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Enzymatic Extraction of Fucoxanthin from Brown Seaweeds

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Original article

Enzymatic extraction of fucoxanthin from brown seaweeds

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Summary Brown seaweeds contain a number of bioactive compounds. The xanthophyll, fucoxanthin, has *in vivo* efficacy against disorders such as type 2 diabetes, obesity and cancer. Organic solvents are traditionally employed to extract fucoxanthin, but carry a toxic chemical and environmental burden. The aim of this study was to optimise a fucoxanthin extraction method using enzymes, water, low-temperature dehydration and mechanical blending, to produce yields comparable to those achieved with an organic solvent (acetone). Response surface methodology was applied, using *Fucus vesiculosus* as a model species. A fucoxanthin yield of 0.657 mg g^{-1} (dry mass) was obtained from *F. vesiculosus* blade using the enzymatic method, equivalent to 94% of the acetone-extracted yield. Optimum extraction parameters were determined to be enzyme-to-water ratio 0.52%, seaweed-to-water ratio 5.37% and enzyme incubation time 3.05 h. These findings may be applied to the development of value-added nutraceutical products from seaweed.

Keywords Brown seaweed bioactives, enzymatic extraction, extraction yield optimisation, fucoxanthin, green chemistry, liquid chromatography coupled with mass spectrometry.

Introduction

Fucoxanthin is a photosynthetic xanthophyll carotenoid found predominantly in brown macroalgal seaweeds (Phaeophyceae) and in microalgal diatoms (Bacillariophyceae) (Durnford, 2003). It also occurs at lower concentrations in golden algae (Chrysophyceae) and Raphidophyceae (Roy *et al.*, 2011; Larkum *et al.*, 2012). Fucoxanthin has many applications in human health as an inhibitor of tumour activity (Hussain *et al.*, 2016; Mei *et al.*, 2017), bacteria (Shannon & Abu-Ghannam, 2016), type 2 diabetes (Miyashita *et al.*, 2011), obesity (Abidov *et al.*, 2010; D'Orazio *et al.*, 2012), oxidative stress (Kong *et al.*, 2016), metabolic syndrome (Nishikawa *et al.*, 2012), Alzheimer's disease (Lin *et al.*, 2016) and UV light-induced damage (Matsui *et al.*, 2016). However, the majority of brown seaweed biomass harvested globally each year is used for animal feed, fertiliser, biofuel production or low-value human food products (Loureiro *et al.*, 2015; Wells *et al.*, 2017). An efficient, green chemical fucoxanthin extraction method may be useful in the development of value-added nutraceutical products from brown seaweed biomass. The extraction of fucoxanthin is traditionally achieved with organic solvents such as acetone, hexane, ethanol, dimethyl sulphoxide or methanol which are yield effective but result in the production of chemical waste as environmental and economic burdens (Kerton & Marriott, 2013; Jose & Archanaa, 2017). The cell wall of brown seaweeds is composed primarily of cellulose (repeating units of β (1→4)-linked D-glucose) and alginate (repeating units of two epimers β-(1→4)-D-mannuronate (M) and α-(1→4)-L-guluronate) which may be degraded with various enzymes such as cellulases or alginate lyase (Deniaud-Bouët *et al.*, 2014; Manns *et al.*, 2016). Enzymolysis, that is the hydrolysis of cell wall polysaccharides with enzymes, coupled with low-temperature drying, and mechanical blending has the potential to produce fucoxanthin yields comparable to those obtained with organic solvents by releasing pigment-containing thylakoids, while obviating the need for chemical waste disposal. Temperature, incubation time, pH and ratio of substrate to enzyme are important parameters to be considered.

The aim of this study was to maximise the solid–liquid extraction yield of fucoxanthin from brown seaweeds using a prolifically available variety, *Fucus vesiculosus*, as a model species. A pretreatment of low-temperature oven drying and mechanical blending was

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Chemical compounds studied in this article: Fucoxanthin (PubChem CID: 5281239); Viscozyme (PubChem SID: 318693935); ammonium acetate (PubChem CID: 517165); sodium acetate trihydrate (PubChem CID: 23665404).

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followed by enzymatic extraction. Response surface methodology (RSM) was applied using three extraction variables: enzyme-to-water ratio, seaweed-to-water ratio and enzyme incubation time. Optimised responses were applied to the holdfast, stipe and blade of nine other commercially available and common brown seaweeds. Preparative thin-layer chromatography (P-TLC) was used to isolate fucoxanthin in the crude extract before characterisation and quantification by high-performance liquid chromatography coupled with mass spectrometry (LC-MS). Fucoxanthin yields were compared to those previously achieved with the same ten species and harvest batch using acetone extraction (Shannon & Abu-Ghannam, 2017).

