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Biorefinery of Red Algae for Multiple High-Value Products

Final Report SSNV 2022

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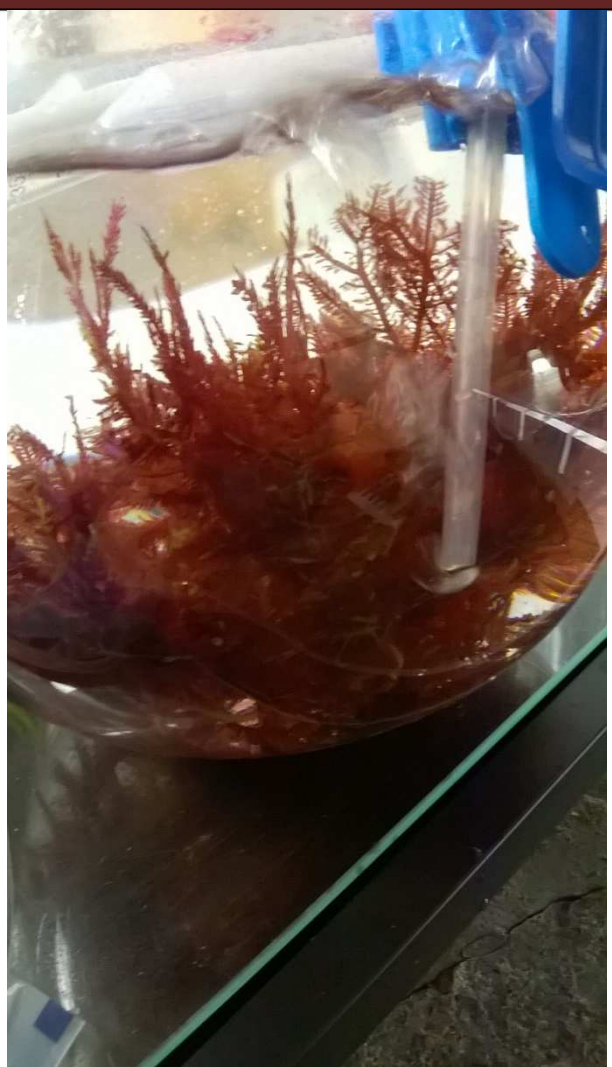

