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In-process epimerisation of alginates from *Saccharina latissima*, *Alaria esculenta* and *Laminaria hyperborea*

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ABSTRACT

Alginates are valued in many industries, due to their versatile properties. These polysaccharides originate from brown algae (Phaeophyceae) and some bacteria of the *Azotobacter* and *Pseudomonas* genera, consisting of 1 → 4 linked β-D-mannuronic acid (M), and its C5-epimer α-L-guluronic acid (G). Several applications rely on a high G-content, which confers good gelling properties. Because of its high natural G-content (F_G = 0.60–0.75), the alginate from *Laminaria hyperborea (LH)* has sustained a thriving industry in Norway. Alginates from other sources can be upgraded with mannuronan C-5 epimerases that convert M to G, and this has been demonstrated in many studies, but not applied in the seaweed industry. The present study demonstrates epimerisation directly in the process of alginate extraction from cultivated *Saccharina latissima (SL)* and *Alaria esculenta (AE)*, and the lamina of *LH*. Unlike conventional epimerisation, which comprises multiple steps, this in-process protocol can decrease the time and costs necessary for alginate upgrading. In-process epimerisation with AlgE1 enzyme enhanced G-content and hydrogel strength in all examined species, with the greatest effect on *SL* (F_G from 0.44 to 0.76, hydrogel Young's modulus from 22 to 34 kPa). As proof of concept, an upscaled in-process epimerisation of alginate from fresh *SL* was successfully demonstrated.

1. Introduction

Alginates are a family of linear, anionic polysaccharides, consisting of 1 → 4 linked β-D-mannuronic acid (M), and its C5-epimer α-L-guluronic acid (G) (Fischer & Dörfel, 1955). The residues are organised in homopolymeric regions (M- and G-blocks), that are interspersed by alternating regions (MG-blocks) (Haug, 1964). Commercial alginates are derived from the extracellular matrix of brown algae, but they can also be found in some encapsulating water- and soil bacteria in the *Azotobacter* and *Pseudomonas* genera (Gorin & Spencer, 1966; Linker & Jones, 1966). Alginates are highly valued in industrial applications such as food, feed, biomaterials, and biomedicine due to their high natural abundance from renewable biomasses, biocompatibility, biodegradability, high intrinsic viscosity, and ability to form thermostable hydrogels in the presence of divalent cations (Andersen et al., 2012; Smidsrød & Haug, 1968).

Each year, 95,000 tons (dry weight) of brown algae are harvested worldwide for alginate production (Peteiro, 2018). By commercial harvest of the wild growing *Laminaria hyperborea (LH)*, Norway alone contributes to 16 % of this biomass (Steen, 2022). This species, particularly the stipe, supplies high-quality alginates due to a high fraction of G (F_G), which is reported to range between 0.60 and 0.75 (Moe et al., 1995; Skjåk-Bræk et al., 2016). More specifically, the stipe alginate has a high fraction of long G-blocks, which provides favourable hydrogel properties such as high mechanical rigidity (Nordgård & Draget, 2021). The alginate from the lamina of *LH* has a lower G-content (F_G = 0.49) than the stipes (Skjåk-Bræk et al., 2016; Tøndervik et al., 2020), and consequently *LH*-lamina alginate has a lower value. *LH* has been harvested commercially in Norway for >80 years, and each year 150,000 tons (wet weight) are harvested with an annual export value of roughly 134 million USD (Johannesen, 2015). However, due to the impact of bottom trawling on coastal ecosystems (Mac Monagail et al., 2017) and inadequate bathymetric conditions for trawling in many areas along the coast, there is an upper limit to annual *LH* outtake. Global demand for alginate is increasing, particularly in biomedical and pharmaceutical applications, such as in wound healing, delivery of bioactive agents and

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cell transplantation (Lee & Mooney, 2012). As a result, new sources of high-quality alginates will be needed to meet the expected demands for the coming years.

Seaweed cultivation is a new, but rapidly growing sector in Europe. Mainly two species are cultivated in Norway, *Saccharina latissima (SL)* and *Alaria esculenta (AE)*. Since 2017 the production volume of these two species has been a few hundred tons (Directorate of Fisheries, 2023), but because of excellent coastal conditions, cold, pristine water with high salinity and nutrient access, this production has the potential to be scaled up to reach millions of tons annually (Skjermo et al., 2020). These two cultivated species offer a new, renewable, and reliable source of biomass suitable for alginate production, with extractable alginate yields ranging from 20 to 25 % of the algal dry weight (Nøkling-Eide et al., 2023). However, when compared to *LH* stipe alginate, the *SL* and *AE* alginates have a lower F_G and shorter G-blocks (Table 2), making them less valuable. As the production costs for cultivated seaweed are still considerably higher than for wild harvested seaweed, targeting high-value applications will be important to make establishment of biorefineries for cultivated seaweed feasible.

Alginates can be enzymatically modified in vitro by mannuronan C-5 epimerases, which converts M to G (Fig. 1), changing the alginate's sequential pattern (Ertesvåg & Skjåk-Bræk, 1999; Petersen et al., 2023). The first mannuronan C-5 epimerase was discovered and isolated from *A. vinelandii* over 50 years ago (Haug & Larsen, 1969), and it has been known for >20 years that these enzymes can be used to upgrade alginates (Ertesvåg et al., 1999). It has been identified 6 extracellular, calcium-dependent epimerases from *A. vinelandii* (AlgE1-6), all with different modular structure and epimerisation pattern in the final product (Ertesvåg et al., 1995; Petersen et al., 2023; Svanem et al., 1999). Molecular size varies in these epimerases which are composed of one or two A-modules that contain the catalytic site and one to seven R-modules that contribute to substrate and calcium binding, creating long G-blocks (AlgE1-1, AlgE6), short G-blocks (AlgE2, AlgE3-1 and AlgE5) or MG-blocks (AlgE4, AlgE1-2, AlgE3-2). AlgEs have been applied to alginates from seaweeds in numerous studies, where the already extracted, precipitated and purified alginates typically are epimerised in a buffer containing NaCl and $CaCl_2$ (Campa et al., 2004; Mørch et al., 2008; Strand et al., 2003; Svanem et al., 2001; Tøndervik et al., 2020).

To our knowledge, epimerisation of seaweed alginates is not done in the industry today, mainly due to the additional cost and time involved in the alginate refining from harvested biomass and the lack of commercially available mannuronan C-5 epimerases. The present study aimed to epimerise alginates from cultivated *SL* and *AE* and the lower-valued lamina of *LH* "in-process", which entails that the epimerases are added directly during the alginate extraction process rather than to precipitated and purified alginates. The hypothesis was that it is possible to obtain an equally effective epimerisation in-process as in buffer, and that the epimerised alginates possess similar chemical and physical properties as high-value alginates. This is to our knowledge the first study describing in-process epimerisation of alginates from *SL*, *AE*, and *LH*.

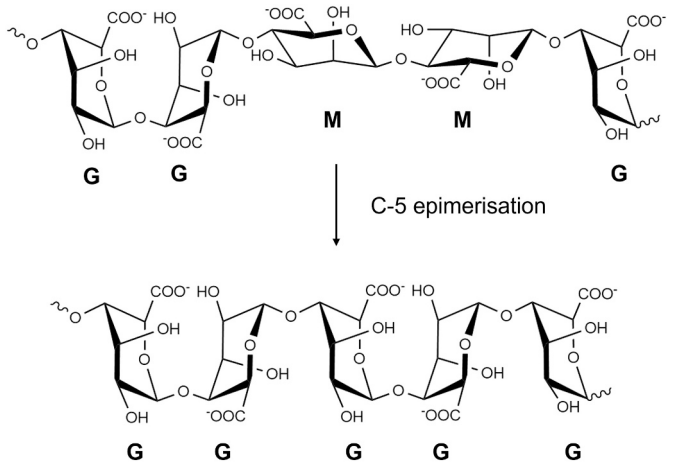

Fig. 1. C-5 epimerisation in alginate includes a $^4C_1$ to $^1C_4$ transition of the epimerised residues, and β-D-mannuronic acid (M) is converted to α-L-guluronic acid (G). In the five residues represented in this figure, the alginate structure changes from GGMMG to GGGGG.