Materials and methods

Chemicals

α-Amylase (from *Aspergillus oryzae* ATCC 14156) was purchased from Megazyme (Ireland). Viscozyme (from *Aspergillus aculeatus* ATCC 36411), Protease (from *Bacillus licheniformis* ATCC 6346), and Celluclast (from *Trichoderma reesei* ATCC 26921) were purchased from Novozyme (Ireland). Ammonium acetate was purchased from BDH Laboratory (UK), sodium acetate trihydrate from Scharlau (Spain) and LC-MS-grade ethanol, methanol and fucoxanthin standard (all-*trans*-fucoxanthin) from Sigma-Aldrich (Ireland).

Samples

Ten common species of Irish brown seaweeds were selected for the study. *Alaria esculenta*, *F. vesiculosus*, *Laminaria digitata, Saccharina latissima, Ascophyllum nodosum, Laminaria hyperborea, Himanthalia elongata, Fucus serratus*, *Saccorhiza polyschides* and *Pelvetia canaliculata* were purchased from Quality Sea Vegetables, Burton Port, Co. Donegal, Rep. of Ireland. Authentication of species was provided by the supplier. Samples were harvested in the mid of July 2015 from the north-western coast of Ireland (54.9823°N, 8.4343°W) at mean monthly air and seawater temperatures of 14.5 °C.

Methods

Sample preparation and dehydration

Fresh, whole seaweeds were placed in a colander and rinsed with cold tap water to remove epiphytes and debris and then patted dry. Each thallus was separated into holdfast, stipe and blade and then sliced into 2-cm pieces. Samples were placed in a single layer on a drying tray in an incubator oven (Innova 42; Mason Technology, Ireland) with an air velocity of 2.0 ± 0.1 ms^{-1} for 12 h at 40 °C in darkness. Dehydration temperature and time were based on a modified version of previously optimised parameters for Irish brown seaweeds in terms of phytochemical preservation (Gupta *et al*., 2011).

Mechanical blending

Dehydrated blade, stipe and holdfast were ground separately using a laboratory-scale blender (Salter EK2002, 1000 Watt, 25 000 RPM, 1 L capacity) for 20 s to a particle size of 1.0 ± 0.2 mm (VWR Digital calliper). Initial trials using blending times from 5 to 90 s found no significant ($P \geq 0.05$) increase in fucoxanthin extraction yields or reduction in particle size after 20 s.

Initial enzyme trials and RSM range determination

Trials with Viscozyme, α-amylase, Protease and Celluclast determined Viscozyme to have significantly greater extraction efficiency for fucoxanthin compared to all other enzymes, and therefore, it was selected for this study. Pre-RSM trials for three extraction variables were carried out in the following ranges: seaweed-to-water ratio 0.1% to 50.0%; enzyme-to-water ratio 0.1% to 50.0%; and enzyme incubation time 0.1 to 24.0 h. Quantification by HPLC determined that no statistically significant increase in *F. vesiculosus* fucoxanthin content occurred outside the following ranges: seaweed-to-water ratio 0.5% to 5.0%; enzyme-to-water ratio 0.5% to 5.0%; and enzyme incubation time 0.5 to 12.0 h. These ranges were therefore used as the upper and lower limits for the RSM design of experiment.

Response surface methodology design and analysis

A 2^3 + star central composite design was applied using Statgraphics Centurion XV (StatPoint Technologies Inc., USA). Sixteen variable combinations in experimental runs were generated by the design. The effects of unexpected variability in the observed responses were minimised by randomisation. Experimental data generated from the design were fitted to a second-order polynomial regression model (eqn 1) where Y is the predicted response (fucoxanthin), and X_1 (enzyme-to-water ratio), X_2 (seaweed-to-water ratio), and X_3 (enzyme incubation time) are the coded values of the independent variables.

$$Y = B_0 + (B_1X_1) + (B_2X_2) + (B_3X_3) + (B_{11}X_1^2) + (B_{22}X_2^2) + (B_{33}X_3^2) + (B_{12}X_1X_2) + (B_{13}X_1X_3) + (B_{23}X_2X_3) \quad (1)$$

Statistical interpretation of RSM experimental data generated by the model was evaluated by the analysis of variance (ANOVA) and coefficient of determination, R^2, measuring goodness of fit of the regression model. Significance of the model and data was determined at the 95.0% confidence level ($\alpha = 0.05$).

Enzymatic extraction procedure

Sodium acetate buffer (4 mL, 0.1 M, pH 4.5) was added to a flask. Dehydrated, ground seaweed was combined with ddH_2O in ratios from 0.5% to 5.0% to a total volume of 16 mL and added to the buffer. The flask was covered with Parafilm and incubated (Innova 42; Mason Technology) at 50 °C, 100 RPM for 10 min. Once the flask contents had reached 50 °C, liquid Viscozyme (100 fungal β-glucanase units g^{-1}) was added in ratios of 0.5% to 5.0% of the total solvent volume. Incubation was carried out at 50 °C, 100 RPM from 0.5 to 12.0 h. The reaction was halted by plunging the flask into a water bath (80 °C, 5 min). The flask was then cooled on ice (5 min). Flask contents were transferred to Nalgene tubes and centrifuged (12 min, 12000 × ***g***, 4 °C) (Sigma 2K15; Mason Technology). The supernatant was retained. The pellet was washed and centrifuged eight times (20 mL). Pooled supernatant was reduced by evaporation (~~Laborota 4002 Heidolph rotary evaporator, Germany~~) at 30 °C to 5 mL. Extracts were frozen to −80 °C and then lyophilised to a powder (24 h, 0.02 mbar, −52 °C) (Labconco freeze-drier, USA). The lyophilised extract was stored (−20 °C, in darkness) until HPLC analysis.

