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Acrylic acid and DMSP lyases in the green algae *Ulva*

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ABSTRACT

Green macroalgae of the genus *Ulva* contain dimethylsulfoniopropionate (DMSP) produced from methionine through a transamination pathway. DMSP is converted by DMSP lyase enzymes (DLs) into acrylic acid (AA), a high-value compound with a vast market as its esters have applications in the production of plastic additives, textiles, sealants, adhesives and surface coatings. The levels of AA produced by *Ulva* species, and the processes to extract it from this biomass, are currently poorly understood. In this study an analytical method was implemented for the simultaneous measurement of AA, DMSP and the pathway intermediates (4-methylthio-2-oxobutyrate, 4-methylthio-2-hydroxybutyrate, and 4-dimethylsulfonio-2-hydroxybutyrate) in *Ulva* biomass. The amounts of these compounds – with a focus on AA – were then quantified after processing the macroalgae with different chemical, thermal, and enzymatic treatments. AA was extracted in the range of 0.22–0.45 % of the algal dry weight. Sequential enzyme treatments showed no higher yields compared to individual enzymatic treatments or chemico-physical extractions, an encouraging result for the application of the treatments in industry. Codon-optimised synthetic genes for two candidate DLs from *Ulva mutabilis* were expressed and purified in *E. coli* with solubility tags, and the biochemical characteristics of these recombinant lyases were investigated. Both enzymes UM021_MBP and UM030_Halo7 had lower activity than their microalgal counterpart Alma1, with whom they share 33.9 % and 39.7 % similarity at amino acid level respectively. UM030_Halo7 appeared more active than UM021_MBP, with K_m and V_m of 7.10 mM and 5.99 mM/min/mg protein respectively. UM030_Halo7 showed a preferred pH of 6.0, an optimal of temperature of 20 °C, was inhibited by NaCl concentration equal or higher than 0.5 M but not by H_2O_2 up to 50 μM. These results advance our understanding of AA biosynthesis in *Ulva* at the molecular level, and could be useful to implement processes for extracting AA from *Ulva* species within a biorefinery framework.

1. Introduction

The sea lettuces of the genus *Ulva* are a group of green macroalgae distributed in coastal environments worldwide. They have great ecological significance, due to their rapid growth and their contribution to coastal biogeochemical cycles [1]. They are responsible for causing the so-called "green tides", massive algal proliferations triggered by high-nutrient concentrations, which seriously affect marine environments, and the socio-economic context in coastal communities [2]. However, *Ulva* biomass has many beneficial applications, such as uses in the food and feed industry, as well as in the pharmaceutical, nutraceutical, biofuel and bioremediation sectors [3]. These seaweeds also represent interesting model organisms for studying different biological aspects, including morphogenesis [4] and multicellularity [5].

One of the key contributions of the sea lettuces is their involvement in the sulfur biogeochemical cycle since they produce high levels of dimethylsulfoniopropionate (DMSP) [6,7]. This compound has important physiological roles as antioxidant, intracellular osmolyte, cryoprotectant, anti-stress molecule, and is an abundant source of sulfur [8,9]; it is produced by the sea lettuce, as well as by some microalgae, bacteria, plants and corals [10]. DMSP in *Ulva* is enzymatically produced via a 4-step transamination pathway starting from methionine and with the production of the intermediate metabolites 4-methylthio-2-oxobutyrate (MTOB), 4-methylthio-2-hydroxybutyrate (MTHB) and 4-

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dimethylsulfonio-2-hydroxybutyrate (DMSHB) [6]. The enzymatic steps are, respectively, transamination, reduction, *S*-methylation and oxidative decarboxylation [6]. Once released in the water, DMSP catabolism can occur either via the microbial demethylation pathway, which produces acrylic acid (AA) and methanethiol, or by cleavage into AA and dimethylsulfide (DMS), catalysed by the enzyme DMSP lyase (DL) [11]. The products of the DMSP catabolism have significant effects on the environment and the biota. DMS participates to the sulfur biogeochemical cycle [12], as it represents approximately 90 % of the sulfur flux from the ocean to the atmosphere [13] and its oxidation products contribute to the process of clouds formation and aerosol albedo [14,15]. The significance of AA is less understood, however various roles have been reported in different organisms, such as antioxidant, antigrasing and antimicrobial functions, and source of carbon for bacterial communities [16–20]. It has been shown that AA (together with DMS produced from the cleavage of DMSP) is 20 to 60 times more effective as an antioxidant than its precursor DMSP [16,18], and that oxidative stressors such as UV light, H_2O_2, Fe or CO_2 limitation, or high Cu^{2+} concentration, can lead to increased production of DMSP and/or its lysis to AA and DMS [19,21–23]. Predators grazing also has an important role in the conversion to AA, since this compound has been shown to have deterrent properties against bacteria and other predators due to its toxicity [17,24].

The enzymatic cleavage of DMSP into AA and DMS has been investigated in marine bacteria, microalgae, green macroalgae, corals and one fungal species [11,25]. In green seaweeds, this activity was described in cell free extract of *U. clathrata* (previously known as *Enteromorpha intestinalis*) [26], and was further investigated by purifying and biochemically characterising an endogenous DL from *U. curvata* [27]. More recently, progress in the characterization of algal DLs have been obtained from microalgae, including the haptophyte *Phaeocystis globosa* [28,29], but mainly from *Emiliania huxleyi* and *Symbiodinium* sp. [30–32]. The work on the coccolithophore *E. huxleyi* provides in-depth molecular and biochemical characterization of Alma1, the most active DL identified in microalgae to date, and for which homologs have been found in algal genomes of organisms known to produce DMS from DMSP, suggesting an important physiological role for this enzyme.

Ulva biomass is currently used as edible food, for animal feed, for the bioremediation of land-based aquaculture, production of bioenergy and pharmacological molecules, and as source of chemicals [3,33]. Its processing provides many useful molecules, including proteins, lipids, minerals, fibres, bioactive compounds and polysaccharides such as ulvan and cellulose [34,35]. Different approaches have been implemented for the treatment of *Ulva* biomass, depending on the products to be extracted and on the targeted applications [33,34,36]. Biorefinery cascading approaches have also been proposed in order to valorise this algal biomass by retrieving many valuable products in the same facility through an economically feasible value chain [33,37,38]. AA is a further molecule that could be worthwhile retrieving from *Ulva* biomass, since it is a commercially important platform chemical used as a building block to produce esters with applications in paper treatment, production of plastic additives, textiles, sealants, adhesives and surface coatings [39]. Its global market size was valued at $12.0 billion in 2020, and it is projected to reach $19.2 billion by 2030. At present, AA is obtained mainly from the oxidation of propane or propylene [40], therefore utilising non-renewable materials and processes with a high carbon footprint. Several bio-based routes from renewable feedstocks and environmentally friendly processes have been explored for its production [40,41], but none have reached the commercial stage yet. To our current knowledge, and despite the value of this compound, the retrieval of AA from *Ulva* biomass, or from any other micro or macroalgal species, has not been attempted.

There is currently limited information on the levels of AA produced by *Ulva* spp. The spatial-temporal patterns and changes of DMSP, DMS and AA in the coastal waters off Qingdao (China) during blooms of *U. prolifera* in 2015 and 2018 were monitored [42,43], but no information is available for quantities present in the algal samples. Similarly, biochemical information on *Ulva* spp. DLs, the enzyme responsible for the formation of AA from DMSP, are scarce and were obtained after purification of an endogenous soluble DL from *U. rigida* [27]. This lack of information may be explained by the difficulty in measuring AA content in algal samples, and by the lack of genomic resources for *Ulva* species. In 2018, the sequencing and analysis of the *U. mutabilis* genome was published [1], identifying candidate genes involved in DMSP biosynthesis and conversion to DMS and AA, including those for two DLs. Phylogenetic analysis revealed that both genes clustered together alongside *Alma1*, and transcriptomic analysis showed differential expression under different environmental conditions. Since then, the presence of six DL homologs has been predicted also in the species *U. prolifera* [8].