2. Material and methods

2.1. Seaweed supply

The two cultivated species used in this study, *SL* and *AE*, were supplied by Seaweed Solutions (Trondheim, Norway), and harvested on the east coast of Frøya (N 63°44.672′, E 8°53.198′), Norway. Sporelings from both species were cultivated on ropes deployed 2 m below the sea surface in January 2022. *SL* and *AE* were harvested on the 6th of June 2022 and the 3rd of May 2022, respectively. The wild growing *LH* was supplied by Nutrimar (Kverva, Norway) and was harvested on the 16th of November 2022 on the west coast of Frøya (N 63°45.185′, E 8°15.928′) at various depths, and stored refrigerated for 2 days. In both harvesting areas, the water is classified as being euhaline (>30 PSU, practical salinity unit) and highly wave exposed. A correct species identification was guaranteed by the presence of a midrib in *AE*, and a thick round stipe in *LH* (Fig. 2). Following harvest, the seaweed lamina was separated from the stipe and holdfast, and the lamina was transported in a cooled container (without seawater) for approximately 3 h, prior to further processing. The seaweed was milled using an industrial meat grinder with a grate hole diameter of 6 mm, prior to freezing (−20 °C).

2.2. Alginate extraction and epimerisation

2.2.1. Alginate extraction

The alginate extractions, including water rinsing-, acid- and alkaline-steps, were done in triplicates. If nothing else indicated, all chemicals used in this study were of analytical grade and purchased from Sigma-Aldrich (supplementary material). The frozen, milled *SL*, *AE* and *LH* lamina were defrosted, and rinsed with fresh water at low pH to release water soluble compounds such as fucoidan and laminarin (Birgersson et al., 2023). 100 g seaweed was added to 800 mL of ion-free water in 1 L flasks, and the pH was adjusted to 4.5 with 1 M HCl. The seaweed was incubated for 1 h under shaking (100 rpm, orbital movement 2.5 cm amplitude) at 50 °C. Then, the water fraction was removed by filtering (mesh size 295 μm) and weighed. The seaweed was then washed with acid as described in Nøkling-Eide et al. (2023): 800 mL 0.2 M HCl was added to the flasks, and the seaweed was incubated for 20 h under shaking (100 rpm, orbital movement 2.5 cm amplitude, Eppendorf® New Brunswick™ Innova® 42/42R Incubator Shaker), at 20 °C and pH 1. After incubation, the acid fraction was removed by filtering (mesh size 295 μm) and weighed. The seaweed was washed with ion-free water for 30 min, before the water was removed by filtering (mesh size 295 μm). To extract alginate, 700 mL ion-free water was added to the seaweed flasks and the pH was adjusted to 7 with 1 M NaOH. The ratio between seaweed and liquid was chosen based on optimal conditions for maximising alginate yields (Nøkling-Eide et al., 2023). The seaweed was incubated under shaking (100 rpm, orbital movement 2.5 cm amplitude) for 1 h at 20 °C. The alginate solution was separated from the residual seaweed by centrifugation (10,628 ×*g*, 15 min, Thermo Scientific™ Sorvall LYNX 6000 Superspeed Centrifuge) followed by filtration (mesh size 295 μm).

2.2.2. Epimerisation

In-process epimerisation reactions were performed with AlgE1 enzyme (supplied by AlgiPharma AS). The activity of AlgE1 was measured to 119 U/mg lyophilised enzyme, as described previously by

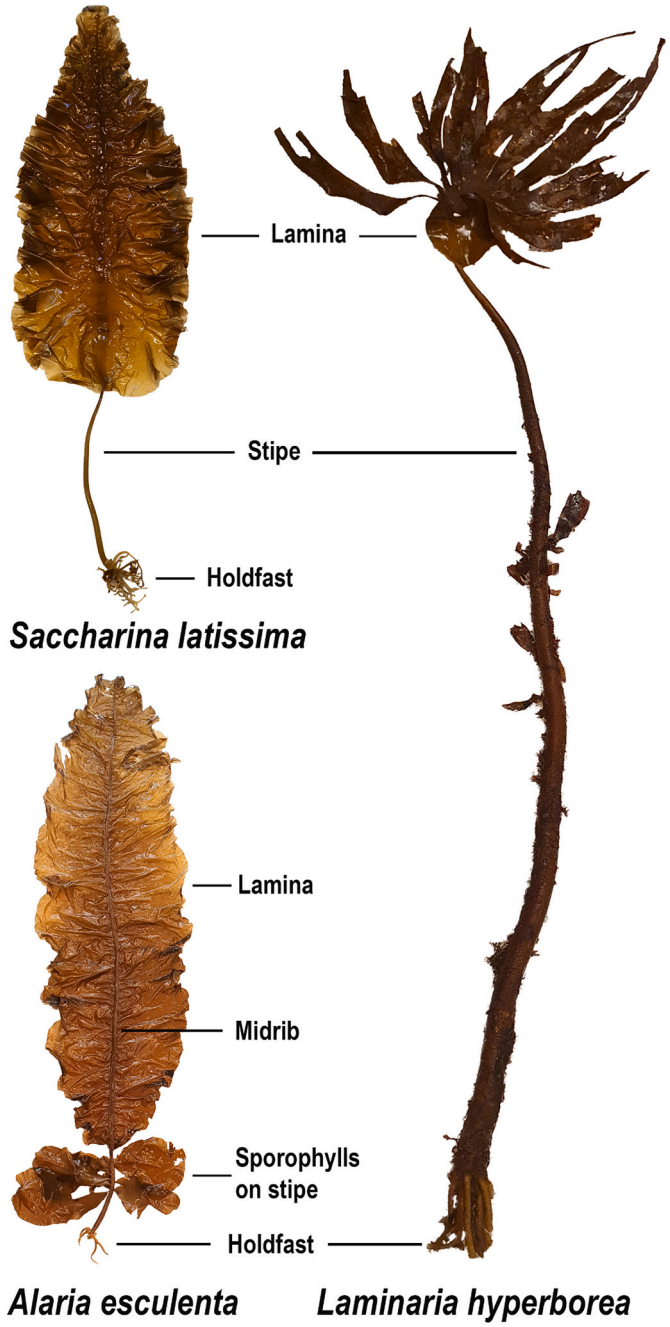

Fig. 2. Illustrative photos of *Saccharina latissima*, *Alaria esculenta* and *Laminaria hyperborea*. Only the lamina of the three species were characterised and epimerised in this study.

Stanisci et al. (2018). For epimerisation in each species, the alginate solutions were distributed in six flasks, half serving as control reactions (175 mL × 3 flasks), and half serving as in-process epimerase reactions with AlgE1 (175 mL × 3 flasks). NaCl (100 mM) and $CaCl_2$ (2.5 mM) were added to all flasks to a total volume of 200 mL, giving alginate concentrations of 0.18, 0.18 and 0.15 % (w/v) in *SL*, *AE*, and *LH*, respectively. Additionally, 2.94 mg AlgE1 was added to the epimerase reaction flasks, corresponding to enzyme concentrations of 939, 1142 and 1147 U/g alginate in *SL*, *AE*, and *LH*, respectively (Table S1, supplementary material). The reactions were carried out under shaking (100 rpm, orbital movement 2.5 cm amplitude, Eppendorf® New Brunswick™ Innova® 42/42R Incubator Shaker) for 60 h, at 20 °C and pH 7.