Report Summary

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Summary: Seaweeds are a renewable natural source of a plethora of valuable compounds that have attracted the attention of scientists in recent years. Following the concepts of sustainability and zero-waste policy, the aim of the present study was to reduce pressures on the environment by the use of a sequential multiproduct biorefinery approach in combination with eco-friendly extraction procedures and the development of a tissue cultivation concept that is up-scalable for future industrial applications, using tissue cultivated *Porphyra umbilicalis* and *Corallina officinalis* from the indoor culture collection of northern Icelandic algae. The applied biorefinery approach used in the present study, resulted in the recovery of four products of commercial value i) a sulphated polysaccharide (sPS) and ii) protein containing fraction (which were separated by precipitation), iii) a PUFA fraction and iv) a carrageenan-rich fraction. The experimental exposure to high velocities (HV) and salinities (HS) in long- (LT) and short-term (ST) trials revealed that *P. umbilicalis* (Pu) contained the highest product amounts in the Pu-LT-HV assay compared to *C. officinalis* (Co). The sPS analysis showed highly sulfated polysaccharides for both tested species, whereas the results of the monosaccharide composition analysis highlighted for *C. officinalis* extracts that they were only constituted of galactose and xylose, while *P. umbilicalis* samples were mainly composed of galactose with lower amounts of mannose and small amounts (< 2.5% DW) of xylose, arabinose, galactose, glucose and rhamnose. The amino acid profiles of the protein fractions elucidated that the most abundant amino acids in fresh collected *P. umbilicalis* (control) were glycine, arginine, leucine, valine and threonine, with concentrations ranging from 6.53-13.60 mg/g DW. In the protein fraction of the sample Pu-LT-HV this dominance shifted to the amino acids asparagine, serine, alanine and glutamine with values ranging from 10.81 to 23.69 mg/g DW. In contrast, the amino acid profile of fresh collected specimens of *C. officinalis* was dominated by asparagine, serine, lysine, arginine and valine. The protein fraction of the sample Co-LT-HV showed the dominance of asparagine, serine, lysine and arginine. The lipid profile of *P. umbilicalis* was dominated by high values of polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (AA, 20:4n-6) in the control as well as experimental assay with concentrations ranging from 982.45-1397.06 µg/g DW and 1029.08-2945.37 µg/g DW, respectively. In contrast, the lipid profile of *C. officinalis* was dominated by unsaturated fatty acids such as palmitic acid (16:0) and saturated fatty acids such as myristic acid (14:0) and stearic acid (18:0). In addition, significant concentrations of docosadienoic acid (22:2n-6) and docosahexaenoic acid (DHA, 22:6n-3) were detected in the high velocity assay of *C. officinalis*. The carrageenan yield of *P. umbilicalis* varied between 13.15 ± 2.01% and 19.82 ± 2.71% DW. The FTIR spectrum confirmed the presence of the characteristic peaks of carrageenan in the recovered fraction of sample Pu-LT-HV from *P. umbilicalis*. Based on the FTIR analysis, the extracted carrageenan is a mixture of κ-, ι-, and λ-carrageenan. In the NMR spectrum only signals for κ-carrageenan and ι-carrageenan were detectable. A phytochemical screening conducted revealed the presence of flavonoids beside others in the residual biomass of *C. officinalis* and *P. umbilicalis*. Most of the valuable fractions obtained in the present study from *C. officinalis* and *P. umbilicalis* were successfully integrated into our solid skin care product series, although for instance proteins and carrageenans are more valuable as food ingredients.

Keywords: *Red algae*, *multiproduct biorefinery*, *tissue cultivation*, *solid skin care products*

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1. Introduction

Seaweeds or macroalgae are multicellular photosynthetic plant-like organisms that grow mainly in coastal areas. Several red seaweed (Rhodophyta) species such as *Porphyra umbilicalis* and *Corallina officinalis* (Fig. 1) are economically relevant because they represent 61% (~18 million tonnes) of the total global seaweed production (Peñuela et al. 2018). They are mainly used to extract agar and carrageenan, but also contain other high-value compounds, in amounts varying significantly between species and seasons (Baghel et al. 2014, Seghetta et al. 2016, Torres et al. 2019a). The presence of some unique bioactive components, not found in terrestrial biomass, makes red seaweed an even more attractive resource (Torres et al. 2019). Most of the current utilization schemes are based on processing a single seaweed into a single product, whereas the rest of the material is discarded (Kerton & Yan 2017). The valorisation of all biomass and co-production of other compounds for the food, feed, pharmaceutical, cosmetic or medicinal industries is necessary to obtain sustainability (Baghel et al. 2015, Barbot et al. 2016, Laurens et al. 2017, Torres et al. 2019a). For an integral utilization of the resources, the sequential separation of all constituents is usually required following a multistage process (Van Hal et al. 2014) (Fig. 2). This biorefinery concept offers a scheme for the production of high value-added components, warrants the maximum utilization of the biomass, reduces the cost of production, offers environmental protection and mitigates climate change impact (e.g., Balina et al. 2018, Torres et al. 2019b, Sadhukhan et al. 2019).