Quantification of fucoxanthin

Preparative thin-layer chromatography

Preparative thin-layer chromatography was used to isolate fucoxanthin from the crude enzymatic seaweed extract according to a protocol optimised in this laboratory by Rajauria & Abu-Ghannam (2013). Lyophilised, crude enzymatic *F. vesiculosus* extract was dissolved in ethanol and pipetted onto the base of a TLC silica plate precoated with fluorescent indicator (Macherey-Nagel ALUGRAM® SIL G/UV_{254}, 0.20 mm, 20 × 20 cm, Germany). A solution of commercial fucoxanthin standard was pipetted alongside the crude extract as a reference. The plate was left in darkness (30 min, 25 °C) to dry. Mobile phase (chloroform:diethyl ether:ethanoic acid:n-hexane (10:3:1:1, v/v/v/v)) was added to a glass TLC developing chamber with lid. The silica plate was developed in the chamber (30 min, 25 °C) in darkness and then removed and allowed to dry (30 min, 25 °C). An orange band with an R_f corresponding to that of the fucoxanthin standard was visible for the enzymatic *F. vesiculosus* extract. The bands were collected separately by cutting with a scalpel and dissolving the silica fragments in methanol (20 mL). The solutions were vortexed (10 min, 100 RPM) in Nalgene tubes, then centrifuged (15 min, 10 000 × ***g***) and washed twice with fresh methanol (20 mL) to remove the fucoxanthin from the silica fragments. Pooled supernatants were syringe-filtered (Sigma-Aldrich Millex Durapore PVDF 0.22-μm pore) and lyophilised to a powder (24 h, 0.02 mbar, −52 °C) (Labconco freeze-drier, USA).

Preparation of P-TLC seaweed extract stock solutions

Stock solutions of seaweed extracts were prepared for HPLC and LC-MS analysis by dissolving lyophilised P-TLC enzymatic extract of seaweed in LC-MS-grade ethanol.

HPLC-guided identification

Initial identification of fucoxanthin in the lyophilised P-TLC enzymatic *F. vesiculosus* extract band corresponding with the fucoxanthin standard was carried out according to a method developed by Sugawara *et al.* (2002). Separation was achieved with HPLC (Alliance-Waters e2695 Separations Module, 400 atm pressure, at 4 °C), using a C18 reverse-phase column (Phenomenex, Luna 4.6 mm × 250 mm, 5 μm particle size) and a UV photodiode array detector (Waters 2998). The mobile phase was acetonitrile:methanol:water (75:15:10, v/v/v/) containing ammonium acetate (0.1%). Mobile phases were filtered (Merck Millipore HVLP 0.45 μm) and sonicated (Branson 5510 Ultrasonic Cleaner). A 25-min isocratic programme was used with a flow rate of 1.0 mL min^{-1}, injection volume 10 μL and 25 °C constant column temperature. Detection was performed at 449 nm. Separation of fucoxanthin was achieved at 14.937 min. The concentration of fucoxanthin in the seaweed extracts was extrapolated from commercial fucoxanthin standard solutions.

LC-MS characterisation of fucoxanthin

The HPLC-generated peaks that corresponded with commercial fucoxanthin standards were further characterised by their positive ions. Molecular characterisation of fucoxanthin in the seaweed extracts was carried out according to a protocol optimised in this laboratory for fucoxanthin by Rajauria *et al.* (2017) using LC-MS (Agilent Technologies 6410 Triple Quad LC-MS, with 1200 series LC and MassHunter Workstation software, USA). The liquid chromatographic conditions were as described in the preceding section (*HPLC guided identification*). Operating conditions for mass spectrometry were as follows: positive ionisation mode, fragmentor voltage 120 V, capillary voltage 3.5 kV and collision energy 10 eV. The nebulising and drying gas used was nitrogen, at a pressure of 50 psi, flow rate 10 L min^{-1} and drying temperature 350 °C, with a capillary current of 35 nA. Mass spectral data were recorded in the mass range of m/z 100–1000 on ESI interface mode.

Moisture content determination

Raw, and after incubator-dried, moisture contents were determined by drying control samples in an oven (Binder, Germany) at 105 °C until a constant mass was obtained.

Statistical analysis

All experiments were conducted in triplicate ($n = 3$) and replicated at least twice. Results are expressed as mean values ± standard deviation. All statistical analyses and data were fitted to models using Statgraphics Centurion XV. The coefficient of determination (R^2) and mean square error (MSE) were used as criteria for adequacy of fit. Multiple range tests were used to determine least significant differences between samples at the 95.0% confidence level ($\alpha = 0.05$).