As a positive control to in-process epimerisation, conventional epimerisation reactions were also performed on precipitated and redissolved alginates at 0.1 % w/v in a buffer containing MOPS (40 mM), NaCl (100 mM), $CaCl_2$ (2.5 mM), at 20 °C, pH 6.9 and AlgE1 at 1000 U/g alginate. The reactions were done in triplicates for all three species, with a corresponding triplicate of enzyme-free controls.

Finally, an upscaled in-process epimerisation of alginates from fresh *SL* was included as a proof of concept. Fresh *SL* harvested 6th of June 2023 was processed in a hydrolysis tank (PilotOrb) with AlgE1 (1762 U/g alginate, Table S1, Supplementary material), using 3 kg biomass in 21 L ion-free water, and adjusted to pH 7 with 1 M NaOH. To understand the initial reaction kinetics, 40 mL alginate was sampled every hour for the first 6 h and analysed further.

After epimerisation, the alginates were precipitated with acid, neutralised, precipitated with 50 % ethanol, and then washed twice in 70 and 96 % ethanol, respectively. The precipitated alginates were lyophilised and weighed prior to characterisation (Christ Beta 1–8 LSCbasic freeze dryer, pressure 1 mbar, condenser temperature −55 °C).

2.3. Characterisation

The seaweed raw material, the water fraction from the alginate extraction, and the precipitated alginates were characterised by several methods described below.

2.3.1. Dry matter and ash analysis

Dry matter (DM) was measured in the raw material of *SL*, *AE* and *LH*, and the water fraction obtained during alginate extraction, to decide the leakage of dry matter in this step. For all species, approximately 5 g fresh, milled biomass, and the lyophilised matter (Christ Beta 1–8 LSCbasic freeze dryer, pressure 1 mbar, condenser temperature − 55 °C) from the rinsing water wash was weighed out in crucibles, in triplicates. The samples were dried at 105 °C in 22 h in a VWR® VENTI-Line® 56 Prime drying oven, and then cooled down to approximately 20 °C in a desiccator. Each crucible was weighed, and the dry matter content calculated based on the seaweed and water fraction wet weight (ww) and Eq. (1):

$$\mathrm{DM}(\%\text{of ww}) = \frac{\text{Total weight after drying} - \text{weight crucible}}{\text{ww}} \times 100\% \quad (1)$$

Ash content was decided in the raw materials of *SL*, *AE*, and *LH* by incinerating the samples in an ashing furnace (L 24/11 BO, Nabertherm) at 550 °C for 12 h. Ash content was calculated based on Eq. (2):

$$\mathrm{Ash}(\%\text{of DM}) = \frac{\text{Total weight after combustion} - \text{weight crucible}}{\text{DM}} \times 100\% \quad (2)$$

2.3.2. Elemental analysis

The content of carbon (C), nitrogen (N), hydrogen (H) and sulfur (S) in the raw material of *SL*, *AE* and *LH* was determined by weighing approximately 5 mg lyophilised sample in tin capsules. The samples were oxidised at 1150 °C and analysed on a Vario-El-Cube CHNS element analyser (Elementar).

Further, the concentrations of Na, Ca, K, Mg, P, Cl, I, Br, Fe, As, Zn, Ba, Mn, Cu, Cd, Se and Pb were measured by an Agilent 8800 Triple Quadrupole ICP-MS (Agilent Technologies, USA) with ISIS (Integrated Sample Introduction System), SPS4 autosampler (Agilent Technologies, USA) and a standard sample introduction system (Micro Mist glass concentric nebulizer, quartz double pass spray chamber, quartz torch with 2.5 mm id and standard nickel cones). He and O_2 were used for cell reactions. All solutions and dilutions were prepared using ultra-purified water 18.2 MΩ from a OmniaTap 10 UV system (Stakpure, Germany) and concentrated nitric acid (65 % HNO_3) purified by a Savillex DST-100 Acid Purification System (Savillex, USA) or 25 % tetramethylammonium hydroxide (TMAH, Acros). Standards for calibration curves

were prepared from single element and mixed standard solutions from Inorganic Ventures, USA. Na, Mg, P, S, K, Ca, Fe, Cu, Zn, As, Se, Cd, Ba and Pb were diluted in 5 % HNO_3 (v/v), while 1 % (v/v) TMAH were used for chlorine (Cl), bromine (Br) and iodine (I). Indium (In) and tellur (Te) were used as internal standards. 200 mg lyophilised seaweed samples were digested with 5 mL 50 % (v/v) nitric acid (HNO_3) at 250 °C in an UltraWAVE microwave oven (Milestone, Italy) and diluted to 5 % (v/v) upon analysis. Samples for Cl, Br and I analysis were extracted in parallel with 5 mL 20 % (v/v) TMAH at 80 °C in a bead bath for 20 h and diluted to 1 % (v/v) upon analysis.

2.3.3. Molecular weight analysis

To decide molecular weight of the extracted alginates, the standard protocol from ASTM (American Society for Testing and Materials) was followed (ASTM F2605-08). Size-exclusion chromatography (SEC) with online multi-angle static laser light scattering (MALS) were performed at ambient temperature on an Agilent 1260 Infinity II system consisting of a solvent reservoir, isocratic pump, automatic sample injector, pre-column (OHpak LB-G 6B guard) and a main column (OHpak LB 806 M). The column outlet was connected to a Dawn HELEOS-II multi-angle laser light scattering photometer (Wyatt, U.S.A.) ($\lambda 0 = 663.8$ nm) followed by RI-501 refractive index detector (Shodex). The eluent was 0.15 M $NaNO_3$ with 0.01 M EDTA (pH = 6.0) and the flow rate was 0.5 mL/min. Alginate samples were dissolved in mobile phase (c = 0.5 mg/mL) for 20 h and filtered (pore size 0.45 μm) before injection. All samples were injected three times, with injection volumes of 50 μL. Data were collected and processed (with dn/dc = 0.150 mL/g and $A_2 = 5.0 \cdot 10^{-3}$ mL·mol/g^2) using Astra (version 7.3.21) software (Wyatt, USA).

2.3.4. ^{1}H NMR characterisation

The alginates were analysed by following the ASTM standard test method for determining the chemical composition, including sequential parameters, of alginate by NMR spectroscopy (ASTM Standard F2259-10, 2012) and as described before (Ertesvåg & Skjåk-Bræk, 1999; Grasdalen, 1983; Grasdalen et al., 1981). In summary, the alginates were partially degraded by stepwise acid hydrolysis to an approximate degree of polymerization (DP) of 70 for ^{1}H NMR analysis. 20 mg alginate was dissolved in ion-free water, pH adjusted to 5.6 with 0.1 M HCl, and hydrolysed at 95 °C for 1 h. The solutions were swiftly cooled to approximately 20 °C and then pH adjusted to 3.8, followed by hydrolysis at 95 °C for 50 min. The solutions were again cooled to approximately 20 °C, neutralised with 0.1 NaOH (pH 6.8–7.5) and lyophilised (Christ Beta 1–8 LSCbasic freeze dryer, pressure 1 mbar, condenser temperature −55 °C).