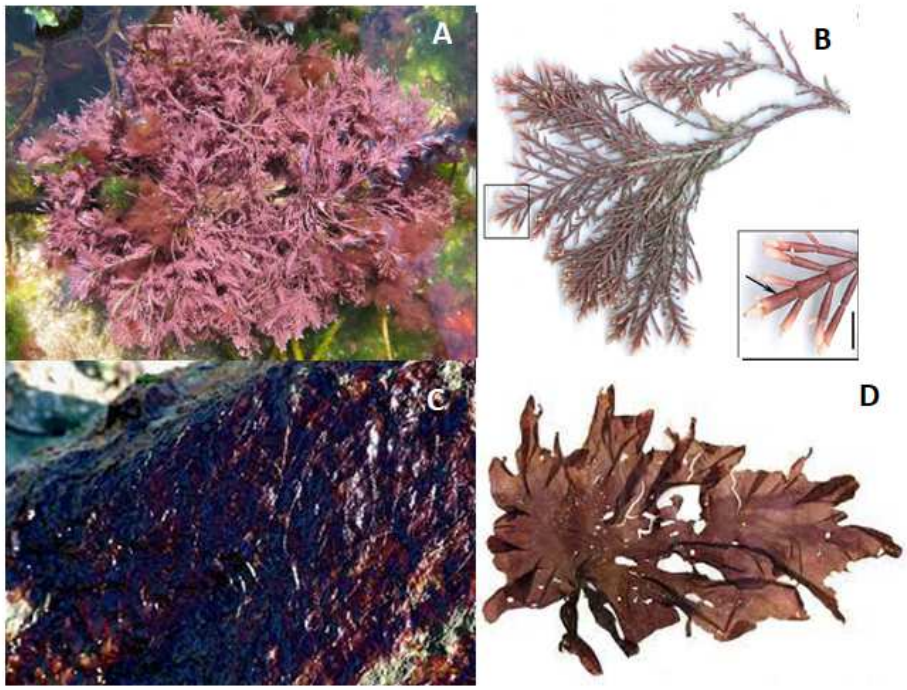

Figure 1. *Corallina officinalis* in a rock pool (A). Representative frond of *C. officinalis*. Inset: apical region of frond branch (scale bar = 1 mm); arrow (Williamson et al. 2014) (B)**.** A commercially valuable maricultured macroalgae is the red seaweed *Porphyra*, which belongs to the family of red algae called Bangiaceae (Wahlström et al. 2018) (C, D).

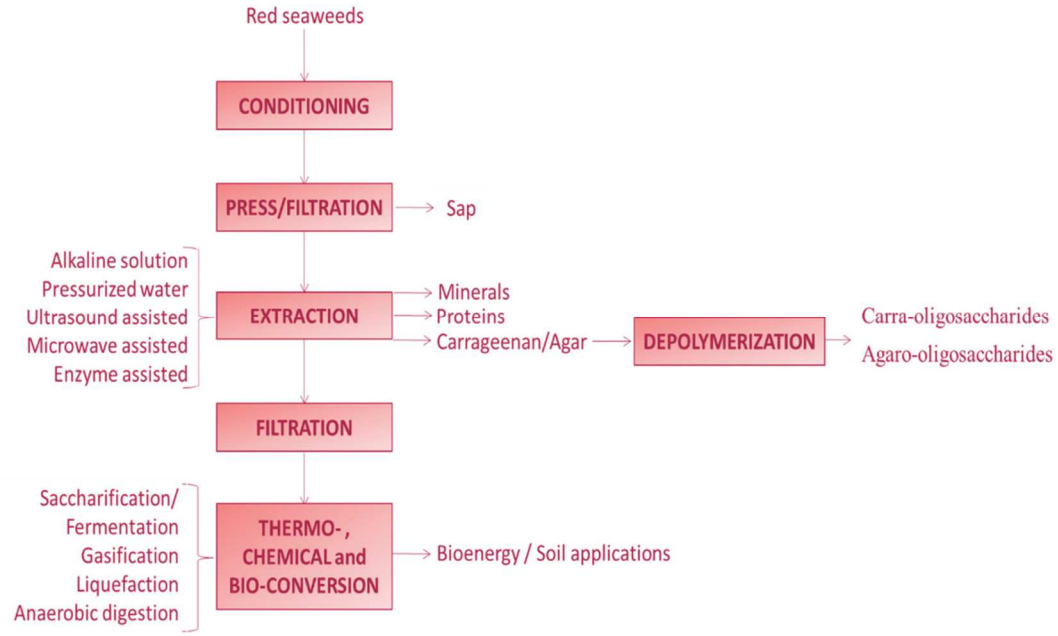

Figure 2. Simplified flow diagram of a red seaweed biorefinery (from Torres et al. 2019a).

