An individual alginate lyase is effective in the disruption of *Laminaria digitata* recalcitrant cell wall

Mónica Costa¹, Luís Pio¹, Pedro Bule¹, Vânia Cardoso², Cristina M. Alfaia¹, Diogo Coelho¹, Joana Brás², Carlos M. G. A. Fontes¹,² & José A. M. Prates¹,²*

In the present study, 199 pre-selected Carbohydrate-Active enZymes (CAZymes) and sulfatases were assessed, either alone or in combination, to evaluate their capacity to disrupt *Laminaria digitata* cell wall, with the consequent release of interesting nutritional compounds. A previously characterized individual alginate lyase, belonging to the family 7 of polysaccharide lyases (PL7) and produced by *Saccharophagus degradans*, was shown to be the most efficient in the in vitro degradation of *L. digitata* cell wall. The alginate lyase treatment, compared to the control, released up to 7.11 g/L of reducing sugars (*p* < 0.001) and 8.59 mmol/100 g dried alga of monosaccharides (*p* < 0.001), and reduced cell wall fluorescence intensity by 39.1% after staining with Calcofluor White (*p* = 0.001). The hydrolysis of gel-forming polymer alginate by the alginate lyase treatment could prevent the trapping of fatty acids and release beneficial monounsaturated fatty acids, particularly 18:1c9 (*p* < 0.001), to the extracellular medium. However, no liberation of proteins (*p* > 0.170) or pigments (*p* > 0.070) was observed. Overall, these results show the ability of an individual alginate lyase, from PL7 family, to partially degrade *L. digitata* cell wall under physiological conditions. Therefore, this CAZyme can potentially improve the bioavailability of *L. digitata* bioactive compounds for monogastric diets, with further application in feed industry.

Due to an increasing interest in the use of macroalgae for food and feedstuffs¹, as well as for pharmaceutical industries, organic fertilizers, eutrophication inhibition, bioremediation and biogas generation², their cultivation has been steadily growing over the last decade. The nutritional profile of macroalgae, although variable among species and depending on growth location and harvesting season¹, consists of numerous vitamins, minerals, pigments, phenolic compounds, carbohydrates and high quality proteins¹. Carbohydrates comprise a high proportion of macroalgae biomass (from 4 to 76% dry matter, DM)³, whereas lipids are usually found in small amounts (<5% DM) with values up to 1.13% DM in brown algae³. However, their lipid profile can be rich in monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids⁴–⁶, which might have beneficial effects on human health⁷.

*Laminaria* sp. are seawater multicellular eukaryotic and autotrophic brown macroalgae, which are amongst the most cultivated seaweeds worldwide, representing the largest biomass in coastal regions⁸. Brown macroalgae have a distinct carbohydrate-rich cell-wall⁹,¹⁰, which comprises up to 45% DM of alginate and fucose-containing sulphated polysaccharides (FCSPs) (fucans or homofucans and fucoids or heterofucans), as well as small amounts of cellulose (1 to 8% DM)¹¹, β-1,3 glucans, unbranched mixed-linkage β-D-glucans (1,3-and-1,4-β-D-glucose residues) masked by alginate¹² and arabinogalactans linked to proteins¹³. The proportion of alginates, fucoids and cellulose was found to be 3:1:1¹⁰. Alginate is a linear polysaccharide composed of only two epimers, β-1,4-D-mannuronic (M) and α-1,4-L-guluronic (G) acids, arranged in heteropolymERIC (MG) or homopolymeric (MM or GG) blocks along the polymer chain¹¹. FCSPs are composed of two different backbones with α-1,3- or alternating α-1,3-/α-1,4-linked L-fucose residues. These motifs form acetylated or branched structures presenting one to three sulphate esters on positions O-2, O-3 or O-4¹¹. Laminarin is a polysaccharide located in intracellular vacuoles that constitutes the carbon storage of macroalgae and is composed of (1,3)-β-D-glucopyranose residues with some 6-O-branching in the main chain and β-1,6-intrachain links¹⁴,¹⁵. These polysaccharides were found

¹CIISA - Centro de Investigação Interdisciplinar Em Sanidade Animal, Faculdade de Medicina Veterinária, Universidade de Lisboa, 1300-477, Lisboa, Portugal. ²NZYTech - Genes and Enzymes, Estrada do Paço Do Lumiar, Campus do Lumiar, Edifício E, 1649-038 Lisboa, Portugal. ³email: japrates@fmv.ulisboa.pt
to have bioactive properties with relevance for potential applications in functional foods and feeds, cosmetics and pharmaceutical products\(^1\)–\(^8\). For instance, alginate was shown to reduce blood pressure and cholesterol and to have antimicrobial and anticancer activities\(^9\); FCSPs were described as antioxidant, anti-inflammatory, immunostimulant, antimicrobial and anticancer compounds\(^11\)–\(^15\), and laminarin was reported as an anticancer, anti-inflammatory, immunostimulatory, anticoagulant and antioxidant\(^14\)\(^,\)\(^15\) agent.

However, cell wall carbohydrates are organized in a complex cross-linked matrix, resulting in a highly recalcitrant cell wall that resists breakage and serves as a natural defence mechanism for algae\(^6\). In fact, alginic cross-links with phenolic compounds and constitutes gel-forming and hydroscopic polymers that control cell wall rigidity\(^11\). These polymers form a network that embed fucose-containing sulphated polysaccharides. The latter are tightly assembled to cellulose microfibrils by cross-linkage\(^11\). The intricate macroalga cell walls have been described to exert anti-nutritional effects for monogastric animals, by trapping valuable nutrients, with a concomitant decrease in the efficiency of feed digestion and absorption\(^16\). The presence of complex polysaccharides in seaweed cell walls can also decrease the rate of algae biomass hydrolysis during the production of renewable energies, thus reducing bioethanol and biogas yields\(^17\).

Mechanical processes, such as hammer mill, are usually applied for incorporation of seaweed in diets for monogastric animals\(^1\). However, mechanical methods are less specific for macroalgae cell disruption than enzymatic procedures. Therefore, the latter can be more advantageous for the release of algae valuable nutritional and bioactive compounds and increase of their bioavailability\(^16\). The effectiveness of exogenous enzymes (i.e., cellulases, xylanases\(^16\)–\(^22\) and a mixture of carbohydrases\(^19\)–\(^22\)) on hydrolysing algae biomass with an increase of protein extraction or digestibility was previously demonstrated for green (i.e., *Ulva rigida*)\(^16\) and red (e.g., *Palmaria palmata*, *Gracilaria* sp. and *Chondrus* sp.)\(^19\)–\(^22\) seaweeds. Other studies reported the use of cellulases, alginate lyases\(^23\)–\(^25\) and a carbohydrase mixture\(^26\) for the degradation of brown macroalgae biomass (*Laminaria digitata*)\(^22\)\(^,\)\(^25\); *Saccharina latissima*\(^24\)\(^,\)\(^25\) and a mixture of different species including *Sargassum* sp.\(^26\) envisaging biotechnological applications. These applications consisted in the production of bioethanol and biogas\(^23\)\(^,\)\(^25\), algae saccharification\(^24\) and extraction of bioactive compounds\(^26\).