Results and discussion

Mathematical modelling and RSM optimisation of enzymatic extraction

A regression equation fitted to the experimental data is shown in eqn 2, where Y is the predicted response (fucoxanthin), and X_1 (enzyme-to-water ratio), X_2 (seaweed-to-water ratio), and X_3 (enzyme incubation time) are the coded values of the independent variables.

$$Y = 0.504431 - (0.0474112 * X_1) + (0.0143882 * X_2) + (0.0289971 * X_3) + (0.00332511 * X_1^2) - (0.000958862 * X_1 * X_2) + (0.00202725 * X_1 * X_3) + (0.0023388 * X_2^2) - (0.0015828 * X_2 * X_3) - (0.00286578 * X_3^2) \quad (2)$$

All three variable effects were found to be significant. Enzyme-to-water ratio was found to have the most significant effect ($P = 0.0057$) on fucoxanthin yield, followed by seaweed-to-water ratio ($P = 0.0192$) and enzyme incubation time ($P = 0.0340$). The combination of factorial levels required to maximise fucoxanthin yield from *F. vesiculosus* blade was determined to be enzyme-to-water ratio 0.52%, seaweed-to-water ratio 5.37% and enzyme incubation time 3.05 h. An optimum value of 0.706 mg g^{-1} (dry mass of *F. vesiculosus* blade enzymatic extract) was predicted. The greatest observed value obtained was 0.657 mg g^{-1}. This is 93.06% of 0.706 mg g^{-1} which is in good agreement with the predicted value. The R-squared statistic (adjusted for degrees of freedom) indicated that the model as fitted explained 91.16% of the variability in fucoxanthin yield. The standard error of the estimate showed the standard deviation of the residuals to be 0.031, with the average value of the residuals expressed as a mean absolute error of 0.014.

A response surface plot (Fig. 1) was constructed according to the modelled experimental data. The effects of two variables (enzyme-to-solvent ratio and seaweed-to-solvent ratio) on fucoxanthin yield are depicted in a three-dimensional surface plot, while the third variable (time) was kept at 6.25 h, the midpoint of the range.

Fucoxanthin quantification of enzymatic extract by HPLC

Using Viscozyme, a fucoxanthin yield of 0.657 mg g^{-1} (dry mass of *F. vesiculosus* blade) was achieved using the enzymatic method. This was equivalent to 93.99% of the previously optimised organic solvent (acetone) extraction yield 0.699 mg g^{-1} (dry mass of *F. vesiculosus* blade) (Shannon & Abu-Ghannam, 2017). RSM was an effective technique for optimising enzymatic extraction conditions as the fitted model explained 91.16% of the variability in fucoxanthin yield. Optimised responses applied to *F. vesiculosus* stipe and holdfast produced fucoxanthin contents equivalent to 90.43% and 88.02%, respectively, of their acetone extracted yields. The comparative Viscozyme-extracted fucoxanthin content of the blade, stipe and holdfast of the ten seaweeds under study is presented in Fig. 2, as quantified by HPLC. The previously optimised acetone extraction yield for each species and thallus region is included in the graph for comparison. Values are the mean of three replicates ± standard deviation. Letters denote least significant difference between columns ($P \leq 0.05$).

In the other nine seaweeds under study, fucoxanthin content of the enzymatic extracts ranged from

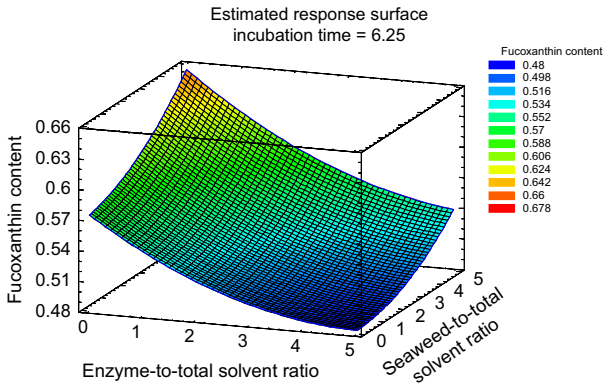

Figure 1 Multiple response surface optimisation plot showing the effects of enzyme and solvent ratios on fucoxanthin yield.

