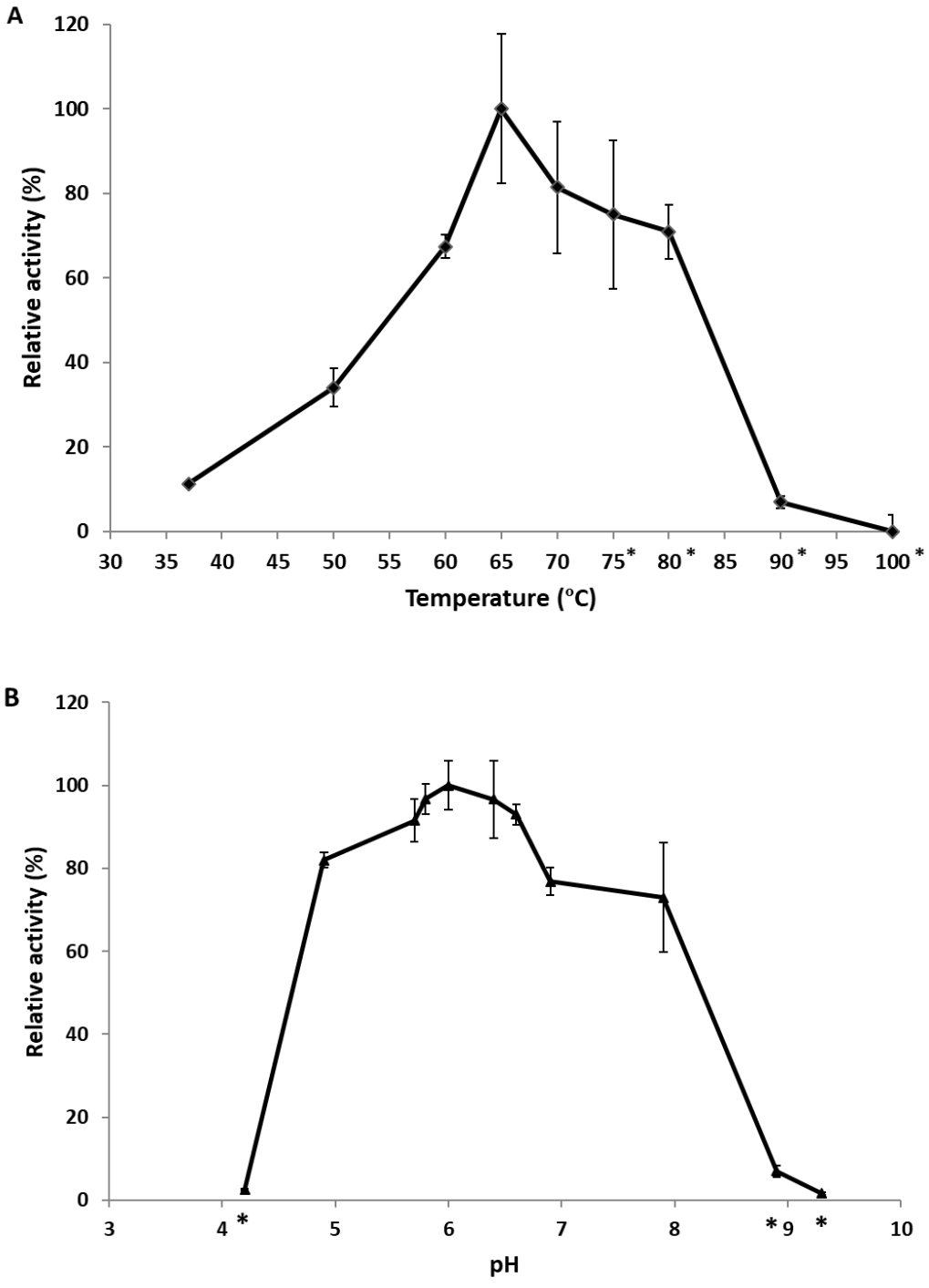


Figure 1 Effect of temperature and pH on the activity of AMOR_PL7A.