Therefore, exogenous Carbohydrate-Active enzymes (CAZymes) could be a suitable option to deconstruct macroalgae cell wall, similarly to what was recently described by our research team for microalgae\(^27\)\(^,\)\(^28\). Moreover, these exogenous enzymes were shown to improve the nutritional value of cereal-based diets\(^29\)\(^,\)\(^30\), with the consequent industrial application as feed additives for poultry and pigs\(^31\). Additionally, the use of sulfatases could be of major importance for the degradation of the recalcitrant structure of branched and sulphated FCSPs, as reported in a recent study\(^32\). Thus, we hypothesised that CAZymes and sulfatases could, individually or in combination, degrade the recalcitrant *L. digitata* cell wall, with the consequent improvement of nutrients bioavailability. Cell wall disruption was assessed by fluorescence microscopy, reducing sugars and oligosaccharides profile after incubation of macroalga with the enzymes. The release of nutritive and bioactive compounds from macroalgae, following the enzyme treatment, was assessed by quantifying proteins, pigments and fatty acids.

**Results**

**CAZymes and sulfatases selection and evaluation of expression and purity of recombinant enzymes.** All of the CAZymes and sulfatases selected for the initial screen were chosen for their activity, either determined or predicted, towards specific compounds of brown macroalgae cell walls, like alginate, FCSPs and cellulose\(^11\). Furthermore, most are produced by marine halophilic bacteria, which are organisms likely adapted to feed on algae biomass. The catalytic activity and the biochemical properties of the majority of the candidates were previously described in the literature and their amino acid sequences can be accessed in Genbank (see Supplementary Table S1). Seventeen of the selected CAZYmes (ID 166 to 174, 184, 189 to 195) did not have their activity and substrate specificity previously characterized, but were selected due to sharing high homology (see Supplementary Table S1 for Genbank accession numbers) to some of the other well-characterized candidates.

In order to evaluate the soluble protein yield for each enzyme, a qualitative scale was used based on the protein concentration (g/L): -, 0.0; +, 0.1 > 2.1; ++, 2.1 > 4.1; ++++, 4.1 > 6.1; ++++ > 6.1 (see Supplementary Table e S1). From the 199 recombinant enzymes, 23 did not express (-), 81 had low expression levels or were mostly insoluble (+) and 95 had good expression and solubility levels (+++, ++ ++, ++ ++ +). Among low expressing enzymes, 4 were slightly degraded (ID 5, 69, 73 and 129). Among high expressing enzymes, 1 had low solubility, 1 was degraded and 3 (ID 147, 151 and 184) had a different SDS-PAGE migration pattern than what was expected from the calculated molecular weight (data not shown). All soluble protein fractions were enriched by a high throughput IMAC protocol before the activity screens (see Supplementary Fig. S1).

**Screening of individual enzymes for Laminaria digitata cell wall disruption.** Each one of the 176 CAZymes and sulfatases with low to high expression levels (see Supplementary Table S1) was individually incubated with a macroalga suspension in phosphate buffered saline (PBS) solution for an evaluation of their ability to degrade *L. digitata* cell wall. The majority of enzymes was unable to deconstruct alga biomass (see Supplementary Table S2), but 8 individual enzymes (ID 6, 18, 20, 21, 22, 28, 29 and 46) had a measurable capacity to degrade the cell wall of *L. digitata*, as shown in Table 1. This ability was assessed by both the release of reducing sugars, as evaluated through the 3,5-dinitrosalicylic acid (DNSA) method, and the decrease of fluorescence intensity from Calcofluor white stained cell walls. The fact that brown macroalgae cell walls form an intricate carbohydrate structure\(^11\) will allow the evaluation of cell wall integrity loss by assessing the reduction of Calcofluor white staining fluorescence intensity, even though the dye only binds specifically to minor compounds of the cell wall (cellulose and, to some extent, mixed-liked 1,3–1,4-β-glucans)\(^12\)\(^,\)\(^33\). Therefore, the data in Table 1 is presented according to two qualitative scales: the first scale is based on the amount of released reducing sugars (g/L):
The eight-enzyme mixture led to a release of reducing sugars of 6.74 g/L, which corresponded to an increase of only 0.57 g/L compared to the three-enzyme mixture. Then, the latter mixture was compared to the activities of each enzyme in the eight-enzyme mixture.

Table 1. Screening of the selected individual CAZymes for Laminaria digitata cell wall disruption. Each enzyme is presented with the project identification number (ID), name, category, EC number, main substrate and qualitative scales of released reducing sugars and fluorescence intensity. The following qualitative scales were defined: (1) amount of released reducing sugars (g/L): −, 0.00 < 2.77; +, 2.77 < 3.99; ++, 3.99 < 5.20; +++ , 5.20 < 6.42, ++++, > 6.42; (2) decrease of fluorescence intensity (%): −, 0.00 < 9.92; +, 9.92 < 20.0; ++, 20.0 < 29.9; +++ , 29.9 < 38.5; ++++, > 38.5. The numeric values of released reducing sugars and decreased fluorescence intensity obtained for alginate lyase (ID 22), the most active enzyme on cell wall disruption, are also presented. *Initial screening, **final incubation.