The current study aimed at filling gaps in the understanding of DMSP and AA production in the green algae *Ulva* spp. To achieve such goals, our work followed three different approaches (Fig. 1): i) the implementation of an analytical method for the simultaneous measurement of DMSP and AA in *Ulva* biomass; ii) the quantification of the amounts of DMSP and AA found in *Ulva* samples using biomass processed through different treatments; iii) the investigation of the biochemical characteristics of candidate DLs identified in the genome of *U. mutabilis*.

2. Materials and methods

2.1. Quantification of DMSP pathway intermediates and AA in Ulva spp. biomass

2.1.1. Hydrophilic interaction chromatography coupled to mass spectrometry (HILIC-MS) analysis

DMSP pathway metabolites MTOB, MTHB, DMSHB, DMSP, and AA were detected and quantified by hydrophilic interaction chromatography coupled to MS (HILIC-MS), implemented using methods adapted from Spielmeyer and Pohnet [44] and Curson et al. [45]. For liquid chromatography, a Waters Acquity I-Class UPLC or a Dionex Ultimate 3000 LC system was used, coupled to a Bruker maXis HD TOF mass spectrometer. HILIC was performed at 40 °C using a Sequant ZIC-HILIC column (100 × 46 mm ID, 5 μM particle size, with a preceding Phenomenex C18 guard column to act as a particulate filter). Data for all compounds was acquired in a single run in negative (1–9 min; AA, MTOB, MTHB) and positive (9–40 min; DMSP, DMSHB) ESI modes. The mass spectrometer source conditions were set to nebulizer 2.5 bar, nanoBooster 2.5 bar, dry gas 8.0 L/min, dry heater 200 °C, charging voltage 2000 V, and end plate 1321 nA. Data was acquired in full scan mode over the *m/z* range 40 to 650 with targeted MS2 (for compound confirmation only). The system was calibrated before use with Agilent ESI-L Low Concentration Tuning Mix to achieve RMS mass errors <0.38 ppm. Mobile phases were as follows: A, 10 mM ammonium acetate in 95:5 (*v*/v) water:acetonitrile; B, 10 mM ammonium acetate in 10:90 (v/v) water:acetonitrile. Flow rate and mobile phase gradients were optimised to minimize run and re-equilibration times, which were finalised to the following settings: 0–2 min isocratic 100 % B 0.5 mL/min; 2–22 min to 35 % B; 22–22.01 min flow increased to 0.8 mL/min; 22.01–28 min isocratic hold at 35 % B; 28–28.1 min to 100 % B; 28.1–38 min isocratic 100 % B; 38–38.31 min flow decreased to 0.5 mL/min; 38.1–40 min isocratic 100 % B. Samples were reconstituted in 90 % acetonitrile and diluted further as required in the same solvent, and 10 μL volumes were injected for analysis. Amounts were quantified against calibration curves constructed using the following authentic standards: MTOB sodium salt (Sigma-Aldrich, K6000); MTHB (Sigma-Aldrich, PH000120); DMSHB sulfate salt (Chem-Space, FCH2937457); DMSP hydrochloride (Sigma-Aldrich, 80828); AA (Sigma-Aldrich, 147230). Calibrations and quantifications were performed in Skyline 22.2.0.351 using extracted adduct ions as described in Fig. S1.

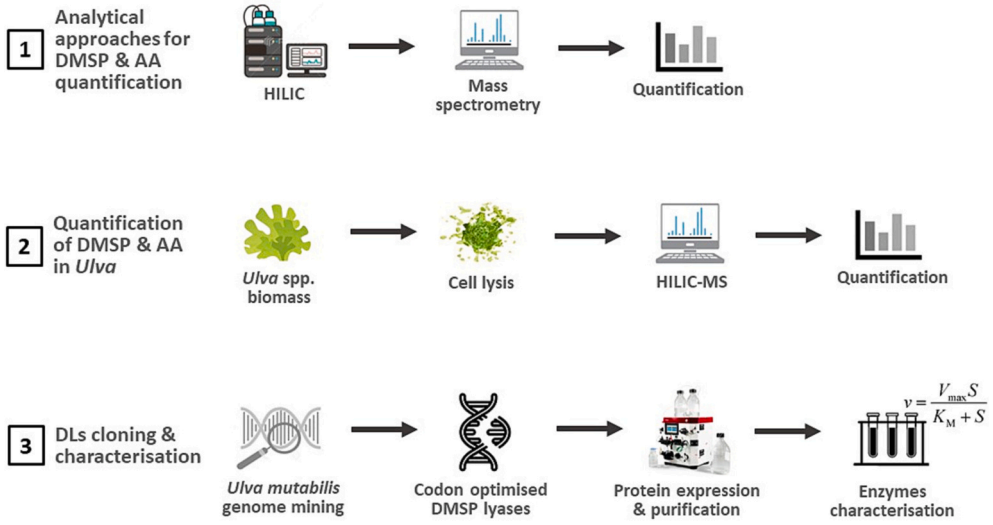

Fig. 1. The experimental set-up. DMSP = dimethylsulfoniopropionate; AA = acrylic acid, DLs = DMSP lyases; HILIC-MS = hydrophilic interaction liquid chromatography coupled to mass spectrometry.

2.1.2. Sonication treatment of Ulva spp.

Samples of ground dried *Ulva armoricana* provided by the Olmix Group (France, https://www.olmix.com/) and of *Ulva rigida* provided by the seaweed farm ALGAplus (Portugal, https://www.algaplus.pt/) within the EU H2020 GENIALG project (https://genialgproject.eu/) were used for metabolite profiling. Olmix seaweeds were harvested from the shore, washed with fresh water, freeze dried and provided as already milled. ALGAplus cultivated algae were washed with filtered sea water, freeze dried, and subsequently milled at the University of York using a Cyclone Mill Twister (Retsh). Three replicates of 100 mg for each type of samples were resuspended in 1 mL of 90 % acetonitrile in a 2 mL tube, sonicated 10 times for 30 s in ice, centrifuged at 18.000 *g* for 3 min, and the supernatant was analysed as described above (Section 2.1.1).

2.1.3. Acid-base treatments of U. rigida

Experiments (named 1 to 9, Table 1) were performed at the Bio-renewables Development Centre (BDC) using a mass to volume ratio of 1:10 and two different systems. Forty grams of *U. rigida* biomass provided by ALGAplus were mixed with 400 mL for the PARR system, while 4 kg were used in 40 L for the AFEX system. The biomass was processed at pH 4.0 using concentrated sulfuric acid, at pH 7.0 using town water, and at pH 9.0 using sodium hydroxide (~5 M aqueous solution). Two temperatures (90 and 120 °C) were tested for each treatment, with an incubation time of 24 h under continuous stirring at 100 rpm in a stainless-steel pressure vessel with oil jacket heating. Treatments at 90 °C were performed once, and treatments at 120 °C in duplicate.

2.1.4. Microwave-assisted treatments of U. rigida

Microwave treatments (named 10 to 12) were tested to facilitate thermal fractionation of the algal biomass, using a microwave reactor (Milestone Ethos Up 1800 W pressure digestion microwave system) run in a temperature control mode to assess the influence of three different final temperatures (100 °C, 130 °C, and 160 °C) at BDC. Five grams of *U. rigida* biomass (ALGAplus) were added to 50 mL of deionized water in a 100 mL PTFE microwave tube, shook gently and left for around 15 min before re-shaking and starting the heating. The mixture was heated over 15 min from ambient temperature to the final temperature without stirring with a maximum microwave power input of 1800 W (varied power during the ramping). After completion of the reactions, the vessels were cooled to ambient temperatures before the separation of liquid and solid phases that were kept at 4 °C for future analysis. Three reactions were run in parallel for each temperature.