Red seaweeds are an excellent raw material for biorefineries, due to the plethora of valuable compounds they contain (Fig. 3). Among macroalgae, red seaweeds accumulate the highest protein contents (up to 45% dry weight =DW) as well as the highest protein digestibility compared to other seaweeds (Álvarez-Viñas et al. 2019). Red seaweeds may be used in dried form as protein sources in food and feed, whereas the bioactive peptides have diverse activities, such as immunomodulatory, antibacterial, antithrombotic, and antihypertensive effects (Murray & FitzGerald 2007, Cian et al. 2019).

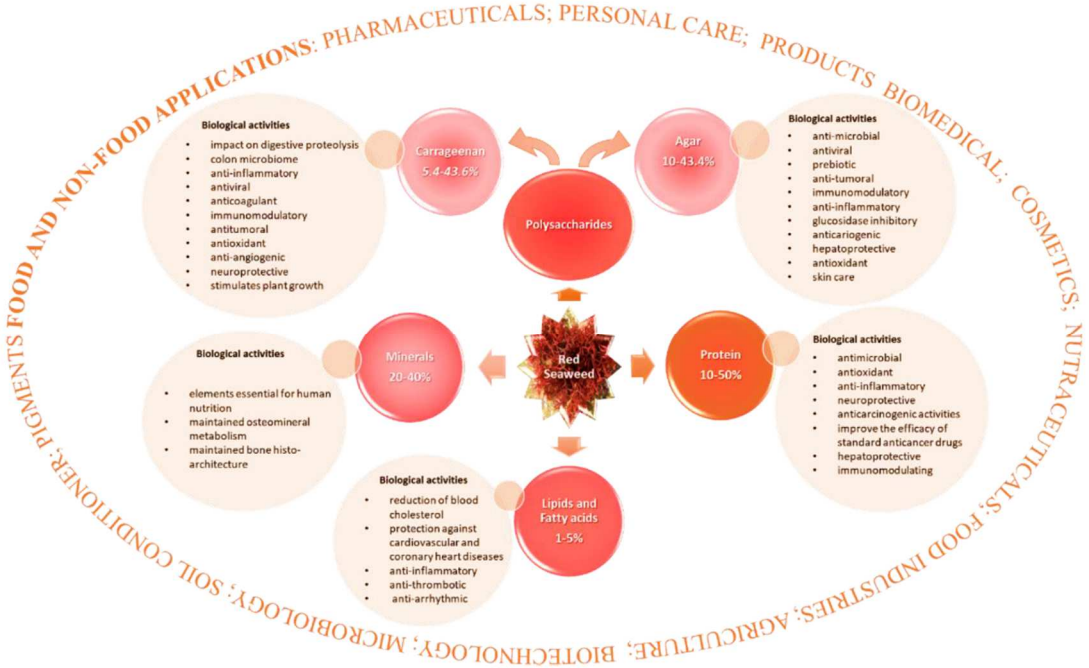

Figure 3. Product overview of red seaweeds and their potential application (from Torres et al. 2019a).