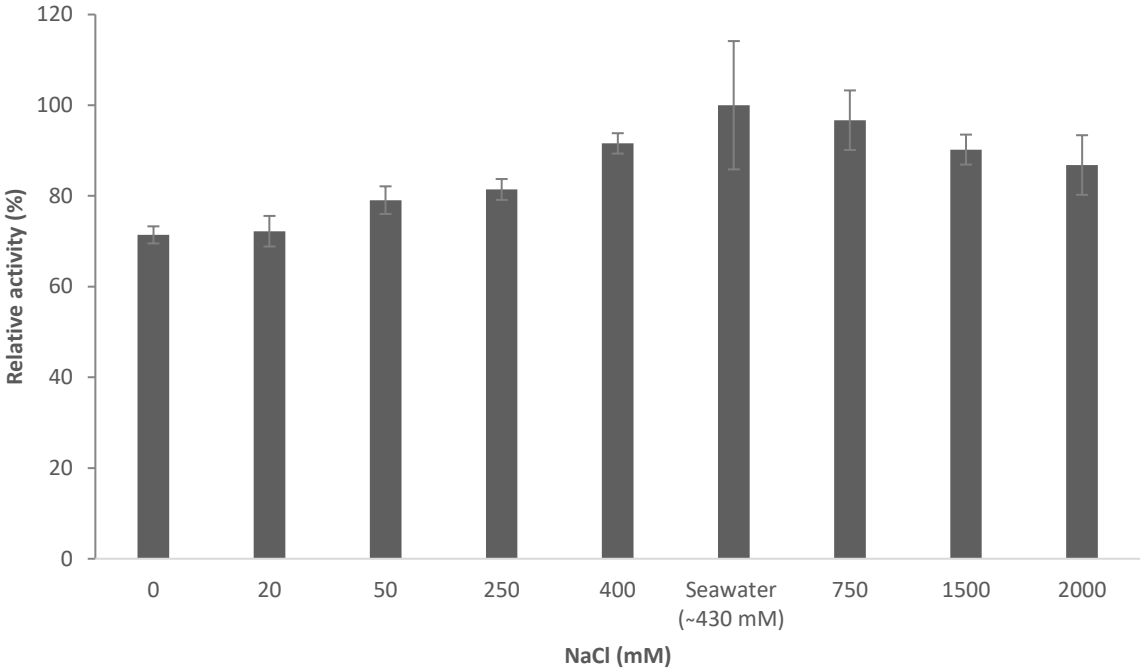


Figure 2 Salt tolerance of AMOR_PL7A at 65°C, pH 6.0.

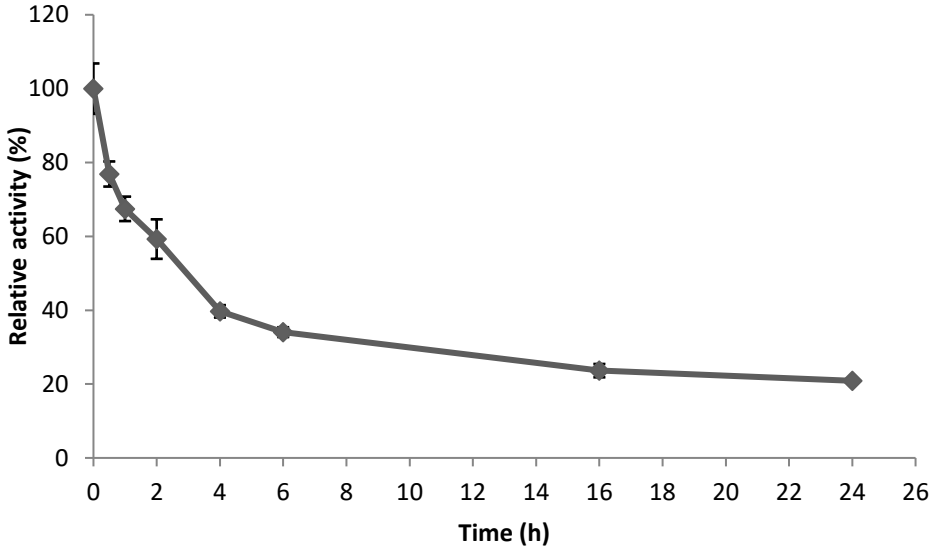


Figure 3 Stability of AMOR_PL7A at 65 °C.