2.1.5. Enzymatic treatments of U. rigida

A first set of enzymatic experiments (named 13 to 16) was performed on ALGAplus *U. rigida* dried biomass in BDC. For this, 1.4 kg of dried biomass was loaded in the 100 L stirred vessel as described above, and 40 L of water was added with stirring at room temperature for 1 h. Hydrated seaweed was then passed through a fruit press straining bag to separate liquid from solid. The solid biomass was then press as described above and the press juice was combined with the drained liquid, forming the mechanical juice that was stored at 4 °C for further analysis. The recovered solids were re-introduced onto the vessel, stirred in 40 L of water, and incubated for 16–18 h at 50 °C and pH 6.5–7.5 in the absence (control condition) or presence of individual enzyme papain (from *Carica papaya*, PanReac AppliChem) or neutrase (Neutrase® 0.8 L, Strem chemicals). Enzymes were added at a final 1 % enzyme:substrate ratio (Table 2). After this, a heat inactivation treatment was conducted for 1 h at 85 °C, the solid and liquid were separated by filtration a mesh bag, and samples from both phases were kept at 4 °C for future analysis. Each treatment was conducted in duplicate.

A second set of enzymatic treatments (named 17 to 22) was

Table 1
Details of the system, pH and temperatures used for acid-base treatments performed with dried *U. rigida* biomass.

Treatment no.	System	pH	Temperature (°C)
1	AFEX	7	90
2	AFEX	4	90
3	AFEX	9	90
4	AFEX	7	120
5	AFEX	7	120
6	PARR	4	120
7	PARR	4	120
8	AFEX	9	120
9	AFEX	9	120

Table 2
Details of the enzymes, pH and temperatures used for each of the individual enzymatic treatment experiment performed with dried *U. rigida* biomass.

Treatment no.	Treatment type	pH	Temperature (°C)
13	Mechanical juice	6.5–7.5	50
14	Papain	6.5–7.5	50
15	Neutrase	6.5–7.5	50
16	No enzyme	6.5–7.5	50

performed with the same *U. rigida* biomass from ALGAplus at BDC. Five different two-enzyme sequential treatments were performed (Table 3), and an extra treatment (named 17) with no enzymes added was used as a control. Enzymes used for the processing of the biomass were: neutrase (see above), ulvan lyase from the bacterium *Formosa agariphila* [46] (not commercially available), papain (see above), amyloglucosidase (AMG300, Murphy & Son Limited), and Cellic® CTE3 (Novozymes). Quantities of added enzymes were as follow (expressed as g per kg of seaweed): neutrase, 25 g; ulvan lyase, 0.06 g; papain, 15 g; amyloglucosidase, 1.2 g; Cellic CTEC3, 15 g. Five kilograms of dried seaweed were added to 20 L of cold water in a 100 L vessel (bespoke 100 L stirred and heat jacketed pressure vessel located in BDC), stirred, and more water was added to make up to 75 L. The stirred vessel was heated to a selected temperature and pH (optimal for the specific enzyme used, see Table 3) for over 1 h (samples taken at this point were called pre-hydrolysates), and then the enzyme A was added and incubated for 18–24 h. The vessel's content was drained through a fruit press straining bag (Straining Bag for Vares 50 Litre Press, Vigo Presses), solids and liquid fraction (named liquid 1) were collected separately and the liquid 1 was frozen at −20 °C. The vessel was washed with fresh water and more solids were collected, added to the previous amount, pressed with a 50 L manual hydraulic press (Vares Fruit Press 50 L, Vigo Presses), the liquid was removed and the obtained solids were weighted. The solids were then added to the stirred vessel together with 20 L of cold water, brought to 75 L with more water, and heated to a set temperature and pH for over 1 h. Enzyme B was then added and incubated for 18–24 h. Again, the contents of the vessels were drained through a mesh bag, the liquid fraction (named liquid 2) was collected and frozen at −20 °C, while the solid fraction was retained and added to more solids obtained by washing the vessels with water, pressed, weighted and frozen. Samples of the pre-hydrolysate, liquid 1 and liquid 2 for each treatment were collected in triplicates and analysed by HILIC-MS to quantify AA and the intermediates of the DMSP pathway, according to the methods described above (Section 2.1.1).

2.2. In silico analysis of DMSP lyases genes in U. mutabilis

Two candidate DL genes, *UM030_0039.1* (XP_005779316.1) and *UM021_0036.1* (XP_029182524.1), were reported after sequencing the genome of *U. mutabilis* [1]. This was checked by using the sequence of *E. huxleyi* Alma1 to perform a Blastp search against the *U. mutabilis* genome using the ORCAE (Online Resource for Community Annotation of Eukaryotes, https://bioinformatics.psb.ugent.be/orcae/) online genome annotation resource [47]. Protein sequence alignment was performed with the online tool Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/, [48]). Molecular weights (MW) were determined with the Expasy ProtParam tool [49]. The presence of signal peptides was assessed with the Signal P-5.0 online tool [50], and disulfide bonds were searched using DiANNA 1.1 web server [51]. Occurrence of transmembrane domains was assessed using DeepTMHMM [52].

Table 3
Details of the enzymes, pH and temperatures used for each of the sequential enzymatic treatment experiment performed with dried *U. rigida* biomass.

Treatment no.	Enzymes	pH	Temperature (°C)
17	Enzyme A: none	7.0	45
	Enzyme B: none	7.0	45
18	Enzyme A: neutrase	7.0	45
	Enzyme B: ulvan lyase	7.0	35
19	Enzyme A: ulvan lyase	7.0	35
	Enzyme B: neutrase	7.0	45
20	Enzyme A: papain	6.0–7.0	65
	Enzyme B: ulvan lyase	7.0	35
21	Enzyme A: neutrase	7.0	35
	Enzyme B: amyloglucosidase	4.2	60
22	Enzyme A: neutrase	7.0	45
	Enzyme B: Cellic CTec3	5.0–5.5	45

2.3. Cloning and expression of recombinant Ulva DLs

Synthetic genes for the two *Ulva* candidate DLs and for Alma1 were codon-optimised for *E. coli* expression by GeneArt (Table S1). Vacuum dried genes were reconstructed following the manufacturer's protocol, to reach a concentration of 40 ng/μL. They were then amplified by PCR using the Phusion Flash High-Fidelity PCR Master Mix (ThermoFisher Scientific) following the manufacturer's instruction, and using the following primers containing the extensions complementary to the vector used for heterologous expression: UM021_0036.1 forward primer AAGTTCTGTTTCAGGGCCCGGAAGGTAAAATGAAAGCACTGAGC and reverse primer ATGGTCTAGAAAGCTTTATGCGGTCAGCTGCTGTGCT; UM030_0039.1 forward primer AAGTTCTGTTTCAGGGCCCGGATACCA ATCTGCTGAAAGCA and reverse primer ATGGTCTAGAAAGCTTTATG CTTCAACCAGCATCTGCAGACC; ALMA1 forward primer AAG TTCTGTTTCAGGGCCCGGGTAATTGTACCAGCCATCCG and reverse primer ATGGTCTAGAAAGCTTTATTTACGTGCGGTAAAAACACCCCAT GCC. The pOPIN suite of protein fusion vectors was used [53], kindly donated by the Oxford Protein Production Facility UK (OPPF-UK). These vectors carry a HIS-tag for purification and different solubility tags among which TRX (thioredoxin), GST (glutathione S-transferase), MBP (maltose binding protein), SUMO, Halo7 and TF (trigger factor) were considered. The In-Fusion HD cloning kit (Takara) was used for integrating genes into the vectors according to the kit's instructions, and the obtained constructs were transformed by heat shock into Stellar competent cells (Clontech Laboratories) following the manufacturer's protocol. Plasmids were isolated from selected bacterial colonies using the Wizard *Plus* SV Miniprep DNA Purification kit (Promega Corporation). Plasmid nucleotide sequences were verified by Sanger sequencing by Eurofins Genomics. The recombinant vectors were then transformed into *E. coli* BL21(DE3)pLySs competent cells (Novagen) by heat shock. An expression screen was performed by growing the bacterial cells in 20 mL of LB supplemented with carbenicillin (50 μg/mL) and chloramphenicol (34 μg/mL) at 37 °C and 200 rpm until they reached the OD_{600} of 0.6–0.8. They were then induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown overnight at 20 °C and 200 rpm. After harvesting, the cells were pelleted, suspended in phosphate buffered saline (PBS) with 0.01 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) and were lysed with the BugBuster Protein Extraction Reagent (Millipore) following the manufacture's protocol. After addition of 5 mM $MgCl_2$ and DNaseI (0.025 U/μL), and filtering through a 0.22 μm filter, an aliquot of both the soluble and insoluble protein content was analysed by SDS-PAGE and western blotting to evaluate the production of the recombinant protein (Table S2).