Approximately 10 mg of the hydrolysed samples were dissolved in 600 μL D_2O (d-99.9 %; Sigma-Aldrich) and dissolved for 20 h. Triethylenetetramine-hexaacetic acid (TTHA) in D_2O (0.3 M, 20 μL) was added as chelator. The samples were centrifuged, and the supernatants were transferred to NMR tubes. 3-(Trimethylsilyl)-propionic-acid sodium salt (TSP) (Aldrich, Milwaukee, WI) in D_2O (1 %, 5 μL) was added for internal chemical shift reference. ^{1}H NMR spectra were recorded at 82 °C on a BRUKER NEO 600 MHz instrument (Bruker BioSpin AG, Fälladen, Switzerland) equipped with 5 mm iProbe TBO. Following signals are integrated for the analysis of the NMR data: ~5.05 ppm (G, H1) = A; ~4.75 ppm (GGM, H5) = B1; ~4.72 ppm (MGM, H5) = B2; ~4.70 ppm (MG, H1) = B3, ~4.65 ppm (MM, H1) = B4;~ 4.45 ppm (GG, H5) = C, where underline indicate uronate residue, H which proton giving rise to the signal, and letter is used as name for the calculation. The calculations for the fractions (F_x) of monomers, diads, and triads in alginate are presented in the supplementary material. The spectra were recorded using TopSpin 4.2 software (Bruker BioSpin) and processed and analysed with TopSpin 4.0.7 software (Bruker BioSpin).

2.3.5. Viscosity measurement

The shear viscosity of the alginates was measured using a Kinexus rheometer (Netzsch, Germany) equipped with an upper double gap bob geometry (25 mm OD) and a lower double gap system cup (D025 C0057 SS). The alginates (triplicates A-C combined) were dissolved in ion-free water to a concentration of 1 % w/v and the viscosity was measured over a shear rate of 0.1–100 s^{-1} with 10 measurements per decade, at 25 °C.

2.3.6. Hydrogel formation and characterisation

To prepare alginates with lower molecular weights suitable for gelling experiments (M_w = 130–150 kDa), the extracted alginates were mechanically depolymerised using a wet pulverizing system (StarBurst Mini HJP-25001H Sugino Machine Ltd., Japan). A 0.2 % alginate solution was forced through a nozzle (d = 100 μm) at high shear rate, by applying pressure (150 MPa) during one pulverizing cycle. The alginate solution collided with a ceramic ball, and the depolymerised product was collected and lyophilised (Christ Beta 1–8 LSCbasic freeze dryer, pressure 1 mbar, condenser temperature −55 °C). The new molecular weights were decided using SEC-MALS (described above).

Alginate gels cylinders were prepared with glucono-δ-lactone (GDL) and dispersed $CaCO_3$, as described by Draget et al. (1989): $CaCO_3$ (4 μm average particle size) was added to 1 % depolymerised alginate solutions to a concentration of 15 mM, followed by degassing with vacuum for 10 min. GDL was added to the suspension at a concentration of 30 mM, followed by careful stirring for 10 s before transferring the solution to casting molds and leaving the gels to set. After 20 h, the gels were saturated with Ca^{2+} by submerging them in a solution of 50 mM $CaCl_2$ in 200 mM NaCl for 24 h at 25 °C. Gel strength (Young's modulus, E) was measured as the force necessary to compress the gels 0.2 mm at a StableMicroSystem TA.XT.plus Texture Analyser with a P/35 flat probe, a 5 kg load cell with trigger force 1 g, and Eq. (3):

$$E = \frac{F/A}{\Delta l / l_0} \tag{3}$$

where F = force applied to the gel, A = surface area of the gel, Δl = compression distance and l_0 = height of the gel. Gel strength was plotted as Young's modulus corrected (E′) with regards to gel weight, and the new and higher alginate concentration after syneresis (Table S2, supplementary material). In this way, the intrinsic gel strength is shown rather than the effect of polymer concentration. 'Rupture strength' was determined with a 30 kg load cell with trigger force 5 g, as the force measured when the gel broke, and 'compression at rupture' as the height of the gel at rupture compared to the initial gel height (l_0). Gel shrinking (syneresis) was determined by weighing the hydrogel after Ca^{2+} saturation (w) and relating this to the original gelling weight (w_0), as described in Eq. (4):

$$\text{Syneresis } (\%) = \frac{w_0 - w}{w_0} \tag{4}$$

3. Results and discussion

3.1. Biomass characterisation

The present study aimed to perform in-process epimerization and subsequent characterisation of alginates from *S. latissima (SL)*, *A. esculenta (AE)* and *L. hyperborea (LH)*, which have differing native alginate structures (Haug, 1964; Nøkling-Eide et al., 2023) and overall chemical compositions. The contents of dry matter, ash, carbon, hydrogen, nitrogen, sulfur, macro elements, micro elements, halides, and heavy metals in the different seaweeds are shown in Table 1 and further elaborated in the supplementary material, Table S3. The *LH* lamina had a considerably higher dry matter content compared to *SL* and *AE*, related to a later harvesting date (November) allowing accumulation of laminarin and mannitol (Devillé et al., 2004; Percival & Ross, 1951; Usov et al., 2001). The high ash concentration of *SL* can be explained by its high potassium content, which is approximately twice the level of *AE* and *LH* (Table S3). Other notable differences between the biomasses were the high content of iodine and bromine in *SL*, and the

Table 1
Harvesting date of the three seaweed biomasses, and their initial dry matter (DM) and ash content, the reduction of dry matter after the water rinsing step (DM red.), and the content of carbon (C), hydrogen (H), nitrogen (N), and sulfur (S).

	A. esculenta	*S. latissima*	*L. hyperborea*
Harvesting date	03.05.2022	06.06.2022	16.11.2022
DM (% of WW)	12.89 ± 0.09	9.51 ± 0.03	30.55 ± 0.37
Ash (% of DM)	31.57 ± 0.19	44.47 ± 0.37	21.46 ± 0.20
C (% of DM)	29.41 ± 0.12	23.36 ± 0.15	33.54 ± 0.19
H (% of DM)	4.65 ± 0.03	4.00 ± 0.01	5.18 ± 0.08
N (% of DM)	2.09 ± 0.07	1.31 ± 0.03	0.84 ± 0.02
S (% of DM)	0.77 ± 0.02	0.67 ± 0.05	1.45 ± 0.09
DM red. (% of DM)[a]	30.79 ± 2.12	35.69 ± 5.50	36.11 ± 3.99

[a] Reduction of dry matter after the water rinsing step. Calculated based on the dry matter content in the liquid phase of 800 mL water rinsing solution, compared to the total dry matter content in the seaweed in these 800 mL.

high content of cadmium in *AE,* which is consistent with previously published literature (Duinker et al., 2020; Hogstad et al., 2023; Kreissig et al., 2021).

The in-process epimerisation protocol applied in this study included a water rinsing step before the acid and alkaline step necessary for alginate extraction (Stanford, 1881), simulating a biorefinery setting where fucoidans, laminarins and other water-soluble components would be extracted prior to alginate (Birgersson et al., 2023). As shown in Table 1, the dry matter of the seaweeds was reduced by 30–36 % after the water rinsing step, indicating a significant loss of minerals and water-soluble carbohydrates from the biomass.

3.2. *In-process epimerisation is species-specific*

Alginates from *SL*, *AE* and *LH* were in the present study epimerised with AlgE1, either in-process or in buffer. Here, the enzyme was found to have varying degrees of effectivity based on the different initial alginate structures in the three species, where the observed disparity was larger when the alginates were epimerised in-process, compared to the buffer control.