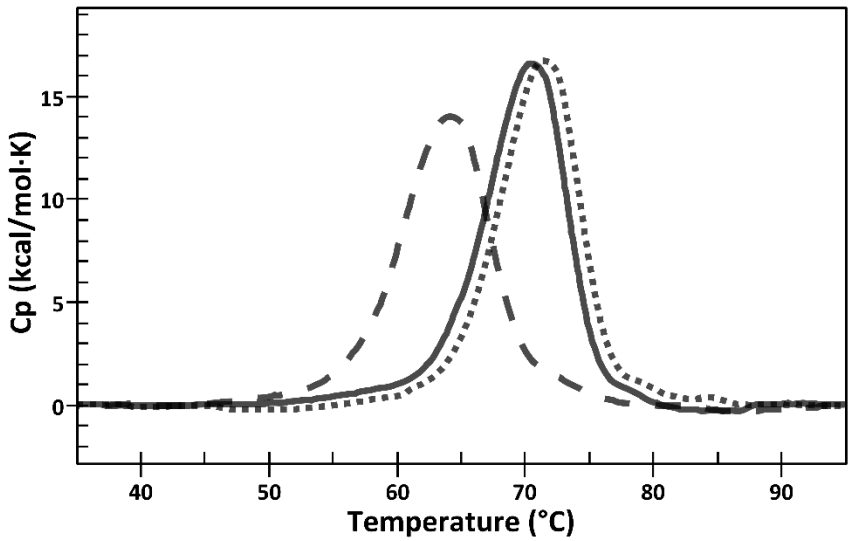


Figure 4 Stability of AMOR_PL7A assessed by Differential Scanning Calorimetry (DSC).