2.4. Purification of recombinant Ulva sp. DMSP lyases

Scaled-up expression was performed using proteins with the tag which gave the best results in terms of quantity of purified protein produced: UM021_0036.1 with the MBP tag (hereafter named UM021_MBP), UM030_0039.1 with the Halo7 tag (UM030_Halo7) and Alma1 with only a His tag and no solubility tag (Alma1) (Table S2). *E. coli* BL21(DE3)pLySs cells containing the constructs were grown in 5 L of LB broth supplemented with carbenicillin (50 μg/mL) and chloramphenicol (34 μg/mL) at 37 °C and 200 rpm until OD_{600} of 0.6–0.8 and then induced with 1 mM IPTG and grown overnight at 20 °C and 200 rpm. After harvesting the cells were pelleted, suspended in his-binding buffer (20 mM sodium phosphate, 500 mM NaCl, pH 7.4, 0.1 % triton) with 0.01 mM AEBSF and were lysed by sonication. After addition of 5 mM $MgCl_2$ and DNaseI (0.02 5 U/μL) the supernatant was affinity purified with either a 5 mL HiTrap TALON crude or a 5 mL HisTrap FF crude column (both from GE Healthcare Life Sciences). The wash step was performed using the his-binding buffer with the addition of 5 mM imidazole and no triton, and bound proteins were eluted using a gradient of 5 to 500 mM imidazole over 20-column volumes. The eluted

fractions showing absorbance at 280 nm were analysed by SDS-PAGE to confirm the presence of the recombinant proteins. Selected fractions were then combined, concentrated to around 1 mL using Microsep TM Advance Centrifugal Devices (Pall Corporation), and run through a size exclusion column (HiLoad 16/600 Superdex 75 pg column, Ge Healthcare Life Science) using phosphate-buffered saline (PBS). The relevant peaks were again verified by SDS-PAGE, concentrated and the protein amount was determined with a nanodrop or using the Bradford method. The removal of the solubility tag was performed using a HRV 3C protease (produced by the Technology Facility at the University of York) at a ratio of 1:10 overnight at 4 °C and gentle shaking. Correct protein folding was assessed via thermal shift assays conducted on purified proteins using a Mx3005P qPCR System (Agilent Technologies) and SYPRO™ Orange Protein Gel Stain (Life Technologies) as a dye. This was diluted 1/100 in deionized water and 3 μL were added to the sample, which also contained 10 μL of the protein to be analysed and 17 μL of the appropriate buffer. The intensity of the fluorescence was measured against a temperature gradient of 25–95 °C and the obtained values were plotted to determine the melting temperature (Tm). This was done by curve fitting using a five parameter sigmoid equation with the Tm measured as the midpoint [54].

2.5. Biochemical characterization of recombinant Ulva DMSP lyases

Assays for DL activity were performed in triplicates with the following reaction mixture (final volume 300 μL): 192 μL of phosphate buffer 100 mM pH 8, 8 μL of DMSP 250 mM, and different quantities of purified proteins depending on which lyase was tested. Reactions were performed at 30 °C for 4 and 24 h, and stopped by boiling for 15 min. The presence of AA at the end of the reaction time was quantified by injecting 10 μL samples in a Waters 2695 HPLC fitted with a reverse-phase XBridge® BEH C18 Column 130 Å (Waters, 5 μm, 4.6 mm × 250 mm). Mobile phase A was H_2O + 0.1 % H_3PO_4 while phase B was 100 % acetonitrile. A flow rate of 1 mL/min was applied and the column was kept at 25 °C. A gradient profile of 30 min was run as follows: 5 min of 100 % A; then a 10 min gradient to drop to 0 % of A (so 100 % B), this held for 10 min; and a return to 100 % A in the last 5 min of the run. The HPLC was coupled to a photodiode array detector (Waters 2996) scanning from 210 nm to 400 nm, and detection of acrylic acid occurs at 210 nm. A standard curve used for the quantification of acrylic acid in enzymatic assay is presented in Fig. S2.

DL kinetic analysis was further conducted for UM030_Halo7 and Alma1 in triplicate and in a final volume of 600 μL reaction mix containing 384 μL of phosphate buffer 100 mM pH 8, 16 μL of DMSP 250 mM, and 22.8 μg of UM030_Halo7 or 12.0 μg of Alma1. Reactions were performed in the same conditions as described above, with samples taken before addition of the enzymes and after 0.5, 1, 2, and 24 h of reaction. Based on the information provided by these experiments, further analysis was focused on UM030_Halo7, using the same 300 μL reaction mix as described above, and with an incubation time of 30 min. Kinetic parameters (V_m and K_m) were determined after measuring specific activities in presence of DMSP concentrations ranging from 0 to 10 mM and by calculation based on Lineweaver-Burk plotting. The effect of pH was determined over a range from 6 to 9 in various buffers at 100 mM final concentrations: phosphate buffer at pH 6, 7 or 8, and Bis-tris propane buffer at pH 8 or 9. Influence of NaCl was examined by testing final concentrations ranging from 0 to 2 M. Similarly, impact of H_2O_2 was tested at final concentrations between 0 and 50 μM. The effect of temperature was assessed by incubating reaction assays between 0 and 40 °C.

3. Results and discussion

3.1. Profiling of DMSP pathway metabolites and of AA in Ulva spp. samples

Ulva spp. are known to produce DMSP and AA, but there is currently limited data on the amounts that can be found in the algal tissues of all the DMSP pathways metabolites, as well as of AA. Furthermore, there is little information on protocols for an efficient extraction of DMSP or AA from the green algal biomass, and for their subsequent quantification. For this reason, the HILIC-MS method previously used for the detection of DMSP only [44,45] was adapted to include quantification of MTOB, MTHB, DMSHB, DMSP and AA in the same analytical run, with consistent results. Peak shapes for the standards' mix were well-defined (despite being broader for DMSHB) and the sensitivity was appropriate, with the limit of quantification (LOQ) sometimes too high only for MTOB and AA (Fig. S1).

This approach was then utilised for the quantification of the metabolites of the DMSP and AA pathway in dried samples of the species *U. rigida* and *U. armoricana* after sonication. No detectable amounts of the intermediates MTOB, MTHB and DMSHB were found in the samples analysed, while both DMSP and AA were measurable (Table 4). AA constituted 1.28 % and 0.86 % of dry weight (DW) of *U. armoricana* and of *U. rigida* respectively. Amounts determined for DMSP were lower, with 0.05 % and 0.06 % DW for both algae. Based on these results, the quantities of biomass were scaled-up and different methods of fractionation were tested to assess their effects on the extraction of DMSP and AA.

3.2. Acid-base treatments of U. rigida

Having considered the nature of the feedstock and products along with technologies on the market, acid and alkaline extractions were conducted under different temperatures to assess their influence on DMSP and AA extraction. No MTOB, MTHB and DMSHB was detected after these treatments. As shown in Table 5, treatments at 120 °C were less efficient for the extraction of DMSP and AA compared to 90 °C, in particular with very low amount of DMSP quantified after incubation at 120 °C. In addition, alkaline pH allowed the recovery of higher amounts of AA, while no specific trend could be identified on the effect of pH on extraction of DMSP.