3.2.1. *Structure of in-process and in-buffer epimerised alginate*

The initial alginate structure differed between the three species analysed in this study (Figs. 3 and 4, Ctrl samples). Before epimerization, *AE* alginates had a higher fraction of guluronic acid, and longer average length of G-blocks ($F_G = 0.53$, $F_{GGG} = 0.35$, $N_{G>1} = 13$) compared to the *LH* lamina ($F_G = 0.46$, $F_{GGG} = 0.22$, $N_{G>1} = 7$) and *SL* ($F_G = 0.46$, $F_{GGG} = 0.23$ $N_{G>1} = 8$) (Table 2). After in-process epimerisation, however, the *AE* alginate obtained the lowest degree of epimerisation, increasing F_G by 13 %, and with no change in the average length of G-blocks ($F_G = 0.61$, $F_{GGG} = 0.39$, $N_{G>1} = 13$) (Fig. 3A, Table 2). The alginate from *SL* on the other hand, showed an increase in F_G by 31 % after epimerisation, demonstrating a very effective in-process epimerisation with AlgE1, further highlighted by longer G-blocks ($F_G = 0.67$, $F_{GGG} = 0.44$, $N_{G>1} = 11$). The in-process AlgE1-epimerised *SL* alginate were thus shown to have a comparable F_G to high-value commercial alginates derived from *LH* stipes ($F_G = 0.60$–0.75) (Moe et al., 1995; Skjåk-Bræk et al., 2016). For the alginate epimerised in-process from *LH* lamina, an increase of 19 % in F_G and longer G-blocks than initial ($F_G = 0.57$, $F_{GGG} = 0.30$, $N_{G>1} = 8$) was observed (Table 2).

During in-process epimerisation, AlgE1 showed species-specific activity on the three alginate types analysed. The in-process AlgE1-epimerisation designed in this study displayed good compatibility with *SL*, resulting in the formation of very long consecutive G-blocks compared to the initial alginate structure. The difference in epimerisation pattern between the three species (Fig. 3A), can be explained by AlgE1's preferred attack mechanism, its affinity to the substrate, and its ability to work processively. Previously it has been shown that AlgE1's affinity to

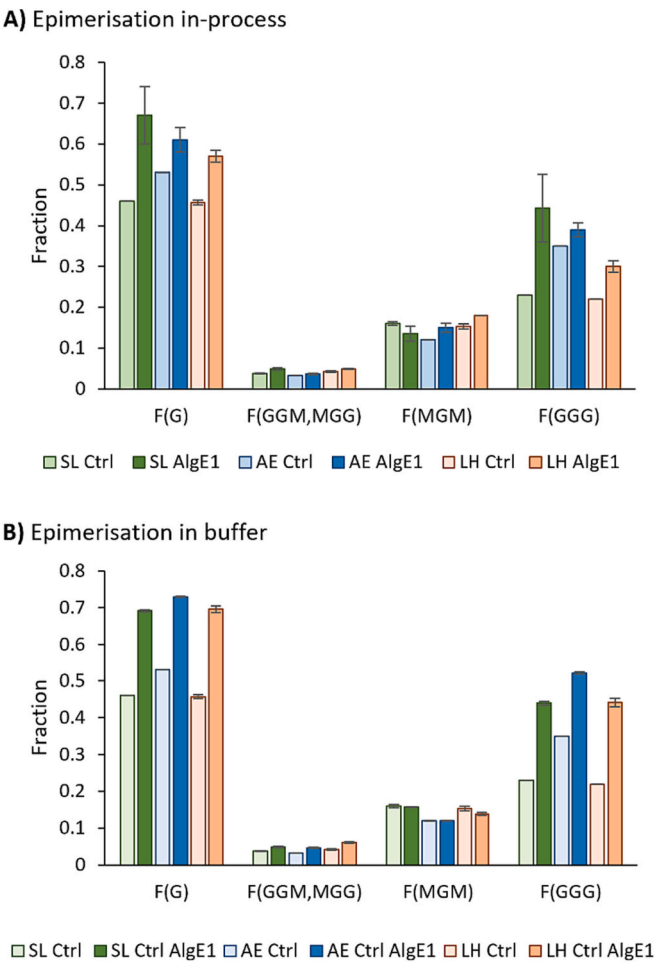

Fig. 3. Average fraction of guluronic acid (F_G), G-blocks ($F_{GGM,\ MGG}$), single guluronic acid residues (F_{MGM}) and the triad of guluronic acid (F_{GGG}), in A) Alginates epimerised in-process, where AlgE1 was applied directly into non precipitated alginate, and B) Alginates epimerised in buffer, where alginate was precipitated twice, washed twice, and then dissolved in a buffer containing 40 mM MOPS, 100 mM NaCl, 2.5 mM $CaCl_2$, with pH 6.9. For each experiment, three controls with no enzyme were included (Ctrl). Data are plotted as means of 3 analysed alginates ± standard deviation.

the substrate depends on already existing G-residues, and how they are distributed (Holtan et al., 2006). The lower fraction of G and shorter G-blocks in *SL* compared to *AE* could represent a preferred substrate for AlgE1, and thus explaining the high degree of epimerisation in-process. However, although the initial alginate structure of *LH* was comparable to that of *SL*, it lacked the same high degree of epimerisation. Thus, one could hypothesise that not only is the initial alginate structure deciding the enzyme's affinity for the substrate, but also the substrate availability during in-process epimerisation.

This hypothesis was supported by the decrease of F_{MGM} (fraction of single Gs) in *SL* after epimerisation, and an increase in *AE* and *LH* (Figs. 3 and 4). AlgE1 consists of two catalytic modules, A1 and A2, which introduces long G-blocks and MG-blocks, respectively (Ertesvåg et al., 1998), and four R-modules that aid in substrate binding and reduce the enzyme's calcium need (Aachmann et al., 2006; Ertesvåg & Valla, 1999). According to this enzyme's suggested mode of action, A2 begins by introducing single Gs in M-blocks, followed by A1 introducing Gs in every second residue in the created MG-blocks. By this approach the enzyme is not required to rotate 180° and dissociate from the alginate chain, and can followingly act in a processive mode (Tøndervik et al., 2013). Following this enzyme mode of action, the F_{MGM} should first increase when the single Gs are introduced by A2, and then decrease