Furthermore, red seaweeds have developed defense mechanisms including the synthesis of photo-protective molecules against UV radiation, such as pigmented compounds (i.e., chlorophyll-a, phycobiliproteins and carotenoids) and mycosporine-like amino acids (Lalegerie et al. 2019). These have potential applications as colorants, UV protectors and antioxidants. The chlorophyll a content of red macroalgae has been reported in the range 0.35-9.8 mg/g DW (Bayu & Handayani 2018, Uribe et al. 2018, Rosemary et al. 2019). These greenish pigments have antioxidant bioactivity and can be converted into compounds with cancer preventive actions (Nunes et al. 2018). The carotenoids of red macroalgae account for 0.2–1.8 mg/g DW (Chan & Matanjun 2017, Bayu & Handayani 2018), predominated by β-carotene, zeaxanthin, lutein and antheraxanthin. Rhodophytes also synthesize phenolic compounds that exhibit free-radical scavenging properties (Øverland et al. 2019), accounting for up to 6 mg gallic acid equivalents/g DW (Tibbetts et al. 2016, Martínez–Hernández et al. 2018). They also contain vitamins such as 2.1–2.7 mg pro-vitamin A/g, 0.05–1.54 mg vitamin B2/g, 3.8–4.8 mg vitamin B6/g, 0.4–1.0 mg vitamin B9/g, 2.5–501 mg vitamin C/ 100 DW, 1.34 mg vitamin E/g DW and 4.61 µg α-tocopherol/g DW (Sakthivel & Pandima Devi 2015, Chan & Matanjun 2017, Martínez–Hernández et al. 2018, Rosemary et al. 2019). Phycobiliproteins, present in red macroalgae in the range of <1–125 mg/g DW, have a great economic potential and can be used in biomedical, food, cosmetics and pharmaceutical applications. Contents in the range 0.8–7 mg R-phycoerythrin/g DW, 0.02–10 mg phycocyanin/g DW have been reported (Francavilla et al. 2013, Baghel et al. 2014, Uribe et al. 2018, Guihéneuf et al. 2018, Álvarez-Viñas et al. 2019).

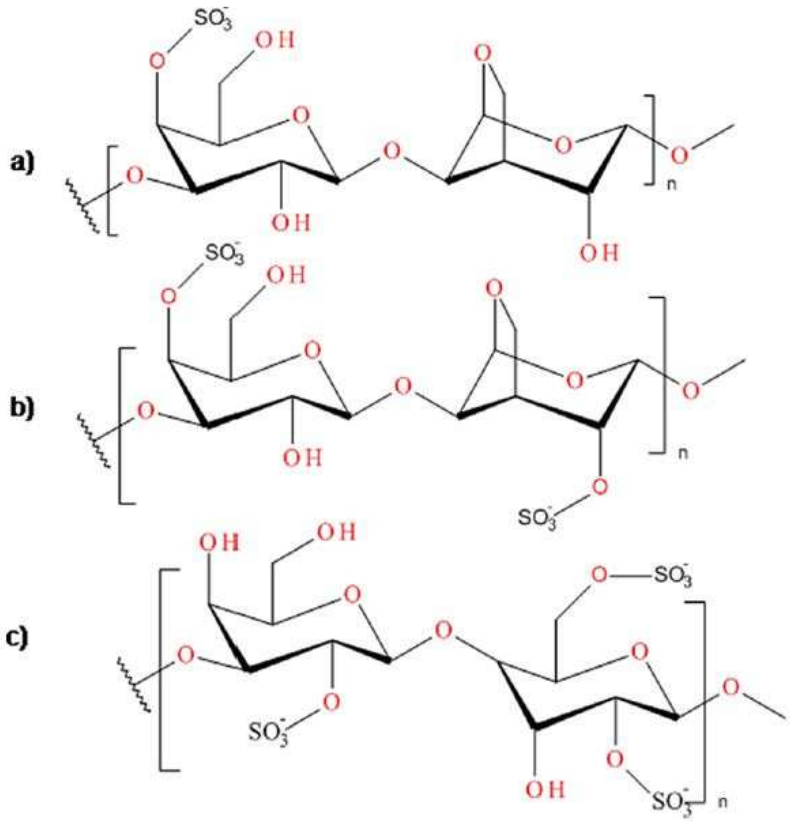

Figure 4. Representative repeating unit motifs of (a) κ, (b) ι, and (c) λ-carrageenan.