| ID   | Name                                      | Category        | EC number       | Main substrate                          | Released reducing sugars scale | Decreased fluorescence intensity scale |
|------|-------------------------------------------|-----------------|-----------------|-----------------------------------------|-------------------------------|----------------------------------------|
| 6    | Cellulase (Cel73;Cell73)                  | Cellulases      | 3.2.1.4         | 1,3–1,4-β-glucans and soluble 1,4-β-glucans | − (0.26 g/L)                  | + (14.6%)                              |
| 18   | Laminarinase A (LamA)                     | 1,3-β-Glucanases| 3.2.1.3/3.2.1.39| Laminarin (1,3–1,4/1,6-β-glucans)       | + + (5.16 g/L)                | ++++ (40.1%)                           |
| 20   | Endo-guluronate lyase (AlyA1;zobellia_1182)| Poly-α-guluronate lyases | 4.2.2.11       | Sodium alginate/β-elimination reaction | + + + + (6.63 g/L)            | ++++ (49.8%)                           |
| 21   | 1,3–1,4-β-glucanase P2 (LacPGluB)         | 1,3–1,4-β-glucanases | 3.2.1.73       | 1,3–1,4-β-glucans                      | + + (5.10 g/L)                | ++++ (51.6%)                           |
| 22   | Alginate lyase / poly-β-mannuronate (Sde_2547) | Alginates lyases | 4.2.2.3        | Alginates and oligoalginates           | + + + + (6.78*-7.11** g/L)    | ++++ (39.1**—46.5% )                   |
| 28   | Cellobiohydrolase (CbhA; Cbh_0413)        | Cellobiohydrolases | 3.2.1.3/3.2.1.91| Amorphous and crystalline cellulose    | + + + + (5.42 g/L)            | ++++ (57.0%)                           |
| 29   | Lytic transglycosylase A (Mba; Mlbh2613)  | Poly-α-guluronate lyases | 4.2.2.n1      | Murein glycan strands and insoluble, high-molecular weight murein sacculi | + + (4.42 g/L)               | ++++ (42.9%)                           |
| 46   | α-L-fucosidase C (ARC; LCABL_29340 possible fragment) | Fucosidases | 3.2.1.51 F1-6Gn | p-nitrophenyl-α-L-fucopyranoside        | − (0.65 g/L)                  | + (22.3%)                              |

Table 2. Evaluation of additive and synergistic effects between the most active CAZymes (project identification number, ID) on the release of reducing sugars from Laminaria digitata cell wall. *Mix 8: ID 6, 18, 20, 21, 22, 28, 29 and 46; Mix 3: ID 18, 22 and 46.

| Enzymes* | Released reducing sugars (g/L) | p-value (Mix 8 versus ID 22) | p-value (Mix 3 versus ID 22) |
|----------|--------------------------------|-------------------------------|-------------------------------|
| Mix 8    | 6.74                          |                               |                               |
| Mix 3    | 6.17                          |                               |                               |
| ID 18    | 4.57                          | 0.001                         |                               |
| ID 22    | 6.25                          |                               | 0.443                         |
| ID 46    | 0.50                          |                               |                               |

Selection of the most active enzymes and assessment of their synergistic action. In order to disclose synergistic actions among individual enzymes, an eight-enzyme mixture based on the initial screening (Table 1), was compared to a three-enzyme mixture (ID 18, 22 and 46) (Table 2, Figs. 1, 2 and 3). These three enzymes were selected based on their organism of origin, thermostability and main substrate. Indeed, laminarinase (ID 18) and alginate lyase (ID 22) were isolated from marine and halophilic bacteria (Thermotoga napolitana44 and Saccharophagus degradans35), respectively, and were described as being thermostable with optimum catalytic activities at 85 to 95 °C44 and 50 °C35, respectively. Although the enzyme with ID 46 was from a non-marine and non-halophilic bacterium (Lactobacillus casei)36, it was relatively thermostable, with an optimum temperature of 42 °C36, and acted towards a main constituent of brown algae cell wall (α-linked L-fucopyranosyl units)37.

The eight-enzyme mixture led to a release of reducing sugars of 6.74 g/L, which corresponded to an increase of only 0.57 g/L compared to the three-enzyme mixture. Then, the latter mixture was compared to the activities...
of each enzyme composing it. It was observed that, when enzyme ID 22 enzyme was individually incubated with *L. digitata* suspension, the value of released reducing sugars was identical (p = 0.443) to the three‐enzyme mixture (6.17 g/L). In contrast, the released reducing sugars by the other individual enzymes (4.57 g/L for enzyme ID 18, and 0.50 g/L for enzyme ID 46) were significantly lower than that of mixture (p < 0.001). Altogether, these results indicate the absence of significant (p > 0.050) additive or synergistic effects among enzymes.

The ratios of released reducing sugars were found to be: alginate lyase *versus* three‐enzyme mixture = 101.3%; alginate lyase *versus* laminarinase = 136.6%, and alginate lyase *versus* fucosidase = 1242%. Regarding the above values, the alginate lyase ID 22 was selected as the most active enzyme for the degradation of *L. digitata* cell wall.

**Effect of alginate lyase on *Laminaria digitata* cell wall integrity.** The extension of released reducing sugars and decreased fluorescence pixels of stained cell walls promoted by the selected alginate lyase (ID 22; Provisional Patent number, INPI, Portugal) are presented in Table 1. The latter is also illustrated in Figs. 1a, b and c. The amount of reducing sugars (7.11 g/L) was significantly increased (p < 0.001), whereas the number of pixels (179 to 109; 39.1% decrease of fluorescence intensity) was significantly reduced (p = 0.001) with the enzyme ID 22, when compared to the control assay.

**Activity, thermostability and proteolysis assays of alginate lyase.** Catalytic activity of alginate lyase (ID 22) was evaluated by both UV spectroscopy, using alginate as substrate at pH 7.5 and 37 °C, and DNSA method. The enzyme showed an activity of 1.52 ± 0.026 AU/min @233 nm and 0.282 ± 0.0025 g reducing sugars/L × min.

The purified (> 90% purity) alginate lyase was tested for its thermostability and proteolysis resistance. For the thermostability assay, the intact protein was subjected to a range of temperatures (30 to 80 °C) (Fig. 4). The enzyme maintained its stability at 37 and 40 °C. Although significant (p < 0.001), only a small variation of protein concentration was found between these two temperatures (0.81 to 0.74 g/L, respectively). However, the stability of alginate lyase declined abruptly between 40 °C and 50 °C, with the enzyme being completely degraded at 50 °C. The proteolytic resistance of alginate lyase is shown in Table 3 and Fig. 5. The enzyme showed partial resistance over the entire assay time.
Effect of alginate lyase on the release of mono- and oligosaccharides from *Laminaria digitata* cell wall. Figure 6 shows the influence of alginate lyase treatment on the release of mono- and oligosaccharides from *L. digitata* cell wall. The composition of mono and oligosaccharides was a mixture of compounds, not individually identified due to its complexity and lack of some commercial standards. With the enzyme treatment, monosaccharide concentrations significantly increased (*p* < 0.001), from 0.02 to 8.64 mmol/100 g dried alga, in relation to the control. Although the amount of oligosaccharides did not significantly differ (*p* = 0.260) between assays, a numerical increase, from 1.19 to 2.57 mmol/100 g dried alga, was found for the alginate treatment. In addition, residual amounts of glucose (8.24 × 10⁻⁴ mmol/100 g dried alga) were released from *L. digitata* biomass with the alginate lyase treatment (data not shown).