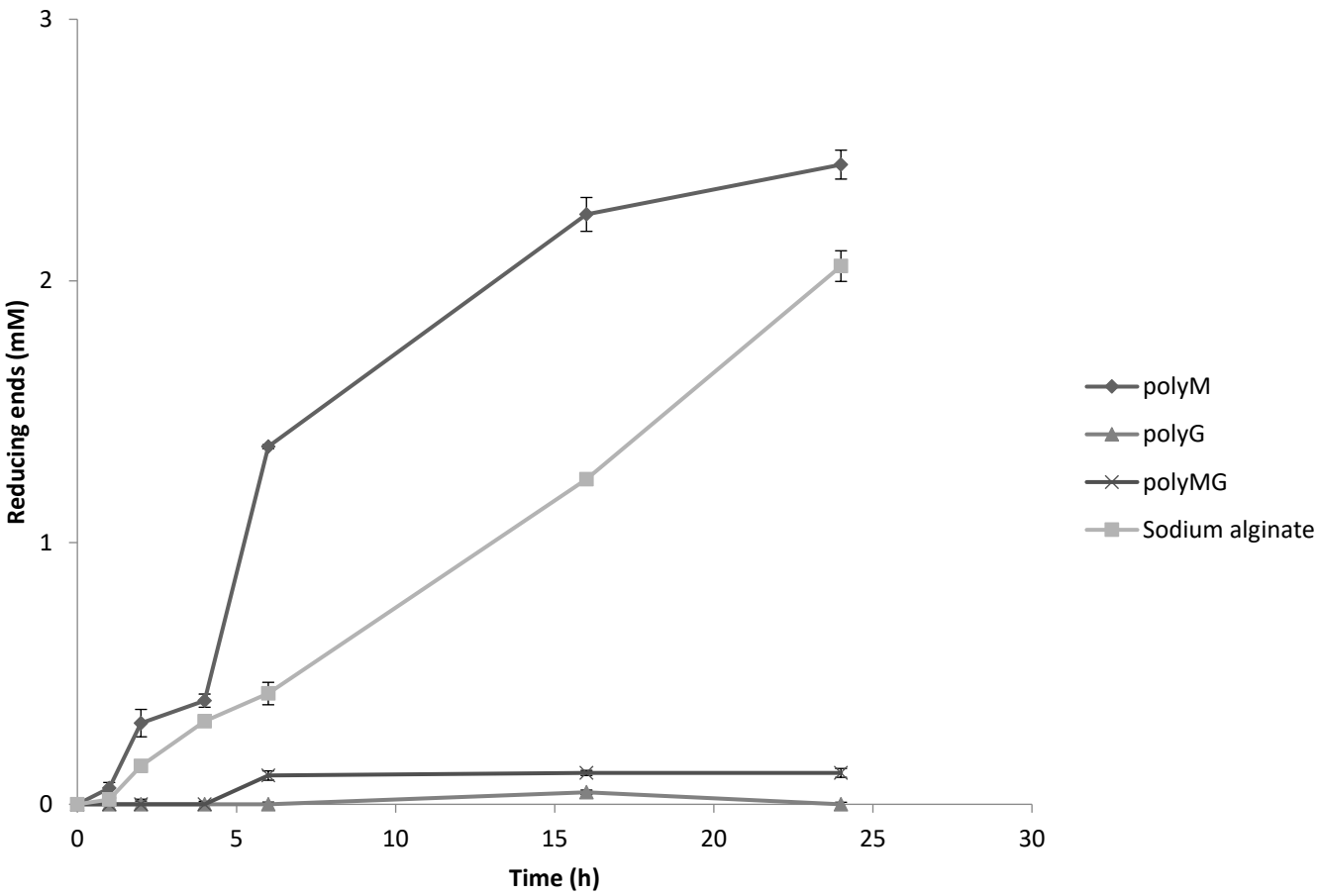


Figure 5 Reducing end formation (mM/mL) from sodium alginate, polyM, polyG