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A cascading biorefinery process targeting sulfated polysaccharides (ulvan) from *Ulva ohnoi*

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ABSTRACT

We evaluated eight biorefinery processes targeting the extraction of ulvan from *Ulva ohnoi*. Using a factorial design the effect of three sequential treatments (aqueous extraction of salt; ethanol extraction of pigments; and $Na_2C_2O_4$ or HCl (0.05 M) extraction of ulvan) were evaluated based on the yield (% dry weight of biomass) and quality (uronic acid, sulfate, protein and ash content, constituent sugar and molecular weight analysis) of ulvan extracted. The aqueous extraction of salt followed by HCl extraction of ulvan gave higher yields (8.2 ± 1.1% *w*/w) and purity of ulvan than equivalent $Na_2C_2O_4$ extracts (4.0 ± 1.0% *w*/w). The total sugar content of HCl extracts (624–670 μg/mg) was higher than $Na_2C_2O_4$ extracts (365–426 μg/mg) as determined by constituent sugar with ulvan specific monosaccharides contributing 94.7–96.2% and 70.1–84.0%, respectively. Ulvan extracted from *U. ohnoi* was 53.1 mol% rhamnose, 27.8 mol% glucuronic acid, 10.1 mol% iduronic acid, and 5.3 mol% xylose with molecular weights ranging from 10.5–312 kDa depending on the biorefinery process employed. Therefore, the extraction of high quality ulvan from *U. ohnoi* is facilitated by an aqueous pre-treatment and subsequent HCl-extraction of ulvan as part of a cascading biorefinery model delivering salt, ulvan, and a protein enriched residual biomass.

1. Introduction

The intensive and targeted cultivation of macroalgae, both marine and freshwater, has been implemented as a mechanism to mitigate impacts from anthropogenic wastewaters. This process has the benefit of remediating contaminants from wastewaters, in particular nitrogen and phosphorous, through incorporation within the macroalgal biomass, which is then harvested and can be used as a bio-resource. Marine macroalgae have been the focus of this process because of their robustness, high productivities, novel biochemical profiles and metabolites, and ability to be cultivated at scale [1]. Species of the macroalgal genus *Ulva* (chlorophyta) are particularly suitable because of their high productivity and resilience to diverse growing conditions. These characteristics specifically facilitate the culture of species of the genus for the bioremediation of wastewaters produced from intensive land-based aquaculture of marine and brackish water fish and invertebrates in temperate and tropical regions [2–4]. Importantly, the algal biomass from this process can be used for applications ranging from animal feed supplements [5,6], fertilisers [7], composts [8,9], foods [10] and dietary supplements and nutraceuticals [11,12]. However, for this process to be cost effective it is essential to obtain the optimum value from the biomass. This has resulted in a focus on biorefinery processes where biomass is used as a feedstock for the production of high-value and other value-added products [1,13].

One product of specific interest in *Ulva* is the soluble fibre ulvan, which is a significant component of the cell wall of the alga [14,15]. Ulvans constitute between 8 and 29% of the dry weight (dw) of *Ulva* depending on species and growth conditions [16]. These complex sulfated polysaccharides are of biomedical interest for applications in tissue engineering, drug delivery and biofilm prevention [17,18]. Ulvans also have antiviral, antioxidant, anticoagulant, antihyperlipidemic and anticancer activity, in addition to immunostimulatory effects [14,15]. Structurally, ulvans are unique with mostly repeating disaccharide units composed of sulfated rhamnose with glucuronic acid, iduronic acid or xylose [15]. The two major disaccharides are designated as aldobiuronic acids; type A: ulvanobiuronic acid 3-sulfate (A_{3s}), a 1,4-linked glucuronic acid with *O*-3-sulfated rhamnose, and type B: ulvanobiuronic acid 3-sulfate (B_{3s}), a 1,4-linked iduronic acid with *O*-3-sulfated rhamnose (Fig. 1). Partially *O*-2-sulfated xylose can also occur in place of uronic acids affording aldobioses, U_{3s} and $U_{2's,3s}$ [15]. Both

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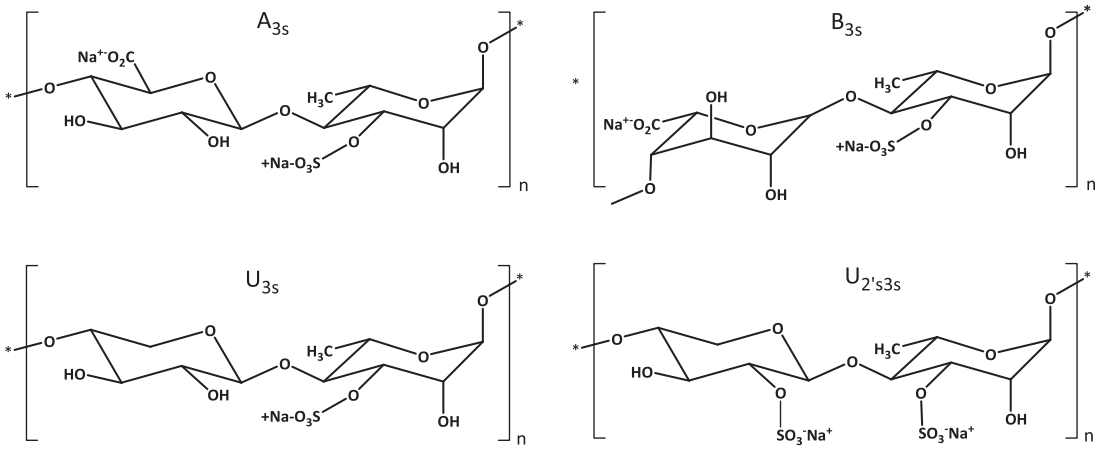

Fig. 1. Main repeating disaccharides in ulvans from species of *Ulva* include aldobiuronic acids (A_{3s} and B_{3s}) and aldobioses (U_{3s} and $U_{2's3s}$) (adapted from Robic et al. [19]).

species and season have demonstrated effects on the chemical structure, macromolecular characteristics, and rheological properties of ulvan extracts [16] and the physical and chemical properties of ulvan are also dependent on extraction methods [19] and stabilisation procedures [20].