**Effect of alginate lyase on the release of proteins and pigments from *Laminaria digitata* biomass.** The influence of alginate lyase (ID 22) treatment on pigment and protein concentrations in the supernatant and residue fractions is presented in Table 4. These results indicate that the hydrolysis of the viscous gel-like structure formed by the polymer alginate¹¹ led to the release of trapped valuable nutrients. The incubation of alga with the enzyme did not trigger (*p* > 0.100) the release of protein from *L. digitata* cells and, thus, a similar protein content was found for the enzyme and control assays (31.1 and 39.9 mg/g alga for the supernatant, and 114 and 91.7 for the residue). Additionally, no significant differences (*p* > 0.071) between assays were observed for chlorophyll, carotenoid and fucoxanthin contents in both centrifugation fractions.

**Effect of alginate lyase on the release of fatty acids from *Laminaria digitata* biomass.** Fatty acid profile in residue and supernatant fractions, after incubation with the alginate lyase (ID 22), was analysed to determine if the enzyme treatment led to the release of fatty acids from *L. digitata* cells to the external environment (Table 4), since these nutrients could have been trapped by the gel-forming structure of alginate. For both supernatant and residue fractions, the percentage of fatty acids was as follows: saturated fatty acids (SFA) > MUFA > PUFA > n-6 PUFA > n-3 PUFA. In the supernatant, the amount of total fatty acids was increased (*p* = 0.001) from 1.30 to 4.22 mg/g dried alga with the alginate lyase treatment. In fact, higher percentages of total MUFA (*p* < 0.001) were found in the presence of enzyme, with a major contribution of 18:1c9 (*p* < 0.001) and additional contributions of 16:1c9, 18:1c11 and 20:1c11. In addition, the percentage of total SFA was significantly decreased (*p* = 0.001) with the alginate lyase treatment, particularly of 12:0, 14:0, 16:0, 18:0, 20:0 and 22:0, as well as the amount of total n-6 PUFA (*p* = 0.038). The 18:2n-6 fatty acid tended to decrease (*p* = 0.053)
with the alginate lyase treatment, although it was found in small percentages for both assays (2.08% for control and 1.35% for the enzyme).

In the residue fraction, the alginate lyase treatment caused no significant differences ($p > 0.096$) either in the amount of total fatty acids or in the percentage of individual fatty acids, leading only to a significant decrease of total SFA ($p = 0.037$) comparatively to the control.

Figure 3. (a) Fluorescence intensity derived from Calcofluor White staining for control assay and fucosidase (FU; ID 46) treatment. No statistical differences were observed ($p = 0.195$). (b) and (c) fluorescence images ($\times 400$) of *Laminaria digitata* suspension stained with Calcofluor White for control assay and fucosidase treatment, respectively.

Figure 4. Thermostability analysis of alginate lyase (ID 22) at different temperatures (30 to 80 °C).
Discussion

A large library of 176 CAZymes and 23 sulfatases was produced to test the hypothesis that some of these enzymes, with well-characterized biochemical characteristics (see Supplementary Table S1), could disrupt the recalcitrant cell wall of *L. digitata* with the consequent increase of nutrients availability. The production of enzymes was done in a high-throughput (HTP) platform and consisted of several steps, including gene synthesis, gene cloning, recombinant protein expression in *E. coli* cells and protein purification. These 199 enzymes were selected based on the polysaccharide matrix composition of macroalgae cell wall, which comprises mainly alginate and

| ID | Time (min) | 15 | 30 | 60 | 90 | 120 |
|----|------------|----|----|----|----|-----|
| 22 |            | +  | +  | +  | +  | +   |

Table 3. Proteolysis resistance for alginate lyase (ID 22) at a concentration of 0.83 g/L, when subjected to the proteolytic action of pancreatin, which was incubated at a final concentration of 2.5 g/L. The reactions were incubated at 37 °C, at regular intervals of 15 min for 120 min. Results are presented at periods of 15, 30, 60, 90 and 120 min of incubation. The qualitative scale of proteolysis resistance is based on SDS-PAGE gels visualisation: -, no resistant (only fragmentation bands); +, partially resistant (protein and fragmentation bands).

**Figure 5.** Proteolysis assay results. Electrophoresis on SDS-PAGE in 14% (w/v) acrylamide gels displaying the fragment bands of alginate lyase (ID 22) (0.83 g/L) after incubation with pancreatin (final concentration of 2.5 g/L). The gel band of 31.8 KDa corresponds to purified protein, whereas the other bands correspond to degraded protein and pancreatin. B: blank (purified undigested ID22) P: purified protein submitted to hydrolysis by pancreatin, M: protein marker.

**Figure 6.** Quantification of released mono- and oligosaccharides for control assay and alginate lyase (AL; ID 22) treatment. Asterisk denotes statistical difference at \( p < 0.001 \).
fucose-containing sulphated polysaccharides, as well as minor amounts of cellulose, putative hemicellulose and mixed-linked β-glucans. Laminarin13–15 is the main carbon storage of brown seaweeds. In addition, the enzyme origin was also attended in the selection, being 121 of them from marine and halophilic bacteria and 41 from thermophilic or hyperthermophilic bacteria.

An individual screening of the enzymes was performed in order to assess their ability to degrade *L. digitata* cell wall, which was evaluated by measuring the release of reducing sugars and the intensity of microscopic fluorescence. Afterwards, the 8 selected recombinant enzymes (see Table 1) were combined and tested in order to obtain a maximum disruption of *L. digitata* cell wall, and then reduced to a combination of 3 enzymes (laminarinase ID 18, alginate lyase ID 22 and α-L-fucosidase ID 46). However, the alginate lyase led to a degradation of macroalga cell wall similar to the simultaneous use of the 3 enzymes. The absence of synergistic and additive effects between enzymes indicates that, as previously reported for brown macroalgae11, the different polysaccharides of *L. digitata* cell wall, namely alginate and FCSFs, are not interlinked within the cell wall structure.