3.3. Microwave-assisted treatments of U. rigida

In addition to the acid-base extraction, microwave hydrothermal treatments were considered to assess the recovery of DMSP and AA. As observed in the previous experiments, no MTOB, MTHB and DMSHB could be quantified. Results presented in Table 6 showed that DMSP represented between 0.15 and 0.67 % of DW, while values for AA were between 0.04 and 0.35 %, with more AA extracted after treatment at high temperature. Although the quantities of these compounds were in the same range as those reported in Table 5, an opposite effect of the experimental temperature was noted, with higher temperatures allowing the retrieval of more AA and DMSP (despite a drop for DMSP at 160 °C). The impact of microwave treatments on the extraction of

Table 4
DMSP and AA amounts quantified in *U. armoricana* and *U. rigida* with the HILIC-MS method. Data presented are means ± S.D. DW = dry weight.

Species	DMSP (mg/l)	AA (mg/l)	DMSP (% DW)	AA (% DW)
U. armoricana	50.41 ± 24.00	1282.64 ± 139.36	0.05 ± 0.02	1.28 ± 0.14
U. rigida	55.98 ± 5.48	856.97 ± 31.88	0.06 ± 0.01	0.86 ± 0.03

Table 5
Concentrations of AA and of DMSP in liquid fractions obtained after acid-base treatments performed with dried *U. rigida* biomass at the BDC. The mean ± SD is reported for each measurement. A "0" value in the column "DMSP (% DW)" indicated that the percentage of DMSP was below 0.005 after calculation.

Treatment no.	Treatment type	DMSP (mg/L)	AA (mg/L)	DMSP (% DW)	AA (% DW)
1	90 °C, pH 7	934.43 ± 80.90	252.96 ± 16.78	0.93 ± 0.08	0.25 ± 0.02
2	90 °C, pH 4	617.86 ± 156.69	310.80 ± 23.27	0.62 ± 0.16	0.31 ± 0.02
3	90 °C, pH 9	556.06 ± 75.22	356.60 ± 7.46	0.56 ± 0.08	0.36 ± 0.01
4	120 °C, pH 7 - run 1	0.06 ± 0.05	149.72 ± 2.23	0	0.15 ± 0.00
5	120 °C, pH 7 - run 2	12.87 ± 0.29	101.88 ± 6.73	0.01 ± 0.00	0.10 ± 0.01
6	120 °C, pH 4 - run 1	0.01 ± 0.02	100.64 ± 3.27	0	0.10 ± 0.00
7	120 °C, pH 4 - run 2	10.96 ± 0.15	67.46 ± 1.87	0.01 ± 0.00	0.07 ± 0.00
8	120 °C, pH 9 - run 1	6.29 ± 0.20	183.48 ± 15.53	0.01 ± 0.00	0.18 ± 0.02
9	120 °C, pH 9 - run 2	4.73 ± 0.41	138.74 ± 10.51	0	0.14 ± 0.01

Table 6
Concentrations of AA and DMSP determined in liquid fractions obtained after different microwave-assisted treatments performed with dried *U. rigida* biomass at the BDC. The mean ± SD is reported for each measurement.

Treatment no.	Treatment type	DMSP (mg/L)	AA (mg/L)	DMSP (% DW)	AA (% DW)
10	Microwave 100 °C	437.86 ± 199.87	42.24 ± 11.97	0.44 ± 0.20	0.04 ± 0.01
11	Microwave 130 °C	668.10 ± 85.16	134.61 ± 23.72	0.67 ± 0.09	0.13 ± 0.02
12	Microwave 160 °C	150.81 ± 185.38	351.85 ± 23.25	0.15 ± 0.19	0.35 ± 0.02

valuable compounds (ulvan, proteins) from *Ulva* biomass has already been assessed, with temperature having a different effect depending on the recovered compounds [55].

3.4. Enzymatic treatments of U. rigida

Enzyme-assisted extraction has recently gained momentum over more traditional processing methods to fractionate compounds of interest from natural resources, including seaweeds, in light of the milder conditions used, the potentially higher efficiency of the process, and the preservation of the properties of the extracted compounds. Therefore, two proteases were tested for the extraction of DMSP and AA. No MTOB, MTHB or DMSHB could be detected after the treatments. Table 7 shows

Table 7
Concentrations of AA and DMSP determined in liquid fractions before and after enzymatic treatments. The mean ± SD is reported for each measurement. The symbol * indicates that AA was detected in only one of the 2 experiments conducted to test this treatment, hence SD is not reported.

Treatment no.	Treatments type	DMSP (mg/L)	AA (mg/L)	DMSP (% DW)	AA (% DW)
13	Mechanical juice	323 ± 27.53	76.68 ± 0.98	0.92 ± 0.08	0.22 ± 0.00
14	Papain	29.91 ± 0.17	6.76 ± 0.57	0.09 ± 0.00	0.02 ± 0.00
15	Neutrase	20.24 ± 2.81	3.11*	0.05 ± 0.01	0.01
16	No enzyme	34.93 ± 1.71	5.56*	0.10 ± 0.01	0.02

that the highest % DW for DMSP (0.92) and AA (0.22) were obtained in the mechanic juice (treatment 13), in ranges similar to more conventional methods. As described in Section 2.1.5, the mechanical juice is obtained before the addition of the enzymes, suggesting that further incubation in presence of individual proteases has only a limited impact on the extraction of additional quantities of both compounds of interest.

To complete the assessment of enzyme-assisted extraction methods, and to assess the use of polysaccharide degrading enzymes and glucosidase in the identification of the most effective protocol for the disruption of the algal cell wall and the release of the metabolites, a sequential two-enzyme treatment was applied on dried algal biomass. MTOB, MTHB and DMSHB were not detected after the treatment, while DMSP and AA could be quantified (Table 8). The amounts of DMSP measured under the different conditions were more homogenous compared to AA in the pre-hydrolysates. DMSP represented approximately 0.20 % of the biomass DW at the end of each sequential treatment, while values for AA ranged between 0.11 and 0.45 %. DMSP contents were higher in the pre-hydrolysates than after the first enzymatic treatment (liquid 1). For AA, the concentrations were similar or higher in liquid 1 when compared with pre-hydrolysates. This suggests that a first enzymatic treatment allowed to extract additional AA, while this was not the case for DMSP, and/or that DMSP may have degraded during the incubation for the first enzymatic treatment. When comparing the concentrations between liquid 1 and 2, lower values were recorded after the second enzymatic treatment for both DMSP and AA, although the extraction of DMSP was more efficient than that of AA after this second treatment. The amounts of DMSP and AA recovered with the different enzyme combinations show no specific trend in relation to the nature of the enzymes used. Importantly, the sequential treatment with two enzymes, compared to the water-only control treatment (treatment 17) did not result in higher yields. This is an important result in the context of a biorefinery approach, as it indicates that the addition of expensive enzymes would probably not be needed, with a consequent reduction of the costs involved in the extraction of AA. Interestingly, when comparing these treatments with the simple water-based extraction presented in Table 4 and performed at smaller scale, it was noticed that, while DMSP was higher in the enzymatic treatments, AA was found in higher amounts in the water-based extraction performed after sonication.