Notably, the fractionation of ulvans from other cell wall components, for example glucuronans, xyloglucans, cellulose and proteins, represents a significant challenge. Conventionally ulvans are extracted at 80–90 °C in aqueous solutions of sodium oxalate or ammonium oxalate to chelate the Ca^{2+} that crosslinks ulvan strands in the cell wall [15,19]. In a seminal study ulvan was extracted from *U. rotunda* (stabilised using a variety of methods prior to extraction), at 85 °C in 0.05 M sodium oxalate, with 25–60% recovery [20]. However, the extracts also contained significant content of proteins (up to 35%) and salts (up to 30%).

Our objective is to develop and assess a cascading biorefinery process for the species *Ulva ohnoi* Hiraoka et Shimada, used for the bioremediation of nutrients (N and P) from intensive land-based aquaculture [2], to extract ulvan while minimizing the content of salts and proteins. To do this we examine the effects of pre-washing the biomass, the pre-extraction of pigments, and alternative methods for the extraction of ulvan, using a factorial design. The initial pre-washing of biomass is targeted to extract salts with a low Na: high K ratio as an initial product for the functional food market while facilitating the improved yield and quality of ulvan [21]. The subsequent pre-extraction of pigments is also targeted to improve quality. Finally, comparison of the extraction of ulvan using sodium oxalate and hydrochloric acid is targeted to optimise both yield and quality. Extracts obtained from these processes are subsequently assessed for product quality in terms of purity and chemical composition while the structure of ulvan extracts are determined using constituent sugar and molecular weight analysis and NMR spectroscopy.

2. General methods

2.1. Cultivation of biomass

Ulva ohnoi Hiraoka et Shimada (Genbank accession number KF195501, strain JCU 1 [2]) is domesticated and was collected from a land-based aquaculture facility near Ayr (19°29′S, 147°28′E), Queensland, Australia, where it is cultivated commercially. Biomass was harvested weekly over three consecutive production cycles ($n = 3$) of 7-days in April 2016. Harvested biomass samples (8 × 100 g fresh weight [fw]) were collected and stored (− 20 °C) in separate zip-lock bags until extraction of ulvan as described in Sections 2.3–2.5. Samples (100 g fw) were also taken from each harvest and dried (60 °C, 24 h) to determine the fresh weight to dry weight ratio (fw:dw) and composition of the starting material (untreated biomass) as described in Section 2.6. Harvest specific fw:dw ratios were used to calculate crude extract yields as g extract per g dw biomass.

2.2. Experimental design

This study tests the assumption that the sequential removal of salts and pigments in a cascading biorefinery process will improve the yield and quality of ulvan extracted in a subsequent step. We use a factorial experimental design to quantify the effect of three extraction treatments; treatment 1 targets the extraction of salts; treatment 2 targets the extraction of pigments; and treatment 3 targets the extraction of ulvan (Fig. 2). During treatment 1, biomass was either subjected to a warm aqueous extraction (Salt Reduced Biomass = SRB) or left untreated (Control Biomass = CB). During treatment 2, biomass was extracted with ethanol (Pigment Reduced Biomass = PRB, and Salt and Pigment Reduced Biomass = SPRB) or left untreated (CB and SRB). During treatment 3, biomass was extracted with 0.05 M $Na_2C_2O_4$ (EP1, EP3, EP5, EP7) or 0.05 M HCl (EP2, EP4, EP6, EP8). To quantify variation between harvests the experiment was conducted in triplicate from three harvests over a 3 week period.

2.3. Treatment 1: extraction of salts

Ulva ohnoi biomass was treated as per Magnusson et al. [21]. Briefly, whole *U. ohnoi* biomass (100 g fw) was submerged in distilled water (1 L; biomass to water ratio of 1:10) at 40 °C for 30 min using a temperature controlled water-bath. Following this the biomass was recovered by filtration through a 200 µm mesh filter.

2.4. Treatment 2: extraction of pigments

Ulva ohnoi biomass was treated using a modification of the methodology by Robic et al. [19]. Residual *U. ohnoi* biomass from previous treatments was suspended in absolute ethanol (1 L) at room temperature for 1 h. The biomass was isolated by filtration (200 µm mesh) and the extraction procedure described above repeated 2 more times.

2.5. Treatment 3: extraction of ulvan

Sodium oxalate extraction method [19]. Residual *U. ohnoi* biomass from previous treatments was suspended in 0.05 M $Na_2C_2O_4$ (1 L) and heated at 85 °C for 1 h. The suspension was filtered (200 µm mesh) prior to vacuum filtration through diatomaceous earth (Celatom®). The extract was then filtered (Whatman® GF/F), concentrated (10 ×) by ultrafiltration (ÄKTA flux 6 fitted with a Xampler 10 kDa NMWC Cartridge), diafiltered (5 volumes of deionised water) and freeze dried.