This enzyme (ID 22) belongs to the family of polysaccharide lyase 7 (PL7) and is produced by *S. degradans* 35. It possesses poly-β-mannuronate (EC 4.2.2.3) and, to a lesser extent, poly-β-guluronate (EC4.2.2.11) lyase activities. The alginate lyase endolytically depolymerize alginate by β-elimination, having both alginate and oligo-alginates

### Table 4
Content (mg/g alga) of total proteins, chlorophylls, carotenoids, fucoxanthins and fatty acids of the supernatant and residue fractions derived from the incubation of *Laminaria digitata* with control and alginate lyase (AL; ID 22) treatment. Two mL of macroalgae suspension was incubated with alginate lyase at a final concentration of 20 mg/L. The control assay took the same amount of PBS. Incubations were done overnight at 37 ºC and 160 rpm. After incubations, supernatant and residue fractions were separated by centrifugation. Only fatty acids whose percentage was > 0.5% are presented. * Values measured in phosphate buffered saline (PBS); ** Values measured after extraction with acetone.
as its main substrates\(^3\). This enzyme (ID 22) showed to be resistant to proteolysis and thermostable until 40 °C, with an abrupt decrease of thermostability between 40 and 50 °C. These results are likely due to the tertiary structure of protein that confers both thermostolerance and an inherent proteinase resistance\(^2\). The instability of this enzyme at high temperatures (> 50 °C) over an increasing period of time was already reported\(^19\), even though a maximum catalytic activity was described as 50 °C\(^25\). This aspect might be explained by the fact that \(S.\) degroedans is a mesophilic, instead of a thermophilic, Gram-negative bacterium. However, this organism is one of the strongest marine biomass degraders, being capable of hydrolysing a great variety of polysaccharides\(^48\).

Furthermore, the efficiency of the recombinant mannuronate-specific alginate lyase from PL7 family (ID 22) on releasing reducing sugars (7.11 g/L) from brown macroalga cell wall was firstly demonstrated herein. In fact, only one previous study\(^23\) reported the recovery of reducing sugars from \(L.\) digitata and \(S.\) latissima biomass (10 and 11 g/L, respectively), through the action of a commercial mannuronate-specific alginate lyase, belonging to the PL5 family (Genbank accession numbers: SUJ15243.1 and SUJ21107.1). This enzyme was produced by \(Sphingobacterium\) multivorans during the enzymatic pre-treatment of algae biomass for biogas production. However, in the present study, a well-characterized endotype PL5 alginate lyase (ID 41) produced by \(Sphingomonas\) sp. A1 (Genbank accession number BAB03312.1), which specifically acts on poly-\(\beta\)-mannuronate regions (EC 4.2.2.3) and depolymerizes alginate into tri- and disaccharides\(^8\), was significantly (\(p < 0.050\)) less efficient at releasing reducing sugars (1.70 g/L) than the 3 analysed enzymes from PL7 family (average of 6.07 g/L). The release of reducing sugars with alginate lyase (ID 22) is possibly related to an increase of monosaccharides and, although not significant, of oligosaccharides released from \(L.\) digitata biomass\(^46\). Although a recombinant alginate lyase with the same catalytic site as the enzyme with ID 22 was previously shown to produce unsaturated oligoalginates (degrees of polymerization of 2 to 5) but not monosaccharides\(^35,40\), a mixture of unidentified monosaccharides, with the same catalytic site as the enzyme with ID 22 was previously shown to produce unsaturated oligoalginates (degrees of polymerization of 2 to 5) but not monosaccharides\(^35,40\), a mixture of unidentified monosaccharides, which was probably composed of uronic acid residues but needs further analysis, was released by the enzyme treatment (ID 22) in the present study. The fact that the residual amount of alginate (dried macroalgae) were obtained with the alginate lyase treatment might indicate that the breaking down of viscous-gel like matrix of alginate lyase (ID 22) released trapped laminarin from the intracellular algae compartment, which became available for a possible autohydrolysis, as previously reported\(^23\). Although, to date, a selective effect of alginate lyase towards carbohydrates from brown seaweed cell wall was never reported, two studies evaluated the release of glucose from brown macroalgae biomass during either bioethanol production\(^23\) or algae saccharification\(^24\). The first described a slight release of glucose from \(S.\) latissima biomass, by using a commercial mannuronate-specific alginate lyase, from an unknown family, produced by \(Sphingobacterium\) spiritivorum. The latter, similarly to the present study, reported no effect of 3 recombinant alginate lyases from PL7 family on the release of glucose from \(S.\) latissima.

In the present study, the decrease of cell wall fluorescence intensity (39.1%) promoted by alginate lyase (ID 22) indicates a partial alga cell wall degradation, similarly to what was observed by our research team for microalgae\(^23,28\). Considering that Calcofluor white preferentially binds to cellulose and, to some extent, mixed-liked 1,3–1,4-\(\beta\)-glucans in macroalgae cell walls rather than to acidic polysaccharides\(^24,25,29\), like alginate and FCSFs in brown algae\(^26\), the degradation of alginates seems to have compromised the whole integrity of the intricate macroalgae cell wall structure with a decreased dye binding by cellulose and mixed-liked glucans. The cell wall disruption was likely due to the ability of enzyme with ID 22 to degrade polymannuronate residues of alginate and consequently compromise cell wall rigidity that is controlled by the gel-forming structure alginate\(^15\). In fact, alginate from \(L.\) digitata cell wall was found to be mainly composed of mannuronic acid (M) instead of guluronic acid (G) residues (M/G ratio between 1.99 and 3.0)\(^15,20\). The preferential activity of alginate lyases from PL7 family on polymannuronic acid rather than on polyguluronic acid or alginate was demonstrated in a recent study\(^45\) when the enzyme produced by a marine fungus \(Paradendryphiella\) salina was incubated with 3 different brown alga species (\(Ascothylum\) nodosum, \(S.\) latissima and \(Fucus\) serratus). However, the activity of PL7 alginate lyase (ID 22) is not specific for polymannuronate as this enzyme can also act on polyguluronic residues of alginate (Kim et al., 2012). Conversely, the catalytic residues of enzyme with ID 41 (Genbank accession number: Q9KWU1) were shown to strictly bind to mannuronic acid residues\(^46\). This latter aspect can help to explain why PL7 alginate lyase (ID 22) was more efficient on degrading \(L.\) digitata cell wall than the PL5 alginate lyase (ID 41), in the present study.

Conversely to carbohydrates, alginate lyase (ID 22) did not release (hydro-) soluble proteins from \(L.\) digitata cells to the external environment. These results can be due to the extracellular presence of phenolic compounds (i.e. phlorotannins) previously cross-linked to alginates\(^21\), which have protein-linkage properties\(^9\), and hydrocolloidal anionic polysaccharides (e.g. algogalactans)\(^8\). These compounds would limit the access and quantification of proteins, through the increased viscosity of extraction medium, a phenomenon that was indeed observed in the supernatants from alginate lyase treatment, and reported for other carbohydrases\(^15\). Their presence was already found to be a limiting factor of protein extraction when cellulase and xylanase acted on \(P.\) palmata\(^19,23,45\). In addition, no release of pigments from \(L.\) digitata cells to the extracellular medium was found with the alginate lyase treatment. The amounts of pigments in control assay residues were slightly different from the values (mg/g dried alga) previously reported for \(Laminaria\) sp.\(^46\) (0.124 versus 0.184 for chlorophyll \(a\), 0.006 versus 0.014 for chlorophyll \(b\), 0.034 versus 0.026 for total carotenoids), which was probably due to the use of different solvents for pigment extraction\(^46\), as well as variations on alga species and harvesting season\(^47\). Similarly to what was reported for microalgae\(^27,28\), the alginate lyase (ID 22) could not disrupt the long parallel lamellae of tree thylakoids in cytoplasmic plastids of brown seaweeds that contain the light harvesting complex with photosynthetic pigments\(^48\). These results are explained by the absence of activity of the enzyme with ID 22 on the lipid and protein-rich plastid membrane, since this enzyme acts specifically on alginate and oligoalginates\(^35\).