Overall comparison of the different biomass processing methods (chemical, microwave, enzymatic) showed limited variations in the maximum yields of AA obtained, i.e. 0.22–0.45 % DW. In contrast, DMSP yields were more variable depending on the protocols used for the treatment of the biomass. Our results are challenging to discuss in view of the current literature on *Ulva* spp. as, to our knowledge, there are no previous reports on the quantification of DMSP and AA in *Ulva* species using the processing techniques described in our study. Indeed, most of the published literature reports DMSP values obtained by measuring using gas chromatography the amount of DMS produced after hydrolysis with NaOH. *U. lactuca* harvested from different locations in the Northern Hemisphere was found to contain 60 to 250 μmol of DMSP per gram of fresh mass corresponding to 8.05 to 33.55 % DW if considering that dry weight represented 10 % of fresh weight [56,57]. The DMSP content of *U. clathrata* was found to be higher after hyperosmotic shock for 72 h (from 80 to 107 mmol per kg of fresh weight), suggesting that DMSP could constitute up to 14.36 % DW as calculated above [26]. *Enteromorpha bulbosa* (now named *Ulva hookeriana*) in the Antarctic was found to contain from 2.0 to 78.6 mmol of DMSP per kg of fresh weight (0.07–10.55 % DW) [58]. Similarly, DMSP contents for *Ulva* sp. and *Ulva compressa* from the West coast of Brittany (France) were reported in the range of 10 to 60 μmol per g of fresh weight (1.34–8.05 % DW) [59]. It is interesting to note that DMSP concentration changed in relation with different light levels, day length, and storage protocols, suggesting that more work is needed to determine the highest amounts of DMSP that could be extracted from *Ulva* species. We hope our results will pave the way for further analysis of DMSP and AA content in green seaweed

Table 8
Concentrations of AA and DMSP determined in liquid fractions before and after sequential enzymatic treatment. The mean ± SD is reported for each measurement, except for total and % DW. To determine the total concentration of DMSP and AA extracted for each scenario, the average concentration of DMSP and AA calculated for liquid 1 and 2 were considered. The total concentration was then used to estimate the % DW. ND = not detected.

Treatment no.	Sample	DMSP (mg/L)	AA (mg/L)	DMSP (% DW)	AA (% DW)
17	Prehydrolysate	103.56 ± 3.41	244.98 ± 48.81	0.16 ± 0.01	0.37 ± 0.07
	Liquid 1 (no enzyme)	73.77 ± 4.11	246.23 ± 16.92	0.11 ± 0.01	0.37 ± 0.03
	Liquid 2 (no enzyme)	57.84 ± 15.78	56.25 ± 34.26	0.09 ± 0.02	0.08 ± 0.05
	Total	65.80	151.24	0.20	0.45
18	Prehydrolysate	112.64 ± 9.75	134.78 ± 19.67	0.17 ± 0.01	0.20 ± 0.03
	Liquid 1 (neutrase)	85.22 ± 2.09	191.85 ± 4.59	0.13 ± 0.01	0.29 ± 0.01
	Liquid 2 (ulvan lyase)	64.68 ± 9.50	60.84 ± 18.67	0.10 ± 0.01	0.09 ± 0.03
	Total	74.95	126.35	0.22	0.38
19	Prehydrolysate	111.12 ± 8.08	121.06 ± 4.57	0.17 ± 0.01	0.18 ± 0.01
	Liquid 1 (ulvan lyase)	56.86 ± 1.88	145.39 ± 20.64	0.09 ± 0.01	0.22 ± 0.03
	Liquid 2 (neutrase)	29.84 ± 4.90	27.24 ± 10.28	0.04 ± 0.01	0.04 ± 0.02
	Total	43.35	86.32	0.13	0.26
20	Prehydrolysate	125.88 ± 4.75	76.77 ± 10.05	0.019 ± 0.01	0.12 ± 0.02
	Liquid 1 (papain)	126.73 ± 2.10	77.01 ± 12.29	0.19 ± 0.01	0.12 ± 0.02
	Liquid 2 (ulvan lyase)	33.46 ± 13.70	ND	0.05 ± 0.02	0
	Total	80.10	37.45	0.24	0.11
21	Prehydrolysate	133.98 ± 3.62	71.82 ± 7.94	0.20 ± 0.01	0.11 ± 0.01
	Liquid 1 (neutrase)	107.3 ± 5.67	111.84 ± 5.39	0.16 ± 0.01	0.17 ± 0.01
	Liquid 2 (amyloglucosidase)	56.90 ± 12.98	3.07 ± 0.24	0.09 ± 0.02	0
	Total	82.26	57.46	0.25	0.17
22	Prehydrolysate (neutrase)	117.29 ± 3.61	89.71 ± 10.63	0.18 ± 0.01	0.13 ± 0.02
	Liquid 1 (Cellic CTec3)	82.94 ± 6.63	115.06 ± 8.62	0.12 ± 0.01	0.17 ± 0.01
	Liquid 2	67.64 ± 6.39	12.08 ± 6.74	0.10 ± 0.01	0.02 ± 0.01
	Total	75.29	63.57	0.23	0.19

biomass, particularly in the context of on-going and future implementation of biorefineries for bloom forming and farmed *Ulva*.

3.5. In silico analysis of U. mutabilis DLs

Analysis of the *U. mutabilis* genome revealed the presence of two candidate DL genes (*UM030_0039.1* and *UM021_0036*) that may have occurred by lateral gene transfer of an *Alma1* type gene from the chromalveolate lineage into the ancestor of *Ulva* and/or other green macroalgae [1]. Pairwise comparison of *Alma1* with *U. mutabilis* homologs showed that UM030_0039.1 and UM021_0036.1 shared 39.7 % and 33.9 % of similarity with the biochemically characterized haptophyte protein, respectively (Fig. S3). The green macroalgal proteins showed 54.3 % of similarity between themselves. Their molecular weight was 36.8 and 40.2 kDa, and they were found to contain four and six disulfide bonds, respectively. Alignment of the two *U. mutabilis* DLs with Alma1 showed conservation of cysteine residues C108 and C265 (numbering from Alma1, Fig. S3), which have been shown to be important for enzymatic activity [30]. None of the *U. mutabilis* DLs harboured a signal peptide, and none of them was predicted to contain transmembrane domains, while the green macroalga *U. clathrata* has been suggested to contain a membrane bound DMSP lyase [26] and *U. curvata* to possess one soluble and three membrane-bound isozymes of DMSP lyase [27].

3.6. Cloning, expression and purification of recombinant Ulva DLs

Both green macroalgal DLs and Alma1, the latter used as positive control for biochemical characterization, were cloned in several vectors of the pOPIN suite to enable production of recombinant proteins with His-tag at their N-terminal end and with different solubility tag at their C-terminus (Table S2). Small scale expression screens were then conducted to identify conditions allowing the production of soluble recombinant proteins. Table S2 shows that soluble Alma1 was observed under all the conditions tested, i.e. without solubility tag and with TRX, GST and MBP tags. Higher production of soluble UM021_0036.1 was obtained with the MBP solubility tag, while soluble UM030_0039.1 was observed only when fused with the Halo7 tag. Based on these results, production was scaled-up for UM021_0036.1 and UM030_0039.1 fused to the MBP and Halo7 solubility tag respectively. These proteins were thereafter referred as UM021_MBP and UM030_Halo7, with a molecular weight of 83 kDa (40 kDa UM021_0036.1 and 43 kDa MBP) and 72 kDa (37 kDa UM030_0039.1 and 35 kDa Halo7) respectively. For both proteins, five litre cultures were used for production of recombinant proteins that were subsequently purified using a combination of affinity and size exclusion chromatography (SEC). The chromatograms for both purification steps are shown in Figs. S4 and S5, together with SDS-page gels showing bands at the expected protein size (Fig. 2), which were also confirmed by western blotting analysis with anti-his antibodies. Fractions T18 to TB3 for UM021_MBP and T19-T23 for UM030_Halo7 were pooled and concentrated before cleaving the solubility tag. Tag cleavage was attempted several times for both *U. mutabilis* DLs, but removal was not complete and therefore proteins with their solubility tag still attached were used. Both tagged proteins were tested for proper folding by differential scanning fluorometry (Fig. S6) and were then used to assess biochemical activity.