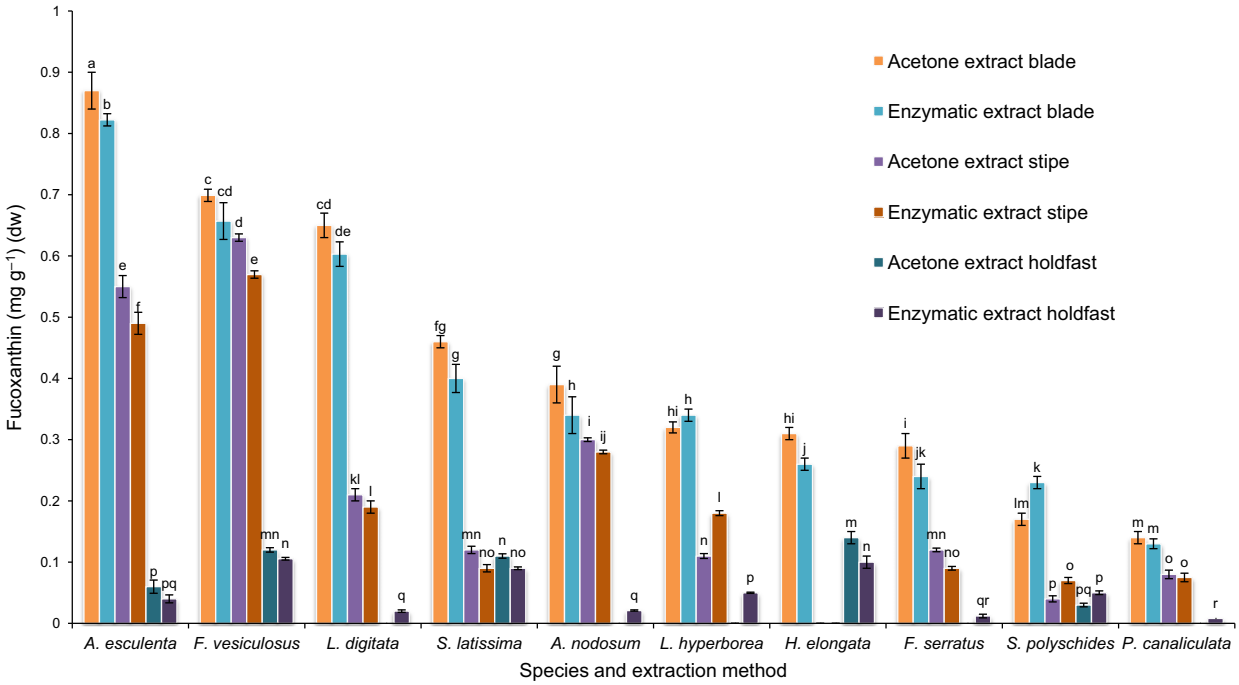

Figure 2 Comparative fucoxanthin content per species, thallus region and method of extraction as quantified by HPLC.

0.008 mg g^{-1} in *P. canaliculata* holdfast to 0.822 mg g^{-1} in *A. esculenta* blade. As expected, the greatest fucoxanthin content was found in the blade of all ten seaweeds, followed by the stipe, and least in holdfast. This is due to the photosynthetic function of the blade and the primarily structural functions of the stipe and holdfast, which contain lower concentrations of light-harvesting pigments.

On average, among all ten species, the optimised Viscozyme extraction yields of fucoxanthin were equivalent to 93.56% (blade), 94.19% (stipe) and 107.96% (holdfast) of their acetone extracted yields. In the case of eight of the ten species, the enzymatic fucoxanthin yield was slightly lower in all thallus regions. However, two of the species, *L. hyperborea* and *S. polyschides*, had enzymatic yields significantly greater than those achieved by acetone extraction. In addition, fucoxanthin was extracted from the holdfasts of five species (*L. digitata*, *A. nodosum*, *L. hyperborea*, *F. serratus* and *P. canaliculata*) in which none was extracted using acetone. This increase is most likely due to the physically impervious nature of some species and thallus regions and the ability of enzymes to hydrolyse bonds that hold cellulose-encased cell components within. *L. hyperborea* and *S. polyschides* were the two most resistant seaweeds in terms of physical texture and experienced the greatest increase in fucoxanthin extraction yield.

The suitability of Viscozyme for cell wall degradation is due to its composition of cellulase, arabanase, β-glucanase, hemicellulase and xylanase which catalyse the cleavage of bonds between β(1→4)-linked D-glucose units (cellulose) while also reducing the viscosity of the reaction mixture (Park *et al.*, 2004; Gupta, 2016).

Fucoxanthin is a carotenoid. Carotenoids are divided into two classes: carotenes and xanthophylls. Carotenes, for example lycopene, are composed of carbon and hydrogen and are non-polar molecules. Xanthophylls, such as fucoxanthin, are composed of carbon, hydrogen and oxygen. The presence of oxygen in xanthophylls makes them more polar than carotenes. Fucoxanthin has six oxygen atoms, within hydroxyl and epoxy groups, allowing partial solubility with polar solvents such as water (Landrum, 2009). The acidic water (pH 4.5) used with Viscozyme in the present study may also have enhanced extraction. Acidified water has previously been used to enhance the extraction of bioactive compounds from brown seaweed. Charoensiddhi *et al.*, 2016b used pH-adjusted water (pH 4.5) to extract laminarin, fucoidan and other polysaccharides from *Ecklonia radiata*. There was a significant increase in total polysaccharide yield at pH 4.5, compared to that obtained at pH 6–8. Similarly, with non-algal biomass, a reduction in pH has been shown to improve the extraction of compounds

such as flavonoids and phenols from plants. For example, Inggrid & Santoso (2016) and Chumsri *et al.* (2008) found that acidified water (pH ≤ 3.0) ruptured vacuoles in plant cell walls, allowing cell-bound compounds to be released. As vacuoles also occur in the cell wall of brown seaweeds (Hurd *et al.*, 2014), the acidic solvent used in the present study may have had a similar effect. It is also probable that the pretreatment of dehydration and mechanical blending aided in releasing fucoxanthin from within chloroplasts due to physical rupturing of the algal cell walls.