Enzyme with ID 22 was able to release fatty acids from alga biomass to the extracellular medium (supernatant), although without a significant effect on the algal incubation residue. To date, only one study\(^49\) suggested the change of fatty acid profile promoted by alginate lyases on brown seaweeds, although using a ligninase lyase.
from PL5 family acting on *Undaria pinnatifida* and with no statistical analysis of data. Thus, the present study
is the first that show the significant effect of a recombinant alginate lyase (PL7) on fatty acid profile of a brown
macroalga (*L. digitata*). It was observed an increase of total MUFA, such as 16:1c9, 18:1c9, 18:1c11 and 20:1c11
fatty acids, and a concomitant decrease of total SFA, including the major 16:0 and 18:0 fatty acids, released to
the supernatant. In fact, oleic acid (18:1c9) was increased by threefold with the alginate lyase treatment. These
results might be explained by the release of phlorotannins to the extraction medium by the action of alginate
lyase, as previously reported51. In fact, tannins were previously shown to inhibit the complete biohydrogenation
of C18 fatty acids in animals50. The latter aspects need to be further exploited due to the benefits that increasing
the release of MUFA, such as 18:1c9, in detriment of SFA, have to human health, particularly on preventing
cardiovascular diseases7.

**Conclusion**

The results obtained in the present study indicate that the sole use of an alginate lyase from PL7 family, under
physiological conditions, can lead to a partial degradation of *L. digitata* cell wall. The disruption of macroalga
cell wall would allow the release of trapped bioactive compounds with important value for biotechnological and
feed industries. The high nutritional value of these compounds may stimulate the use of exogenous enzymes, as
novel biocatalysts, to supplement diets containing *L. digitata* for monogastric animals. Further work is currently
in progress in our research laboratories to assess the effectiveness of using this alginate lyase as a supplement for
monogastric diets with high incorporation levels (10–15% of diet weight) of *L. digitata*.

**Methods**

**Macroalga production.** The low heat-dried brown macroalgae *L. digitata* was obtained from Algolesko
Company (Plobannalec-Lesconil, Brittany, France), where it was cultivated in seawater offshore ponds and bio-
logically certified by Ecocert. Before it was used for the in vitro incubations, the macroalgae was ground in a
knife mill (Grindomix GM 200, Retsch GmbH, Germany), sieved through a woven wire mesh sieve with a diam-
eter of 63 µm (Retsch GmbH, Germany) and stored at -20 °C.

**High-throughput gene synthesis, cloning and protein expression/purification of recombinant
enzymes.** One-hundred and seventy-six CAZymes with high potential for degradation of macroalgae cell
wall were selected from a diverse repertoire, including glycoside hydrolases (GH), pectate lyases (PL) and carbo-
hydrate esterases (CE). In addition, 23 sulfatases were selected for screening, as they are also likely involved in
the degradation of sulphated polysaccharides from macroalgae cell walls51. The generation of 199 recombinant plasmids, as well as the expression and purification of the corresponding enzymes, followed the procedures described in previous studies27,28. One-hundred and sixty-six coding genes for the selected enzymes were synthesised in vitro using NZYGene Synthesis kit (Nzytech, Portugal), whereas the other 33 coding genes were synthesised by Twist Bioscience (San Francisco, CA, USA). The sequence of each enzyme is presented in Supplementary Table S1. After optimisation of synthetic genes for cloning and subsequent expression in *Escherichia coli*, 166 genes were directly cloned into the bacterial expression vector pHTP1 (Nzy-
tech, Portugal) using NZYEasy Cloning & Expression kit I (Nzytech, Portugal), whereas 33 genes were cloned in
pET-29b (+) (Twist Bioscience, San Francisco, CA, USA). The generated recombinant plasmids were subjected to
inducible T7 promoter control, while encoding the 199 enzymes fused to an N-terminal His6-tag that facilitates
purification through Immobilised Metal Affinity Chromatography (IMAC). All recombinant plasmids were
sequenced to ensure that no mutations accumulated during gene synthesis and were used to transform *E. coli*
BL21 (DE3) cells in 24 deep-well plates, followed by protein production, cell harvesting and protein purification.
An IMAC high-throughput method based on an automated protocol that allows the purification of 96 proteins
simultaneously was performed, as previously reported52 but with some modifications. Briefly, 1 mL of crude
cell lysates were incubated with 200 µL of 25% Ni2+ Sepharose 6 Fast flow resin (GE Healthcare, IL, USA) and
transferred into 96-well filter plates (Macherey–Nagel, Duren, Germany53). The wells were washed twice with
1 mL of 10 mM Imidazole buffer (10 mM Imidazole, 50 mM NaHepes, 1 M NaCl, 5 mM CaCl2, at pH = 7.5) and
once with 1 mL of 35 mM Imidazole buffer (35 mM Imidazole, 50 mM NaHepes, 1 M NaCl, 5 mM CaCl2, at
pH = 7.5). Then, the proteins were eluted with 300 µL of 300 mM Imidazole buffer (300 mM Imidazole, 50 mM
NaHepes, 1 M NaCl, 5 mM CaCl2, at pH = 7.5). All steps of protein purification from cell-free extracts were
automated on a Tecan robot (Tecan, Switzerland), containing a vacuum manifold. Homogeneity of purified
proteins and molecular mass of recombinant enzymes were determined by SDS-PAGE in 14% (w/v) acrylamide
gels and compared to a low molecular weight (LMW) protein marker (18.5 to 96 KDa) (Nzytech, Portugal). The
proteins and molecular mass of recombinant enzymes were determined by SDS-PAGE in 14% (w/v) acrylamide
gels and compared to a low molecular weight (LMW) protein marker (18.5 to 96 KDa) (Nzytech, Portugal). The
level of protein expression was determined accordingly (see Supplementary Table S1).