3.7. Biochemical characterization of U. mutabilis DLs

To assess the activity of recombinant DLs, quantification of AA produced under different conditions was done using high-performance liquid chromatography (HPLC). Alma1 was used as a positive control to assess DL activity since the activity was not tested by DMS production as done in previous works [27,30,60], but by direct quantification of AA produced. Experiments were conducted at pH 8 as it was the optimum pH determined for *U. curvata* endogenous soluble DMSP lyase [27]. Table 9 showed that production of AA occured with all the recombinant proteins tested, and higher production was determined in presence of Alma1 compared to *U. mutabilis* DLs despite lower amounts of protein used, with limited variations observed between 4 and 24 h of incubation.

Among the two *U. mutabilis* DLs tested, UM030_HALO7 was the most active. Interestingly, the gene coding for UM030_0039.1 was observed to be transcribed at a level higher than its paralogs UM021_0039.1 in algal samples grown at 18 °C under xenic and axenic conditions [1].

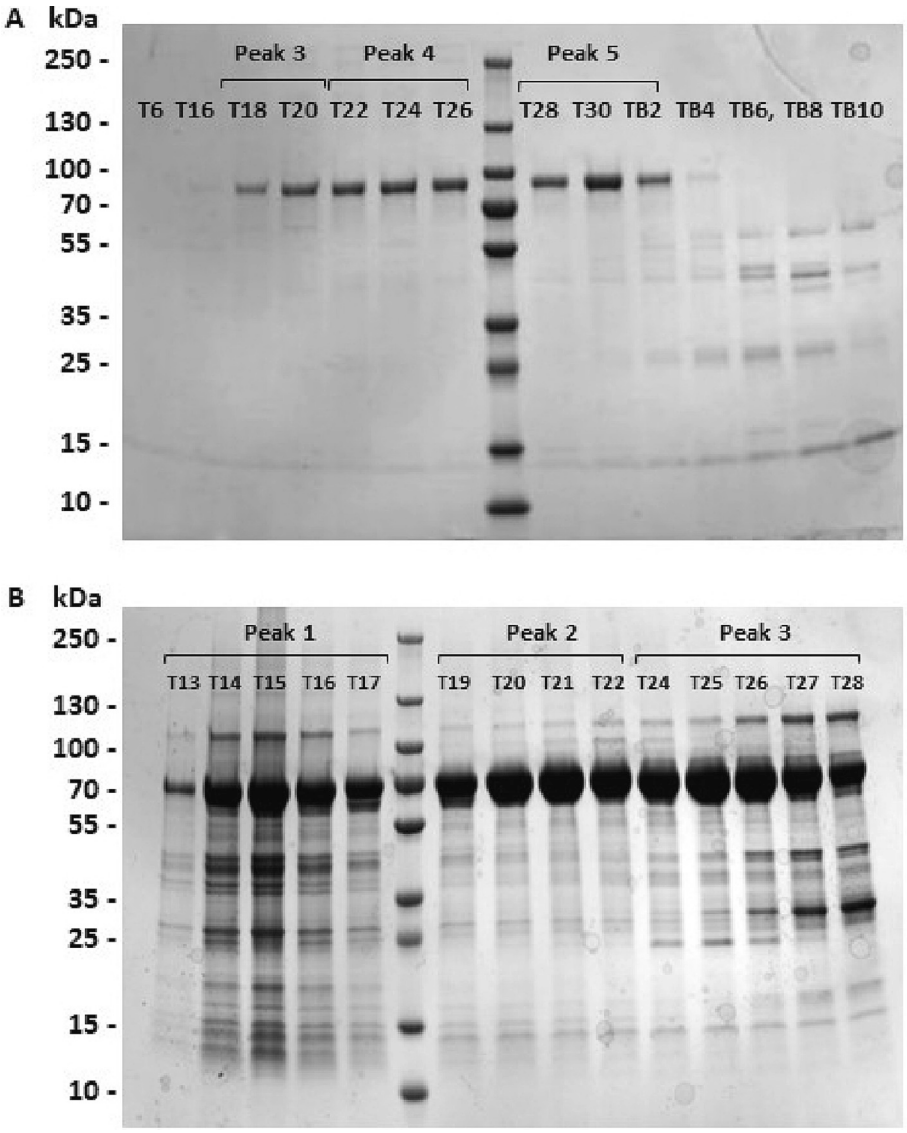

Fig. 2. Purification of recombinant *U. mutabilis* DLs by SEC. A) Results for UMO21_MBP. Several peaks were observed after SEC (Fig. S4), and representative fractions corresponding to peaks 3, 4 and 5 were loaded on an SDS-PAGE gel. B) Results for UM030_Halo7. Representative fractions from peaks 1, 2 and 3 obtained from SEC (Fig. S5) were loaded on an SDS-PAGE gel.

Table 9
Assessment of AA production by recombinant Alma1 and *U. mutabilis* DLs. Specific DL activities were calculated after 4 h of incubation.

	Quantity of protein used	Concentration of AA (mM)		Specific DL activities (μM/min/mg)
		After 4 h	After 24 h	
UM021-MBP	84 μg	0.56 ± 0.11	0.33 ± 0.03	27.68 ± 5.33
UM030-HALO7	11.4 μg	4.98 ± 1.32	3.98 ± 0.10	1818.31 ± 481.76
Alma 1	6 μg	5.92 ± 0.47	5.72 ± 0.36	4111.66 ± 327.13

These differences in gene expression and biochemical activities suggest potential distinct physiological roles and/or regulation for both DLs. Considering these results, subsequent biochemical analysis was conducted with UM030_Halo7 as the representative *U. mutabilis* DL. Kinetic analysis of AA production for both UM030_Halo7 and Alma1 showed that all the production of AA by Alma1 occurred in the first 30 min of the reaction, while almost half of the AA produced by UM030_Halo7 occurred during the same period of time and then continued at a slower pace during the rest of the incubation (Fig. 3A). Therefore, further enzymatic assays were incubated for 30 min. Taking into consideration that previous biochemical analysis of *U. curvata* soluble DL was conducted at pH 8 [27], this pH was used to establish the K_m and V_m of UM030_Halo7. This recombinant protein exhibited typical Michaelis–Menten kinetics when assayed with increasing concentrations of DMSP (Fig. 3B). Apparent K_m and V_m for this substrate were calculated from a Lineweaver–Burk plot and values of 7.10 mM and 5.99 mM/min/mg protein were obtained, respectively. This K_m was similar to the value calculated for Alma1, i.e. K_m of 9 mM [30], but different from results of previous analysis of native soluble *Ulva* DL (K_m = 0.52 mM [27]) and of DL enzymes from the haptophytes *Phaeocystis* spp. (1.77 and 2.31 mM, [28]). The influence of pH on UM030_Halo7 activity was tested in presence of phosphate buffers at pH 6 to 8, and Bis-tris propane buffers at pH 8 and 9. Higher production of AA was observed at pH 6, with decreasing production of AA as pH increased, supported by the AA concentration at pH 9 being almost half the value determined at pH 6 (Fig. 3C). Analysis of DL activity in cell free crude extracts of the green