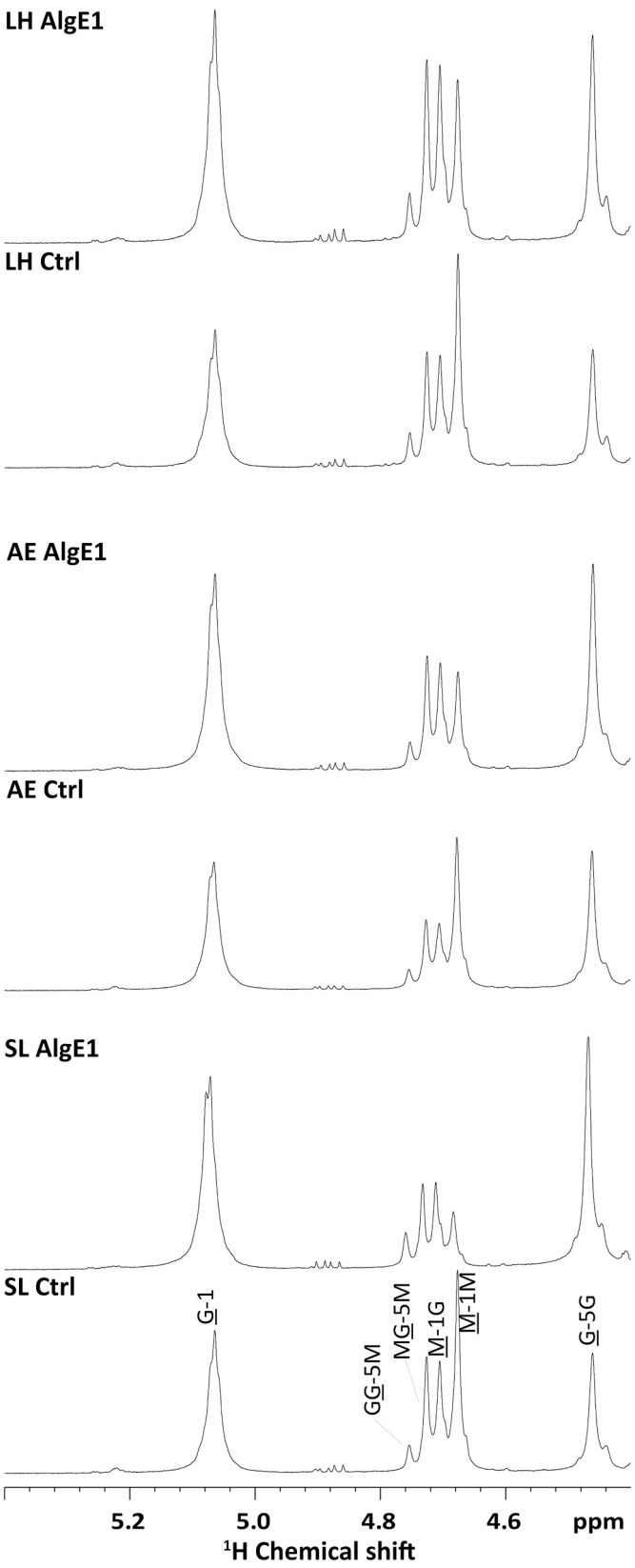

Fig. 4. ^{1}H NMR spectra for the anomeric region of the initial alginate (Ctrl) and in-process epimerised alginate (AlgE1) from *Saccharina latissima* (SL), *Alaria esculenta* (AE), *and Laminaria hyperborea* (LH) recorded at 83 °C on a 600 MHz instrument. The characteristic chemical shifts of the anomeric protons are indicated at the top of the panel. G is guluronate, M is mannuronate, # indicates proton number in alginate sugar ring, and underlined indicates the residue giving rise to the signal.

when the "gaps" are filled in by A1. Since the in-process epimerisation lasted 60 h, it is reasonable to believe that the NMR data presented here represents a completed reaction. As a result, the increase in F_{MGM} following epimerisation could indicate hampering of end-point epimerisation in *AE* and *LH*.

The sequential parameters from epimerisation in buffer (Fig. 3B) supported the hypothesis that the epimerisation has been prevented from reaching endpoint in-process. When epimerised in buffer, the alginates from *AE* and *LH* reached a similar degree of epimerisation as *SL* in-process, and the interspecific differences were less conspicuous. Before epimerisation in buffer, calcium has been removed from the system twice: First during the acid wash of the seaweed before extraction, and then during precipitation by acid. In the in-process epimerisation, calcium has only been removed in the first washing step. Thus, remaining calcium and other counterions could lead the alginates to partly gel, making them less accessible during in-process epimerisation and preventing the processivity of AlgE1 (Stanisci et al., 2018). *AE* had longer G-blocks compared to *SL* to begin with, and thus gelling was expected to occur faster in the in-process solution due to higher affinity for Ca^{2+} (Smidsrød, 1974), making the reaction stop sooner resulting in a lower final degree of epimerisation. One way to avoid this limitation could be to use smaller enzymes during in-process epimerisation, that can penetrate and carry out catalytic activity further into the alginate gel matrixes (Stanisci et al., 2018). AlgE1 with a molecular size of 147.2 kDa, is one of the largest AlgE-epimerases that have been isolated from *A. vinelandii*, and there are several other options also creating long G-blocks, such as AlgE6 (MW ~ 90.2 kDa) or hybrid enzymes consisting of modules from different epimerases (Stanisci et al., 2018). The situation with coextracted impurities, such as excessive calcium, is avoided when the alginates are precipitated, washed, and dissolved in a suitable buffer.

3.3. Molecular weight of in-process epimerised alginate

The alginate extraction protocol applied in this study was under ambient conditions to minimise depolymerization during processing. Table 3 summarises the molecular weight (MW) averages (M_n and M_w) and dispersity index ($Đ$) of the alginates extracted from the lamina of *SL*, *AE*, and *LH*, and showed that the addition of AlgE1 in the extraction process had no effect on MW, which was also evident in the similar concentration profiles for non-epimerised and in-process epimerised alginates in the chromatograms recorded by SEC-MALS (Fig. S2, Supplementary material). Because the extracted alginates had a high MW, they were mechanically depolymerised before further gelling tests, which also resulted in a lower $Đ$ and thus a narrower chain size distribution (Table 3).

The shear viscosities, measured on 1 % w/v solutions in water at 20 °C, were consistent with the SEC-MALS data (Table 3). The alginates with the highest MW were the ones with highest shear viscosity, and the viscosity decreased in the order *AE* > *LH* > *SL* (Fig. 5). In this study, the shear viscosity of the epimerised alginates was similar compared to the non-epimerised controls (Fig. 5). Epimerases requires a certain degree of polymerization (DP) to associate with the alginate substrate into a complex. Previously it has been shown that to form a complex with PolyMG, AlgE1 needs a pentamer, and an octamer to form a complex with PolyM (Holtan et al., 2006). An alginate sample with high MW-averages (M_n and M_w) will therefore ensure a high degree of enzyme-substrate-association, whereas a sample with more depolymerised alginates could consequently result in lower activity. Thus, it is a huge advantage that alginate depolymerization is avoided, which can be achieved by minimal post extraction processing of the alginates.

3.4. Mechanical properties of in-process epimerised alginates

Rheological characterisation of the alginate hydrogels showed that epimerisation resulted in stronger and more brittle hydrogels for *SL*, *AE* and *LH* alginates compared to their non epimerised controls (Fig. 6).

Table 2
Average fraction of guluronic acid (F_G), mannuronic acid (F_M), all possible diads, G-blocks ($F_{GGM,\ MGG}$), single guluronic acid residues (F_{MGM}), the triad of guluronic acid (F_{GGG}), average length of alternating sequences (F_{MGM}/F_{GGM}) and the average length of G-blocks ($N_{G>1}$) in the non-epimerised alginates (Ctrl), the alginates epimerised in-process (Process) and the alginates epimerised in buffer (Buffer) from *S. latissima (SL)*, *A. esculenta (AE)* and *L. hyperborea (LH)*. The fractions are based on the characteristic signals for M and G residues obtained from the anomeric region in the NMR spectra (Fig. 4). For full ^{1}H NMR spectra, see supplementary material (Fig. S1).

Alginate	F_G	F_M	F_{GG}	$F_{GM,\ MG}$	F_{MM}	$F_{GGM,\ MGG}$	F_{MGM}	F_{GGG}	F_{MGM}/F_{GGM}	$N_{G>1}$
S. latissima										
SL Ctrl	0.46	0.54	0.27	0.19	0.34	0.04	0.16	0.23	4.18	8
SL process	0.67	0.33	0.49	0.17	0.15	0.05	0.14	0.44	2.79	11
SL buffer	0.69	0.31	0.49	0.20	0.11	0.05	0.16	0.44	3.20	11
A. esculenta										
AE Ctrl	0.53	0.47	0.38	0.15	0.32	0.03	0.12	0.35	3.65	13
AE process	0.61	0.39	0.43	0.18	0.21	0.04	0.15	0.39	4.09	13
AE buffer	0.73	0.27	0.57	0.16	0.11	0.05	0.12	0.52	2.40	13
L. hyperborea										
LH Ctrl	0.46	0.54	0.26	0.19	0.35	0.04	0.15	0.22	3.63	7
LH process	0.57	0.43	0.35	0.22	0.21	0.05	0.18	0.30	3.61	8
LH buffer	0.70	0.30	0.50	0.19	0.11	0.06	0.14	0.44	2.33	9

Table 3
Number average molecular weight (M_n), weight average molecular weight (M_w) and dispersity ($Đ$) of the alginates extracted from *S. latissima (SL)*, *A. esculenta (AE)* and *L. hyperborea (LH)* lamina, before and after mechanical depolymerization using a wet pulverizing system (StarBurst Mini HJP-25001H Sugino Machine Ltd., Japan). Ctrl indicates the alginates treated with no enzyme, and AlgE1 indicates the alginates epimerised in-process with AlgE1.