Red seaweed dietary fibres are mostly composed of sulfated polysaccharide galactans (a polymer of galactose) (Fonseca et al. 2008**,** Cunha & Grenha 2016). The structures of polysaccharides and their sulfate contents are highly variable between species (Amorim et al. 2011), contributing to the various

facets of their pharmacological ability (Manlusoc et al. 2019). The sulfate content of algal polysaccharides determines the biological potency especially their anticoagulant and antioxidant activities (Zhang et al. 2003). Carrageenan is a family of water-soluble polysaccharides composed of repeating units of partially sulfated disaccharides. Carrageenan exists as different isomers, of which the three most common isomers are κ-, ι-, and λ-carrageenan, where each monomer unit contains one, two, and three sulfate groups, respectively (Arman & Qader 2012) (Fig. 4). The degree of sulfation of the carrageenan strongly affects its properties. The ability of carrageenans to form cross-linked gels in the presence of K+ or Ca2+ ions is one reason why these biomacromolecules are gaining increasing attention (Hilliou et al. 2006) and why they have been investigated as potential hydrogel scaffolds in drug delivery and biomedical applications (Popa et al. 2011, Vázquez-Delfín et al. 2014) κ-Carrageenan forms firm and elastic cross-linked gels in aqueous solutions in the presence of K+ ions and more brittle gels in the presence of Ca2+ ions; ι-carrageenan forms soft gels in aqueous solution in the presence of Ca2+ ions. However, λ- carrageenan does not form any gels in the presence of metal ions (Wang et al. 2005, Wahlström et al. 2018). Despite the low lipid content, red seaweeds are rich in nutritionally important PUFAs (65.6% of total fatty acids) (Kumari et al. 2013). Their ω-3: ω-6 ratio is higher than in terrestrial sources and could be used as nutraceuticals for their anti-hypercholesterolemic, antioxidant, anticancer, anti-diabetic, antihypertensive and anti-inflammatory activities (Kumari et al. 2013, Chan & Matanjun 2017). Beside this, red seaweeds have a high content (8–55 g/100 g DW) of essential minerals and trace elements (Rupérez 2002, Kumar et al. 2011), with high proportions of sulfate (1.3–5.9%). They could be potential ingredients for functional foods, providing a supplementation in deficitary elements in diets (Circuncisão et al. 2018). Rhodophycean species contain important macrominerals, 6.1–21.9 g K/100 g DW, 1.8–8.1 g Na/100 g, 0.2–0.9 g Ca/100 g DW and 0.2–0.5 g Mg/100 g DW (Matanjun et al. 2009, Kumar et al. 2011, Mondal et al. 2013, Baghel et al. 2014). Among the trace minerals Fe is the most abundant followed by Zn, Cu and Se (Nunes et al. 2018). The low Na/K ratio in these seaweeds is beneficial for instance in relation to hypertension (Mondal et al. 2013).

Cultivation of seaweeds has been well established throughout Asia, and there is now growing interest in the cultivation of macroalgae in Europe to meet future resource needs. If this industry is to become established throughout Europe, then balancing the associated environmental risks with potential benefits will be necessary to ensure the carrying capacity of the receiving environments are not exceeded and conservation objects are not undermined. Particularly, seaweed beds and kelp forests of cold-temperate regions around the world represent highly diverse, dynamic and complex ecosystems (Mann 1982, Dayton 1985). Even well managed seaweed harvesting puts substantial ecological pressure on natural algal beds by increasing disturbance levels and removing resources from the ecosystem. Seaweeds in suspended cultivation remove inorganic nutrients from the marine environment during growth (Kerrison et al. 2015, Marinho et al. 2015). Positive remedial effects will occur when the quantity and proportion of nutrients removed are equal to those added by anthropogenic activities (Seghetta et al. 2016). However, undesirable effects could occur if nutrient removal by cultivation results in concentrations which fall below that required for natural primary productivity, and large-scale culture of macroalgae will extract proportionate amounts of nutrients from the surrounding water body (Lüning & Pang 2003). At large-scales, changes to planktonic communities are likely as phytoplankton will experience increased competition for light and nutrients from cultivated species. Changes in primary productivity will affect the trophic flow through affected marine food webs (Cambell et al. 2019). In this context, the cultivation of seaweeds should be

prioritised in areas with excess nutrients such as aquaculture sites in integrated multitrophic aquaculture (IMTA) approaches or communities with untreated municipal wastewater flows into the ocean. For instance, it has been shown that seaweed biomass produced with aquaculture eluents contains 2–4-fold more protein than that grown in wild cultures, because seaweeds assimilate the fish eluents, which are rich in dissolved ammonia and phosphate. However, the cultivation in an IMTA or other excess nutrient scenarios would not solve the problem of variations in the seaweed biochemical composition due to changes in the environmental conditions and therefore the amount and quality of the potential products deriving from the harvested species.