and polyMG.

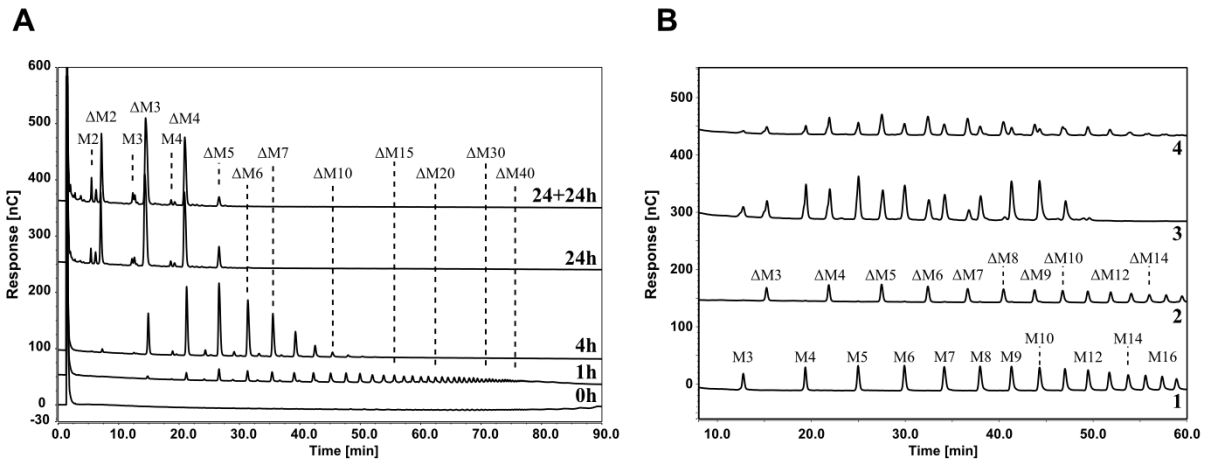
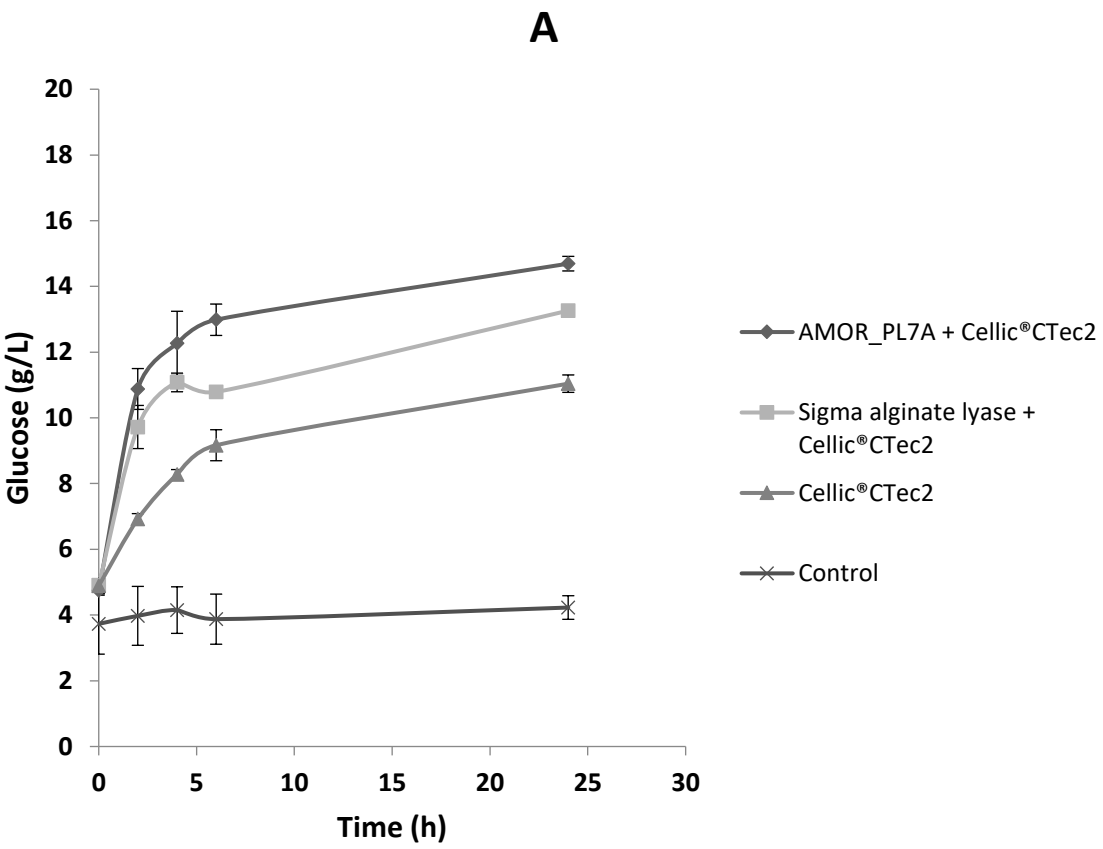