Species	Treatment	Before/after depolymerisation	M_n (kDa)	M_w (kDa)	$Đ$ (M_w/M_n)
SL	Ctrl	Before	255 ± 11	593 ± 25	2.3
	AlgE1		244 ± 13	571 ± 30	2.4
AE	Ctrl		394 ± 23	871 ± 11	2.2
	AlgE1		379 ± 2	867 ± 8	2.3
LH	Ctrl		356 ± 9	755 ± 16	2.1
	AlgE1		352 ± 18	754 ± 22	2.1
SL	Ctrl	After	114 ± 3	149 ± 2	1.3
	AlgE1		99 ± 6	135 ± 7	1.4
AE	Ctrl		111 ± 5	153 ± 4	1.4
	AlgE1		112 ± 5	156 ± 2	1.4
LH	Ctrl		112 ± 1	160 ± 4	1.4
	AlgE1		104 ± 1	150 ± 0	1.4

Young's modulus and rupture strength are used as measures of gel strength and gel elasticity, respectively (Skjåk-Bræk et al., 2016). Due to the cavity formed by two diaxially linked G residues next to each other, the affinity towards divalent ions is increasing with G residues. Strong associations are cooperatively formed between a crosslinking divalent cation and two stretches of G-blocks, in extended "egg-box" structures (Grant et al., 1973; Skjåk-Bræk et al., 1986; Smidsrød et al., 1973). Thus, in Ca-alginate hydrogels, the gels containing alginates with many and long G-blocks will be stronger than gels formed by alginates with fewer and shorter G-blocks. The increased strength of the calcium saturated hydrogels in this experiment was due to the increase in G-blocks after epimerisation and was most prominent in the alginates from *SL* (Fig. 6A), which also had highest degree of epimerisation (Table 2).

The rupture strength, which can be seen as the resistance to gel breakage (Aarstad et al., 2017), typically increases with decreasing G-content (Martinsen et al., 1989). G-rich hydrogels are strong, but also more brittle. With lower G-content, the hydrogels become more flexible, and can tolerate higher applied force and be more deformed before they collapse. This characteristic was observed for *SL* (Fig. 6B), where epimerisation decreased the rupture strength. However, for the other two species, the rupture strength of the hydrogels formed by epimerised alginates was either higher *(AE)* or similar *(LH)* compared to no epimerisation. This can be explained by the epimerisation pattern in *AE* and *LH*, where an increase in $F_{GM,\ MG}$ and F_{MGM} and no decrease in the average length of alternating sequences (F_{MGM}/F_{GGM}) was observed (Table 2). It has been proposed that alternating sequences increase the flexibility of alginate hydrogels, due to an unwinding of Ca-ions when MG/MG-junctions receive pressure, in contrast to GG-junctions that break (Donati et al., 2005; Donati et al., 2009; Mørch et al., 2008). Such an unwinding of Ca-ions in hydrogels characterised by many alternating

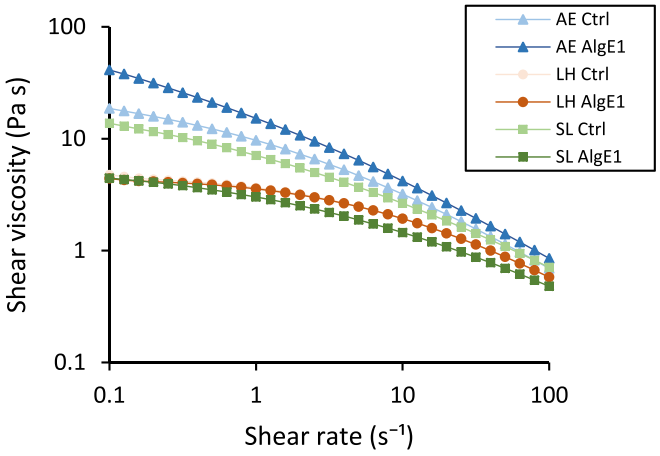

Fig. 5. Shear viscosity of alginates extracted from *A. esculenta (AE)*, *S. latissima (SL)*, and *L. hyperborea (LH)*. Alginates are epimerised in the extraction process (AlgE1) or not epimerised (Ctrl). Note: The curve of *LH* Ctrl is placed right under the curve of *LH* AlgE1, and thus hardly visible in the plot.

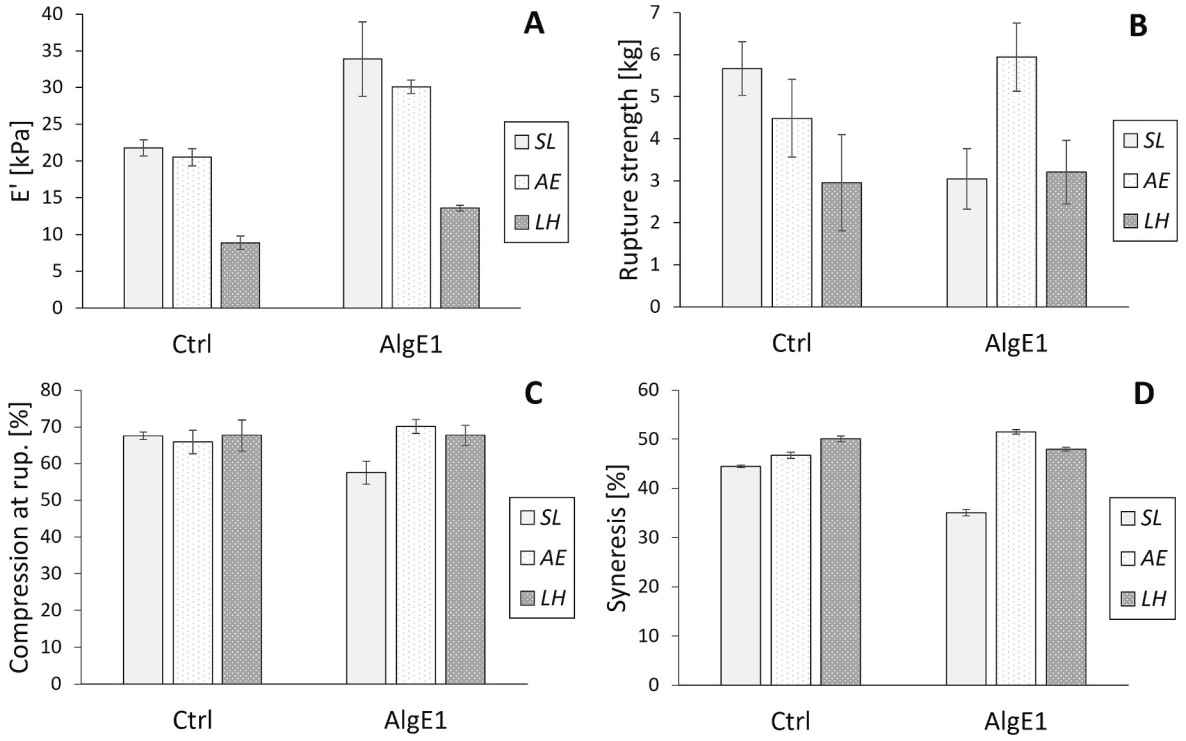

Fig. 6. Mechanical properties of alginate gels (1 % *w/v*) prepared with 15 mM $CaCO_3$ and 30 mM GDL (glucono-δ-lactone), followed by a calcium saturation by dialysis against a solution of 50 mM $CaCl_2$ in 200 mM NaCl for 24 h at 25 °C. Alginates are extracted from the lamina of *S. latissima (SL)*, *A. esculenta (AE)* and *L. hyperborea (LH)*. Ctrl = Alginates not epimerised, AlgE1 = Alginates epimerised in the extraction process with AlgE1. A) Corrected Young's modulus (E') with regards to gel weight after syneresis, and followingly new alginate concentration (Table S2, supplementary material), as a measure of gel strength, B) rupture strength as a measure of gel elasticity, C) Compression at rupture, and D) Syneresis as a measure of gel shrinking. Data are plotted as means of 6 gels ± standard deviation.

sequences is accompanied by higher syneresis (Mørch et al., 2008), as was observed in the present study with higher syneresis in *AE* and *LH* compared to *SL* (Fig. 6D).