Enzymes have been used in the extraction of useful bioactives from algae. Wang *et al.* (2010) used Umamizyme, an endo- and exo-peptidase complex derived from *A. oryzae*, to enhance the recovery of polyphenols and other antioxidant compounds from the red seaweed *Palmaria palmata*. Heo *et al.* (2005, 2003) used inexpensive commercial enzyme preparations such Celluclast, Viscozyme, Flavourzyme, Alcalase and Kojizyme to produce antioxidant hydrolysates from brown seaweeds. They found that several of the hydrolysates had a greater free radical scavenging activity and lipid peroxidation inhibitory effect than synthetic commercial antioxidants α-tocopherol, BHA and BHT.

However, little has been published on the use of enzymes for fucoxanthin extraction specifically. A study by Billakanti *et al.* (2013) reported a 9.3% increase in fucoxanthin yield, compared to solvent-only treatment, from *Undaria pinnatifida* using an enzyme pretreatment of alginate lyase, derived from *Flavobacterium multivorum*, followed by dimethyl ether and ethanol extraction. Optimum parameters for the alginate lyase pretreatment were 37 °C, for 2 h, pH 6.2, 5% (*w*/*v*) solids, with 0.05% enzyme. However, the same volume of dimethyl ether and ethanol used for solvent-only extraction was required after enzymatic pretreatment to achieve the 9.3% fucoxanthin increase. Qin *et al.* (2013) used an equal ratio of cellulosase [*sic*] and pectinase to increase fucoxanthin yields from *Laminaria japonica*. Extraction was carried out at pH 5.0, 50 °C, 0.30% enzyme, for 80 min. A fucoxanthin yield of 0.183 mg g^{-1} (wet mass) was achieved, which was 26.5% greater than traditional organic solvent extraction yield; however, the organic solvent or method used was not specified.

Extraction of algal bioactives through the application of enzymes has the potential to increase yield and safety (Charoensiddhi *et al.*, 2016a; Abu-Ghannam & Shannon, 2017). The presence of cellulose in the cell walls of brown seaweed limits the efficiency of organic solvent extraction (Kim, 2011; Kim & Chojnacka, 2015). The fucoxanthin yields of less than 1 mg g^{-1} in the present study are in line with, and in some cases, greater-than-published results for fucoxanthin in the ten species under study (Ramus *et al.*, 1977; Stengel & Dring, 1998; Schmid & Stengel, 2015). Human clinical trials have found doses of only 0.5–2.4 mg per day to be effective in the treatment of diseases such as obesity and metabolic syndrome (Abidov *et al.*, 2010; Oryza, 2011).

With an aim of reducing or eliminating acetone usage in the present study, mechanical processing in the form of fine blending was applied as a pretreatment before extraction. Dehydration prior to mechanical blending produces a smaller particle size compared to blending of raw, wet seaweed, due to the brittle nature of the dried thalli. This reduction of particle size further aids in the extraction of fucoxanthin by rupturing the membrane-bound thylakoids within the algal cells. As lyophilisation via freeze-drying is a high-energy consumption process, the aim was to dehydrate the seaweed using a low-energy-consuming method, such as oven incubation at 40 °C. Predrying of brown macroalgae has been incorporated as part of other optimisation methods to increase enzymatic extraction yields. For example, Puspita *et al.* (2017) dried three species of Sargassum away from direct sunlight (temperature not specified) for 7 days before extraction of phenolic compounds with a combination of proteases and carbohydrases. Olivares-Molina & Fernández (2016) air-dried *Lessonia nigrescens*, *Macrocystis pyrifera* and *Durvillaea antarctica* at room temperature for 5 days prior to α-amylase and cellulase extraction of phlorotannins; Adalbjörnsson & Jónsdóttir (2015) used freeze-drying (number of hours not specified) before multienzymatic extraction of polyphenols. However, the shorter drying conditions of only 12 h (at 40 °C) optimised in the present study are more suitable for fucoxanthin-specific extraction as this xanthophyll is degraded via oxidation in prolonged exposure to air (Zhao *et al.*, 2014). In addition, oven incubation at 40 °C only consumes approximately 1.6 MJ h^{-1} kg^{-1} in energy, compared to 4.1 MJ h^{-1} kg^{-1} for freeze-drying (Rudy, 2009), which is a 61% reduction in energy usage. An approximate cost analysis of the proposed extraction technique, at laboratory scale, for the model species is as follows. One kilogram of raw *F. vesiculosus* (€1.75 kg^{-1}) has a mean dry mass of 288 g of which 0.0657% (0.657 mg g^{-1}) was quantified by HPLC to be fucoxanthin. This is 0.189 g fucoxanthin per raw kilogram. The volume of Viscozyme (€0.05 cent mL^{-1}) required to treat 288 g of dried seaweed according to the optimised parameters is 28.15 mL, costing €1.41. Therefore, a total raw material cost of €3.16 kg^{-1} (seaweed plus Viscozyme) would produce 0.189 g fucoxanthin or €16.72 to produce one gram of fucoxanthin. Current market prices for fucoxanthin vary widely. For example, analytical grade fucoxanthin (≥95% purity) retails at €4450 g^{-1}(Sigma-Aldrich, 2018). Online seaweed producers based in Asia offer organic solvent-extracted