Figure 6 Degradation of polyM by AMOR_PL7A.


A
Glucose (g/L)
Time (h)
0
2
4
6
8
10
12
14
16
18
20
0
5
10
15
20
25
30
AMOR_PL7A + Cellic®CTec2
Sigma alginate lyase + Cellic®CTec2
Cellic®CTec2
Control

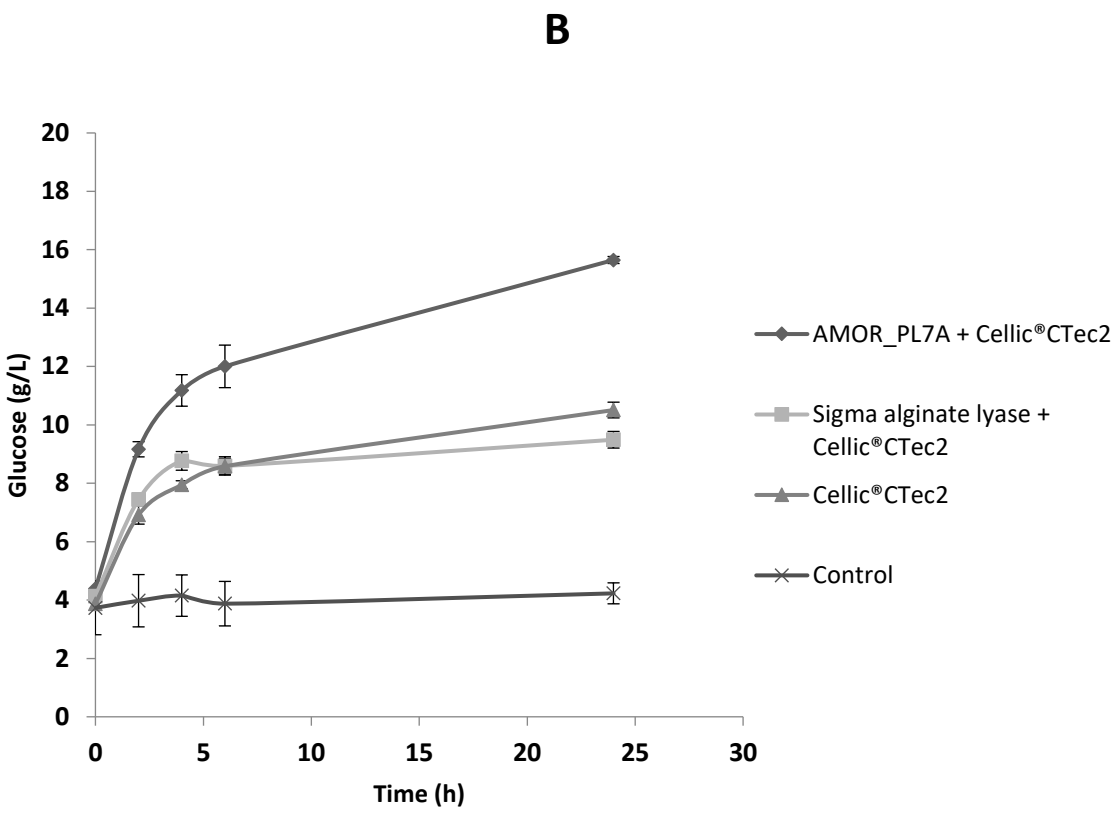


Figure 7 Enzymatic release of glucose.

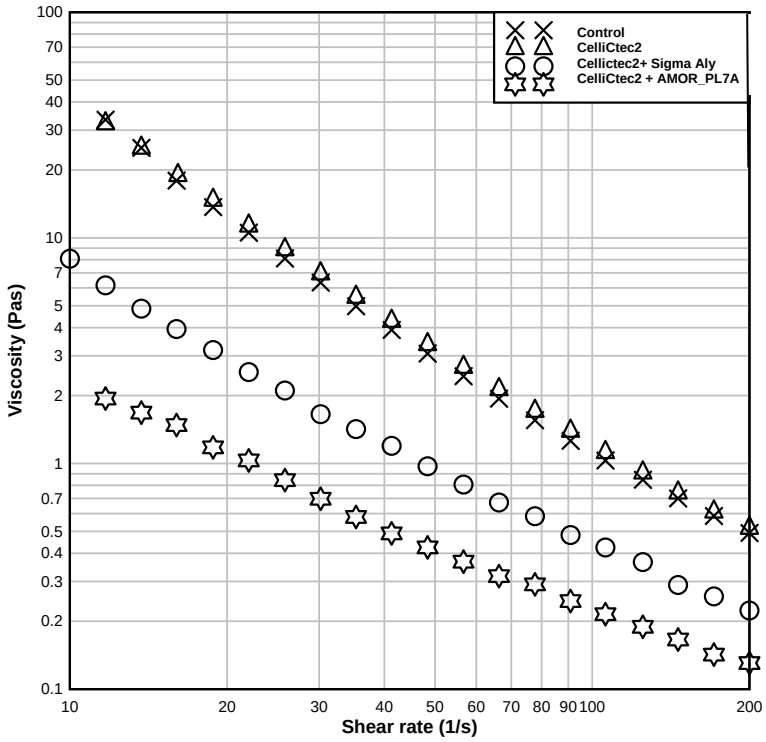
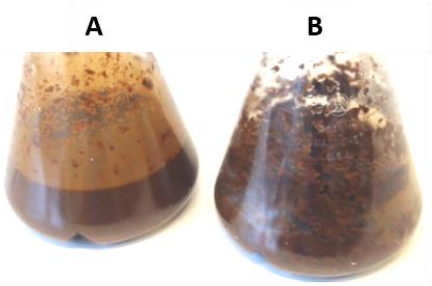


Figure 8 Viscosity (Pas) of hydrolyzed seaweed at different shear rates (1/s).

Toc graphic



Reduction of viscosity in seaweed hydrolyzed with an alginate lyase AMOR_PL7A (A) and control without (B).