The ability to form thermostable hydrogels in the presence of calcium makes alginates a unique gelling agent and scaffold material in several applications, including food products, and as biomaterials in tissue engineering and cell immobilization applications (Clementi, 1997). Although alginates represent a highly diverse group of biopolymers, and their chemical composition can be tailored towards specific applications, most high-value applications rely on high-G alginates (Andersen et al., 2012). Traditionally, stipe alginate from *LH* has been applied in many applications, due to high F_G and gel strength. The Young's modulus values obtained for in-process epimerised *SL*-alginate in this study, was within the range of those values reported for stipe alginate from *LH* (E = 25–29 kPa), making it a promising material for already well-established applications (Mørch et al., 2008).

3.5. Upscaled in-process epimerisation of alginates from Saccharina latissima

Because the initial alginate structures of different seaweed species vary (Fig. 3), in-process epimerisation protocols should be established species-specifically, taking epimerase size, mode of action, and enzyme concentration into account. In the present study in-process epimerisation was achieved in all three species studied but was most effective in *SL* which was therefore selected for a larger scale demonstration using fresh biomass (Fig. 7).

A successful upscaled in-process epimerisation of *SL*-alginate was demonstrated, as the F_G increased from 0.44 to 0.76 in the PilotOrb (Figs. 8, S3, S4 and Table S4, supplementary material), and CHNS-analysis indicated low degree of coextracted contaminants such as protein and fucoidan (C: 30.97 ± 0.14 %, H: 4.41 ± 0.09 %, N: 0.29 ± 0.05 %, S: 0.15 ± 0.13 %). F_{GGG} increased from 0.22 to 0.55, indicating

Fig. 7. Upscaled in-process epimerisation on *SL*-biomass in PilotOrb. A) Milled *Saccharina latissima*-biomass in 21 L of ion-free water with pH adjusted to 7 (after acid wash of biomass), and B) Extracted alginate solution added AlgE1.

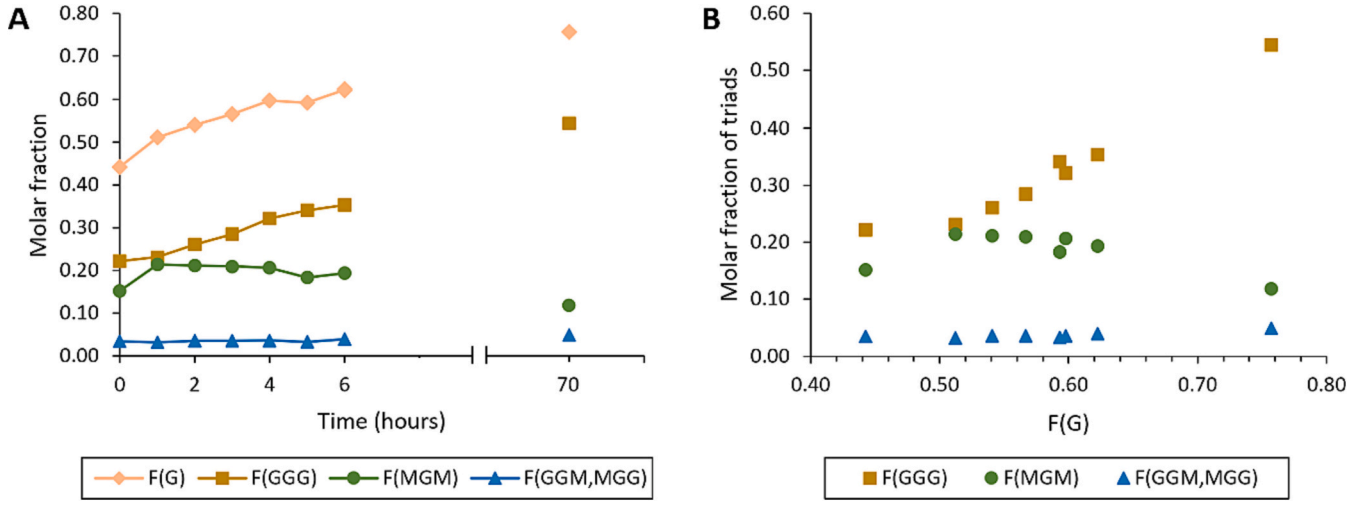

Fig. 8. Compositional structure of *Saccharina latissima* alginate after in-process epimerisation in PilotOrb. A) Average fraction of guluronic acid (F_G), G-blocks ($F_{GGM, MGG}$), single guluronic acid residues (F_{MGM}) and the triad of guluronic acid (F_{GGG}) plotted as a function of reaction time. B) The fraction of triads (F_{GGG}, F_{MGM}, $F_{GGM, MGG}$) plotted as a function of F_G.

formation of long G-blocks. The unchanged fraction of G-blocks ($F_{GGM, MGG}$) during the reaction suggested that the long G-blocks were generated either by filling gaps in MG-regions or merging two existing G-blocks, rather than by the formation of new G-blocks. The alginate was sampled hourly the first six reaction hours and analysed, and a steady increase in F_G and F_{GGG} was observed throughout the reaction. The fraction of single G units (F_{MGM}) increased at the start of the reaction before decreasing (Figs. 8, S4), indicating that the A2 module of AlgE1 was introducing single G units in M-rich regions, followed by A1 filling in G in MG-regions. Compared with the lab-scale trials, the upscaled experiment resulted in a higher degree of epimerisation but also a reduction in the molecular weight after 70 h (M_w from 700 to 221 kDa). As the pH and temperatures were unchanged, both of these differences can be attributed to more rigorous mechanical agitation in the PilotOrb system increasing mixing and dispersal of aggregates, while increasing the shear forces and mechanical depolymerization in the alginate solution.

4. Conclusions

The study demonstrated that in-process epimerisation can offer a new and effective method for upgrading alginates from cultivated seaweeds and underutilised fractions of wild seaweeds. The fraction of guluronic acid, G-block length, and gel rigidity increased with in-process epimerisation, while the alginate molecular weight and viscosity remained high during processing. With an expected increase in demand, cultivated *SL* and *AE*, alongside the underutilised *LH* lamina can thus provide a future supply to complement today's production of high-quality alginates. Especially in-process epimerised *SL* alginates showed an increase in G-content and gel rigidity to levels comparable to the highly valued alginates from *LH* stipe. The in-process protocol applied in this study included the relatively large mannuronan C-5 epimerase AlgE1, and was best suited to *SL*, where it also functioned on fresh harvested biomass in pilot scale. Future studies should include techno-economic analyses to evaluate feasibility of commercial production, and dependence on enzyme concentration in the reaction. Further, other epimerases including enzyme hybrids should be studied for different seaweed species. The initial alginate structure clearly affects the degree of epimerisation obtained, and with extended knowledge on enzyme-substrate complexes, species-specific in-process protocols can be designed and evaluated. With enhanced understanding in some of the features laid out, the future perspective of this study enables an enlarged alginate industry based on numerous seaweed species, including cultivated ones, and thallus parts that traditionally are regarded as waste streams.

CRediT authorship contribution statement

Katharina Nøkling-Eide: Conceptualization, Investigation, Formal Analysis, Visualization, Writing – Original Draft Preparation. **Finn Lillelund Aachmann:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Anne Tøndervik:** Conceptualization, Writing – review & editing. **Øystein Arlov:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Håvard Sletta:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carbpol.2023.121557.

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