**Preparation of macroalga cell suspension.** The preparation of *L. digitata* suspension at 20 g/L in PBS
solution, including a pre-wash step, centrifugation and algae re-suspension, was done using the procedure previ-
ously described for microalgae27.

**Enzymatic cell wall disruption.** The cell wall disruption assay was performed in triplicate and with an
enzyme concentration of 20 µg/mL of incubation volume, as previously reported27, but with the following
changes: the incubation of the 24 well microplate (VWR Chemicals, West Chester, PA, USA) containing mac-
roalgae suspension and alginate lyase was performed overnight at 160 rpm. Then, the microplate was centrifuged
per-oligosaccharides was based on a standard curve, using a range of concentrations from 0.025 to 0.60 mM of glucose, mannose plus xylose. Additional monosaccharides, including ribose, fructose, rhamnose, fucose, as well as its derivatives, such as galactosamine, glucosamine, sorbitol and glucoronic acid were also used for comparison.

Fluorescence microscopic observations. The pellets from the enzyme cell wall disruption assay, were resuspended in 0.5 mL of PBS solution for the initial screening or 1 mL of PBS for the assays with the most active selected enzyme. The suspension was mixed by pipetting to ensure that the algae pellets were suspended evenly. The re-suspended macroalgae biomass concentration was 40 mg/mL. The fluorochrome Calcofluor White (Sigma-Aldrich, St. Louis, Mo, USA), that binds to the cell wall, was added at 10 µL to the suspensions on adhesion slides (SuperFrost Plus Menzel Gläser, Thermo Scientific, Braunschweig, Germany), followed by a solution of 10% KOH (VWR Chemicals, West Chester, PA, USA), in a proportion of 1:1:1. The fluorescence microscopic procedures were done as previously reported. Cells were observed with an epifluorescence microscope and images were captured with a Leica DFC-340FX (Leica, Wetzlar, Germany) camera system, in order to determine the fluorescence intensity, expressed as arbitrary units, by using the Image J software (NIH, Bethesda, MA, USA).

Determination of catalytic activity of alginate lyase. The catalytic activity of alginate lyase was analysed by two different methods: UV spectroscopy and determination of reducing sugars. The UV spectroscopy analysis followed the procedures described in a previous report, with some modifications. Briefly, a mixture of 1 mL of PBS solution containing 1% NaCl (pH = 7.5), 0.5 mL of alginate acid from brown algae (Sigma-Aldrich, Darmstadt, Germany) and 5 µL of alginic acid (ID 22) at 4.37 mg/mL were mixed in a quartz cuvette. The mixture was previously dissolved in a PBS solution at a concentration of 0.3%. The increase in absorbance at 233 nm was continuously recorded during 1 h in an UV/Vis Spectrophotometer (Pharmacia LKB Ultrospec III spectrophotometer, Gemini, Apeldoorn, Netherlands), at 37 °C, to verify linearity. The enzyme activity was maximum during 2 min. The maximum activity was reported in absorbance units (AU) defined as the increase in absorbance units per minute. The determination of reducing sugars was done after stopping the enzyme reaction by using the DNSA reagent with subsequent heating of the samples, following the procedures previously described for the measurement of reducing sugars.

Determination of protein content. After L. digitata suspension and incubation with control and alginate assays, the N content in lyophilised supernatant and residue fractions was quantified by the Kjeldahl method, assuming that no nitrogen from the media interfere with the assay. The crude protein was calculated as N × 4.9257.

Pigment analysis. The content of chlorophyll a, chlorophyll b, total carotenoids and pheophytins were quantified in supernatant and residue fractions from L. digitata suspension, after control and alginate assays, as described by Hynstova et al., with slight modifications previously reported, except that total carotenoids also...
included the amount of fucoxanthin. The fucoxanthin content was quantified in the same way as the other pigments, but using the following formula described in a recent study:\(^5\)

\[
C_{\text{fucoxanthin}} = 6.39 \times A_{445} - 5.18 \times A_{663},
\]

where \(C_{\text{fucoxanthin}}\) is the concentration of fucoxanthin (mg/ml), \(A_{445}\) is the absorbance at \(\lambda_{\text{max}} 445\) nm and \(A_{663}\) is the absorbance at \(\lambda_{\text{max}} 663\) nm.

**Determination of fatty acid composition.** Fatty acids from the lyophilised supernatants and pellets of *L. digitata* after control and alginate assays were extracted as already described for microalgae\(^7\). Fatty acids were esterified to form methyl esters (FAME) by acid-catalysis based on the procedure described in a previous report\(^4\), but using 5 ml of acetylchloride-methanol solution (1.25 M Sigma-Aldrich, St. Louis, Mo, USA) for up to 24.3 mg of sample. The analysis of FAME was done following procedures previously reported\(^7\), except for the quantification of total FAME, that was carried out using nonadecanoic acid (19:0) as internal standard. Each fatty acid was expressed as a percentage of the sum of identified fatty acids (% total fatty acids). The fatty acid present in a percentage inferior to 0.5% were included as others in Table 3.

**Statistical analysis.** Data were analysed using the Generalised Linear Mixed (GLM) model of the SAS software package (version 9.4; SAS Institute Inc., Cary, NC, USA), except data from the thermostability experiment, which were analysed using the MIXED procedure of SAS. Normality was checked using Shapiro–Wilks test. All experiments were conducted in triplicate, except for the initial screening where experiments were conducted in duplicate. The error bars on figures indicate the standard error of the mean (SEM). Results are presented as mean and SEM, and were considered significantly different when \(p<0.05\).

**Data availability** All data generated during this study are included in this published article. The datasets generated during the current study are available from the corresponding author on demand.