fucoxanthin for a very low average price of \$200 kg^{-1} (\$0.20 g^{-1}), with product specifications claiming purities ranging from 5% to 80% fucoxanthin (Alibaba, 2018). However, the chemical solvents used to extract fucoxanthin are not specified and may not be considered safe for human consumption under the international Food Chemicals Codex, European Food Safety Authority Commission Directive 2010/67/EU or Sections 201 and 409 of the U.S. Federal Food, Drug, and Cosmetic Act (FDA, 2002; EU, 2010; USP, 2018).

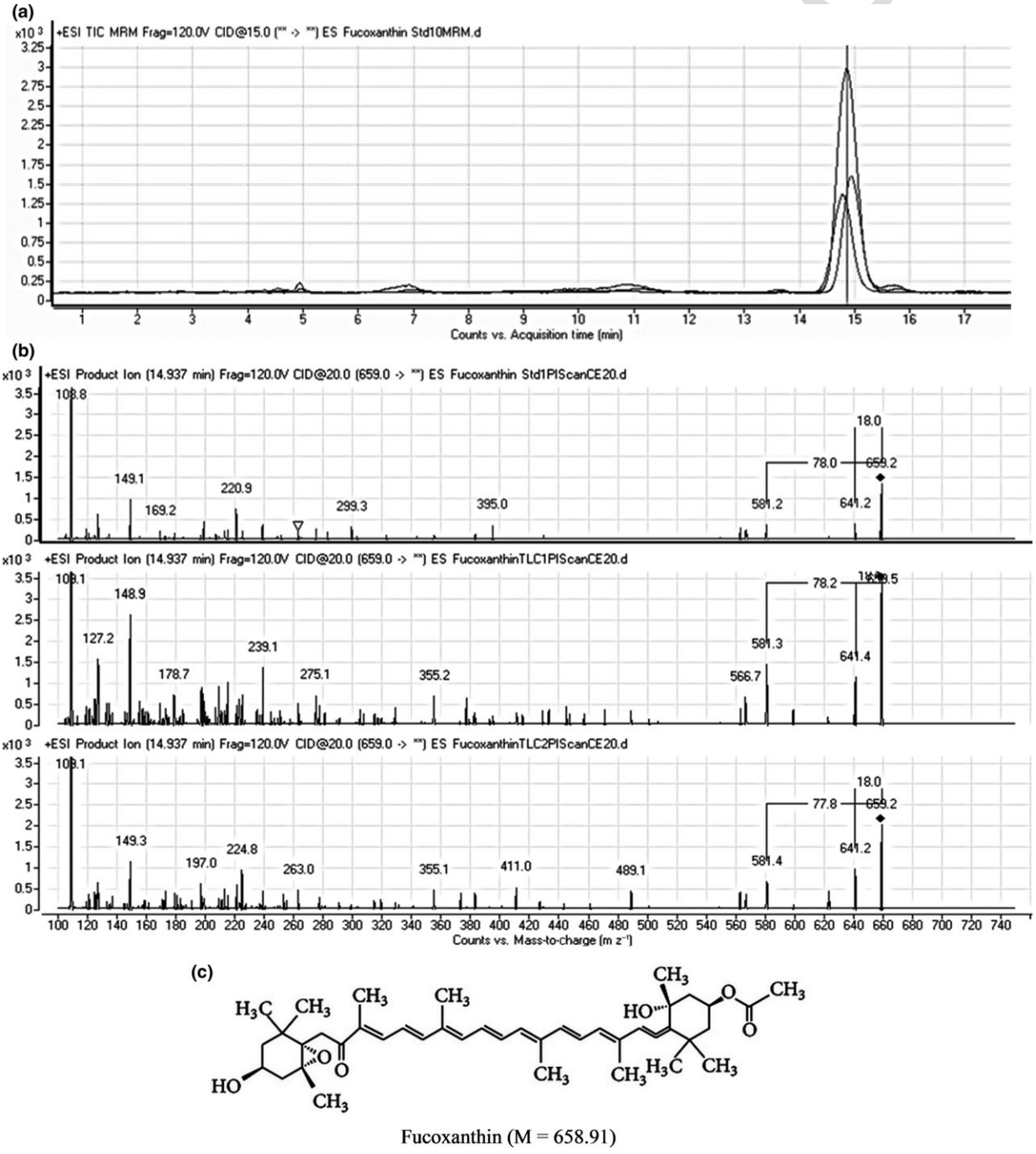

Figure 3 LC-MS spectra of fucoxanthin standard and two enzymatic extracts of *Fucus vesiculosus*.