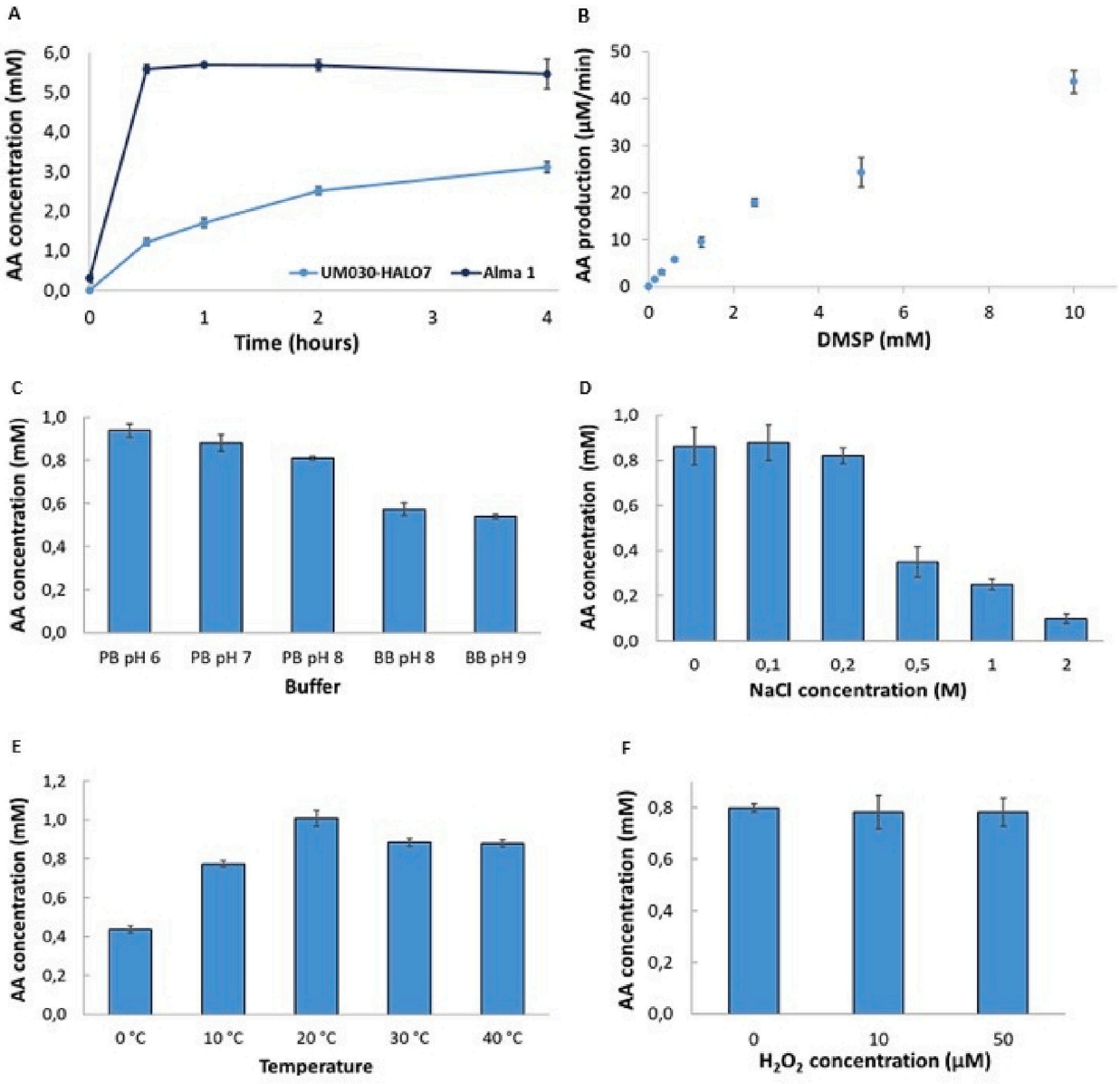

Fig. 3. Biochemical characterization of UM030_Halo7. Each condition was tested in triplicate. A) Kinetic analysis of acrylic acid production. Quantities of proteins used: 22.8 µg for UM030_Halo7 and 12 µg for Alma1. B) Michaelis-Menten plot. C) DL activity measured at different pH values ranging from 6 up to 9; PB = phosphate buffer, BB = Bis-Tris propane buffer. D) DL activity tested at increasing concentration of NaCl. E) Effect of temperature on DL activity. F) Effect of H_2O_2 concentration on DL activity.

macroalga *E. clathrata* showed a similar pH optimum of 6.2 to 6.4 [26]. The influence of different concentrations of NaCl was also tested. Clear changes were observed when passing from a NaCl concentration of 0.2 M to 0.5 M, as the amount of AA produced was less than half of the original value observed in the absence of NaCl or at molarities of 0.1 and 0.2 (Fig. 3D). This behaviour was similar to the 40 % reduction in DL activity observed for the soluble *U. curvata* enzyme between 0.3 and 0.6 M of NaCl [27], but different when comparing with the *E. clathrata* DL activity, which did not vary in presence of NaCl 5.0 and 500 mM [26]. At concentrations of NaCl higher than 0.5 M, a stronger reduction of AA production was observed, suggesting that recombinant UM030_Halo7 may have become sensitive as the NaCl concentration reached that of seawater. This contrasted with Alma1, which showed normal DL activity in presence of 2.0 M NaCl. The optimal reaction temperature for UM030_Halo7 was 20 °C (Fig. 3E), while it was 25 °C for *E. clathrata* [26]. Finally, the influence of H_2O_2 was tested as it was shown that Alma1 was a redox-sensitive enzyme [30]. In contrast to what was observed with the *Emiliania* DL, UM030_Halo7 was not inhibited by H_2O_2 up to 50 µM (Fig. 3F). The difference of sensitivity to NaCl and H_2O_2 between Alma1 and UM030_Halo7 suggests that the two DLs may act under different physiological conditions, despite belonging to the same Asp/Glu/hydantoin racemase superfamily. Therefore, it would be interesting to test the influence of the recently developed 2-bromo-3-(dimethylsulfonio)-propionate inhibitor that inhibits DLs of the Alma enzyme family, but not any of the other known families of DLs [31]. It would also be advantageous to decipher the tertiary structure of algal DLs in order to compare the mechanisms of action and potentially explain the difference observed in the enzymatic properties. The recent development of a molecular toolkit for transgenic studies in the green seaweed *U. mutabilis* [61], and of a genome editing protocol for *U. prolifera* [62] represent useful tools for future studies aimed at advancing knowledge on the biological roles of DLs in green seaweeds.

4. Conclusions

We have shown that it is possible to extract AA from *Ulva* biomass by means of simple processing of this feedstock. In the context of a biorefinery approach for the valorisation of *Ulva* biomass, our results indicate that uncomplicated extraction methods could permit the retrieval of AA, an important compound with numerous downstream applications in several industries. The utilisation of gentle enzymatic treatments, which allowed AA retrieval in quantities similar to the chemical and thermal treatments, would mean that the left-over biomass could still be utilised for further processing to extract both protein and non-protein constituents for the commercialisation of valuable products such as animal feed, fertilisers, chemicals and biofuels [33,36–38]. However, further work is needed to optimize extraction of AA and of other different desired molecules in the context of *Ulva* biorefining.

The characterization of algal DLs may also promote the development of alternative routes for the biosynthesis of AA using organisms that accumulate its precursor DMSP at high concentration, including cyanobacteria [63] that could be developed as new microbial chassis. Indeed, there is the need to increase the bioproduction of acrylates, as AA and its esters are key building blocks for diverse high-value oligomers and polymers in the current chemical industry [64], and it is incumbent on the industry to steer away from current carbon-heavy modes of production. In addition, advances in the knowledge of the molecular basis of the DMSP biosynthetic pathways and its cleavage into DMS and AA in different algae represent an opportunity to compare the ecological and biological role(s) of these biochemical systems across organisms inhabiting different marine ecosystems.

CRediT authorship contribution statement

Giovanna Pesante: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Edith Forestier:** Formal analysis, Investigation, Methodology, Writing – review & editing. **Swen Langer:** Formal analysis, Investigation, Methodology, Visualization, Writing – review & editing. **Andrew Danby:** Formal analysis, Investigation, Methodology, Writing – review & editing. **John Angus:** Formal analysis, Investigation, Methodology, Writing – review & editing. **Mark Gronnow:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – review & editing. **Joseph P. Bennett:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – review & editing. **Tony R. Larson:** Conceptualization, Formal analysis, Investigation, Methodology, Writing – review & editing. **Thierry Tonon:** Conceptualization, Formal analysis, Visualization, Writing – original draft, Writing – review & editing.

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.algal.2023.103176.

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