Received: 6 January 2021; Accepted: 16 April 2021
Published online: 06 May 2021

**References**

1. Makkar, H. P. S. et al. Seaweeds for livestock diets: A review. *Anim. Feed Sci. Tech.* **212**, 1–17. [https://doi.org/10.1016/j.anifeedsct.2015.09.018](https://doi.org/10.1016/j.anifeedsct.2015.09.018) (2016).
2. Holdt, S. L. & Kraan, S. Bioactive compounds in seaweed: functional food applications and legislation. *J. Appl. Phycol.* **23**, 543–597. [https://doi.org/10.1007/s10811-010-9632-5](https://doi.org/10.1007/s10811-010-9632-5) (2011).
3. Costa, M., Cardoso, C., Afonso, C., Bandarra, N. M. & Prates, J. A. M. Current knowledge and future perspectives of the use of Ulva rigida, Gracilaria sp, Fucus vesiculosus and Saccharina latissima as functional ingredients. *Int. J. Mol. Sci.* [https://doi.org/10.3390/ijms19102987](https://doi.org/10.3390/ijms19102987) (2018).
4. Campos, A. M. et al. Azorean macroalgae (*Petalonia brunhiae*, *Halopteris scoparia* and *Osmundea pinnatifida*): bioprospection: a study of fatty acid profiles and bioactivity. *Int. J. Food Sci. Technol.* **54**, 880–890. [https://doi.org/10.1111/ifs.14010](https://doi.org/10.1111/ifs.14010) (2019).
5. Givens, I. Animal nutrition and lipids in animal products and their contribution to human intake and health. *Nutraients* **1**, 71–82. [https://doi.org/10.3390/nu10101071](https://doi.org/10.3390/nu10101071) (2009).
6. FAO. World review in *The State of World Fisheries and Aquaculture. Meeting the sustainable development goals* (ed. FAO) 25, 74 (FAO, 2018).
7. Popper, Z. A. et al. Evolution and diversity of plant cell walls from algae to flowering plants. *Annu. Rev. Plant Biol.* **62**, 567–590. [https://doi.org/10.1146/annurev-arplant-042110-103809](https://doi.org/10.1146/annurev-arplant-042110-103809) (2011).
8. Michel, G., Tonon, T., Scornet, D., Cock, J. M. & Kloareg, B. The cell wall polysaccharide metabolism of the brown alga Ectocarpus siliculosus Insights into the evolution of extracellular matrix polysaccharides in Eukaryotes. *New Phytol.* **188**, 82–97. [https://doi.org/10.1111/nph.1469-8137.2010.03374.x](https://doi.org/10.1111/nph.1469-8137.2010.03374.x) (2010).
9. Adams, J. M., Gallagher, J. A. & Donnison, I. S. Fermentation study on *Saccharina latissima* and *Palmaria palmata* at a large scale. *J. Appl. Phycol.* **32**, 3429–3446. [https://doi.org/10.1007/s10811-019-02185-2](https://doi.org/10.1007/s10811-019-02185-2) (2020).
10. Givens, I. Animal nutrition and lipids in animal products and their contribution to human intake and health. *Nutraients* **1**, 71–82. [https://doi.org/10.3390/nu10101071](https://doi.org/10.3390/nu10101071) (2009).
11. Popper, Z. A. et al. Evolution and diversity of plant cell walls from algae to flowering plants. *Annu. Rev. Plant Biol.* **62**, 567–590. [https://doi.org/10.1146/annurev-arplant-042110-103809](https://doi.org/10.1146/annurev-arplant-042110-103809) (2011).
12. Michel, G., Tonon, T., Scornet, D., Cock, J. M. & Kloareg, B. The cell wall polysaccharide metabolism of the brown alga Ectocarpus siliculosus Insights into the evolution of extracellular matrix polysaccharides in Eukaryotes. *New Phytol.* **188**, 82–97. [https://doi.org/10.1111/nph.1469-8137.2010.03374.x](https://doi.org/10.1111/nph.1469-8137.2010.03374.x) (2010).
13. Dobrincic, A. et al. Advanced technologies for the extraction of marine brown algal polysaccharides. *Mar. Drugs* [https://doi.org/10.3390/md18030168](https://doi.org/10.3390/md18030168) (2020).
14. Overland, M., Mykland, L. T. & Skrede, A. Marine macroalgae as sources of protein and bioactive compounds in feed for monogastric animals. *J. Sci. Food Agr.* **99**, 13–24. [https://doi.org/10.1002/jsfa.9143](https://doi.org/10.1002/jsfa.9143) (2019).
15. Adams, J. M., Gallagher, J. A. & Donnison, I. S. Fermentation study on *Saccharina latissima* for bioethanol production considering variable pre-treatments. *J. Appl. Phycol.* **21**, 569–574. [https://doi.org/10.1007/s10811-008-9384-7](https://doi.org/10.1007/s10811-008-9384-7) (2009).
16. Batista, S. et al. Use of technological processing of seaweed and microalgae as strategy to improve their apparent digestibility coefficients in European seabass (Dicentrarchus labrax) juveniles. *J. Appl. Phycol.* **32**, 3429–3446. [https://doi.org/10.1007/s10811-020-02185-2](https://doi.org/10.1007/s10811-020-02185-2) (2020).
17. Fleurence, J., Massiani, L., Guyader, O. & Mabeau, S. Use of enzymatic cell-wall degradation for improvement of protein extraction from *Chondrus crispus*, *Gracilaria verrucosa* and *Palmaria palmata*. *J. Appl. Phycol.* **7**, 393–397. [https://doi.org/10.1007/BF00003796](https://doi.org/10.1007/BF00003796) (1995).
18. Harnedy, P. A. & FitzGerald, R. J. Extraction of protein from the macroalga *Palmaria palmata*. *Food Sci. Technol.* **51**, 375–382. [https://doi.org/10.1016/j.jlt.2012.09.023](https://doi.org/10.1016/j.jlt.2012.09.023) (2013).
58. Hynstova, V. et al. Separation, identification and quantification of carotenoids and chlorophylls in dietary supplements containing Chlorella vulgaris and Spirulina platensis using high performance thin layer chromatography. J. Pharm. Biomed. Anal. 148, 108–118. https://doi.org/10.1016/j.jpba.2017.09.018 (2018).

59. Wang, L. J. et al. A rapid method for the determination of fucoxanthin in diatom. Mar. Drugs 16, 33. https://doi.org/10.3390/md161010033 (2018).

Acknowledgments
This study was supported by Fundação para a Ciência e a Tecnologia (FCT, Lisbon, Portugal; grant PTDC/CAL-ZOO/30238/2017), and CIISA (Project UIDB/00276/2020) and a PhD fellowship to DC (SFRH/BD/126198/2016).

Author contributions
M.C. prepared macroalgae biomass for the subsequent experiments. P.B. and C.M.G.A.F. constructed the data-bank with CAZYmes and sulfatases. M.C. and P.B. generated recombinant plasmids. V.C. and J.B. were responsible for high-throughput production of recombinant enzymes. M.C. and L.P. preformed the incubations and analysed reducing sugars and fluorescence intensity of algal cell walls, as well as pigment and protein contents. M.C. and C.M.A. analysed fat composition of macroalgae samples, and C.M.A. analysed oligosaccharides amount. M.C. and D.C. performed statistics. D.C. assisted in the execution of the experiments. M.C. performed the manuscript preparation and literature review. J.A.M.P. contributed to the overall research design, interpretation and discussion of the experimental results. All authors have revised, edited and approved the final manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-89278-1.

Correspondence and requests for materials should be addressed to J.A.M.P.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021