Viscozyme is suitable for the preparation of extracts for human consumption as it is compliant with purity specifications set out by the FAO and WHO (FAO/WHO, 2010) and is derived from the non-toxic fungus *A. aculeatus*.

Although enzymatic extraction is a green chemical technique, it can have some limitations such as lower yields, low selectivity and longer treatment times in comparison with traditional organic solvent extraction. Other non-conventional technologies have shown efficacy in the extraction of bioactive compounds from algae (Barba *et al*., 2015). These include ultrafiltration by molecular weight cut-off membrane filters (Zhu *et al*., 2017), pulsed electric fields, supercritical fluids, microwave, ultrasound and accelerated solvent extraction. For example, Parniakov *et al*. (2015) observed a ninefold increase in total chlorophyll yield from the alga *Nannochloropsis* using ultrasound-assisted water, ethanol and dimethyl sulphoxide extraction. Ultrasound, using solvents such as food-grade ethanol, can also be coupled with technologies like supercritical carbon dioxide extraction. Food-grade ethanol and carbon dioxide are less expensive than some enzyme preparations and are suitable for extracting algal bioactives for nutritional and shelf-life enhancement in food products (Roohinejad *et al*., 2017). However, the initial instrumental set-up costs of these techniques may be cost-prohibitive for small-scale seaweed producers, compared to enzymatic flask incubation.

Moisture content determination

The moisture content of the blade, stipe and holdfast of each species before and after oven incubation (12 h, 40 °C) is detailed in Table S1.

Identification of fucoxanthin by LC-MS

To confirm the presence of fucoxanthin in enzymatic extracts, LC-MS characterisation was used after initial HPLC-guided identification. The HPLC peak from the purified enzymatic extract of *F. vesiculosus* (14.937 min) was compared to the corresponding commercial fucoxanthin standard peak and further characterised by its positive ions. Figure 3 shows the LC-ESI-MS spectra of (i) HPLC chromatogram of fucoxanthin standard overlaid with two P-TLC enzymatic extracts of *F. vesiculosus* in full-scan mode from m/z 100 to 1000 atomic mass units (amu), ~~(ii)~~ ESI-MS spectrum of the three peaks depicted in A showing the selected ion 658.91 and its major fragments due to the loss of 18 and 78 amu and (iii) fucoxanthin molecular structure. Fucoxanthin is known to have a molecular mass of 658.91 g. A protonated parent ion (molecular mass (M)+H) was observed in the fucoxanthin standard at m/z 659. An identical ion was observed in the *F. vesiculosus* enzymatic extracts. Two daughter ions were also present as major ESI-MS fragments. These can be seen in all three samples at m/z 641 and m/z 581. The ion at m/z 641 corresponds to the loss of water $[M+H-18]^+$. This is characteristic for a compound such as fucoxanthin which contains a hydroxyl group. The second most abundant ion was at m/z 581, which is due to the loss of acetic acid and water $[M+H-18-60]^+$ from the parent fucoxanthin ion. These findings are in full agreement with reported mass fragmentation data for fucoxanthin (Avula *et al*., 2015; Zhu *et al*., 2016; Rajauria *et al*., 2017), thus confirming its presence in the seaweed extract.

Conclusion

In this work, an effective and sustainable process to extract fucoxanthin from brown seaweeds was optimised. The cellulose content and structure in the cell walls of brown seaweeds limit the efficiency of organic solvent extraction. The application of enzymes could provide a solution by achieving a considerable level of hydrolysis of cellulose in the macroalgal cell walls as presented in this study. In addition, the application of enzyme-based fucoxanthin extraction has advantages over organic solvent extraction. It is a green chemical process with no toxic waste; it converts water-insoluble raw materials into partially water-soluble materials and is a relatively low-cost technology considering the cost-effectiveness of the enzymes utilised. From an application perspective, based on reported clinical trials, the fucoxanthin yields achieved in this study offer potential for complementary treatments of global epidemic nutrition-related disorders such as type 2 diabetes, obesity, cancer and metabolic syndrome. These findings may be applied to the development of value-added nutraceutical products from seaweed biomass.

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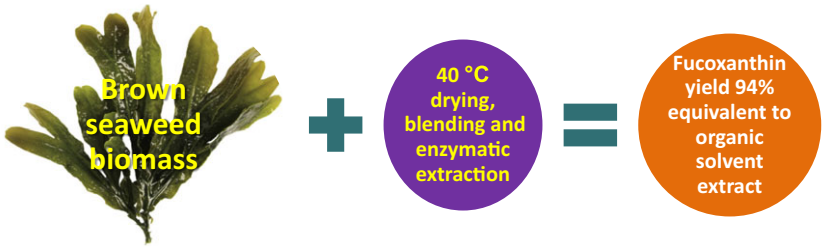
Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Moisture content before and after oven incubation for 12 h at 40 °C.

Graphical Abstract

The contents of this page will be used as part of the graphical abstract of html only. It will not be published as part of main.



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