Tissue culture is a plant multiplication technique using artificial medium in a controlled and sterilized environmental condition. Some methods used in seaweed tissue culture are protoplasm culture, spore culture, and somatic embryogenesis (Rorrer & Cheney 2004). In general, the traditional rationale for establishing cell and tissue cultures from seaweeds has been strain improvement and micropropagation for mariculture, and not the development of culture platforms suitable for agitated bioreactors or tank cultures (Rorrer et al. 1995). Particularly liquid cell suspension cultures derived from marine plants have the potential to biosynthesize for instance novel bio-medicinal compounds in a controlled environment (Rorrer et al. 1995). Macroalgal cell biotechnology is still in its infancy compared with terrestrial plant cell biotechnology. There are more than 85 species of seaweeds for which tissue culture aspects have been reported. Although the initial aim of these techniques focuses mostly on genetic improvement and clonal propagation of seaweeds for mariculture, recently the scope of these techniques has been extended for use in bioprocess technology for production of high value chemicals of immense importance in the pharmaceutical and nutraceutical sectors. Recently, there has been a phenomenal interest in intensifying seaweed tissue and cell culture research to maximize the add-on value of seaweed resources (Reddy et al. 2008) but also to mitigate climate change derived and increasing CO_2 level (ocean acidification) impacts on commercially valuable species such as *Corallina officinalis* (Hofmann et al. 2012).

Figure 4. *Asparagopsis* is one of the main red seaweed species being investigated for aquaculture, due to its ability to reduce methane emissions from the cattle industry (© The Australian Seaweed Institute).

2. Objectives and Aims of the Study

Following the concepts of sustainability and zero-waste policy, the aim of the present study is to reduce pressures on the environment by use of a multiproduct biorefinery approach in combination with eco-friendly extraction procedures and development of a tissue cultivation concept that is up-scalable for future industrial applications.

To enrich the sulfated polysaccharide fraction, long- and short-term term cultivated tissues and fronds of *Porphyra umbilicalis* and *Corallina officinalis*, from the culture collection of northern Icelandic microalgae and seaweeds located in an external BioPol ehf facility in Skagaströnd, will be subjected to the experimental exposure to high salinities and velocities as preselected stress factors (Scholz et al. 2021). Subsequently, a biorefinery approach using enzymatic- and microwave-assisted extraction processes will be tested in the present project, resulting in overall three fractions and finally four products:

I) a water-soluble extract rich in proteins and sulfated polysaccharides (sPS),
II) a lipid fraction with high quantities of polyunsaturated fatty acids and
III) a pure carrageenan fraction in food grade quality.

Residue biomass will be screened for further valuable compounds, which will be if present integrated into the future biorefinery concept. The first fraction will be further separated to obtain purified sPS and protein.

From selected samples, the following compounds and fractions will be analysed:

- the chemical composition of the sPS, including total carbohydrate and monosaccharide composition as well as total phenolic and sulfate contents.
- the composition of the amino and fatty acids.
- the carrageenan fraction will be structural characterised.

In addition, the following bioactivities of the sPS and carrageenan fractions will be tested:

- antioxidant activities.
- elastase and collagenase inhibitory activities.
- effects on human keratinocytes viabilities in MTT assays; and
- effects on the viability of 3T3-L1 cells tested in adipocyte differentiation assays.

In the final part of the project, tests will be conducted with the goal to integrate selected fractions into solid skin care products to facilitate specific properties such as moisturizing, anti-inflammatory and/or anti-aging and replace as far possible conventional components.

A project overview is depicted in Fig. 5.

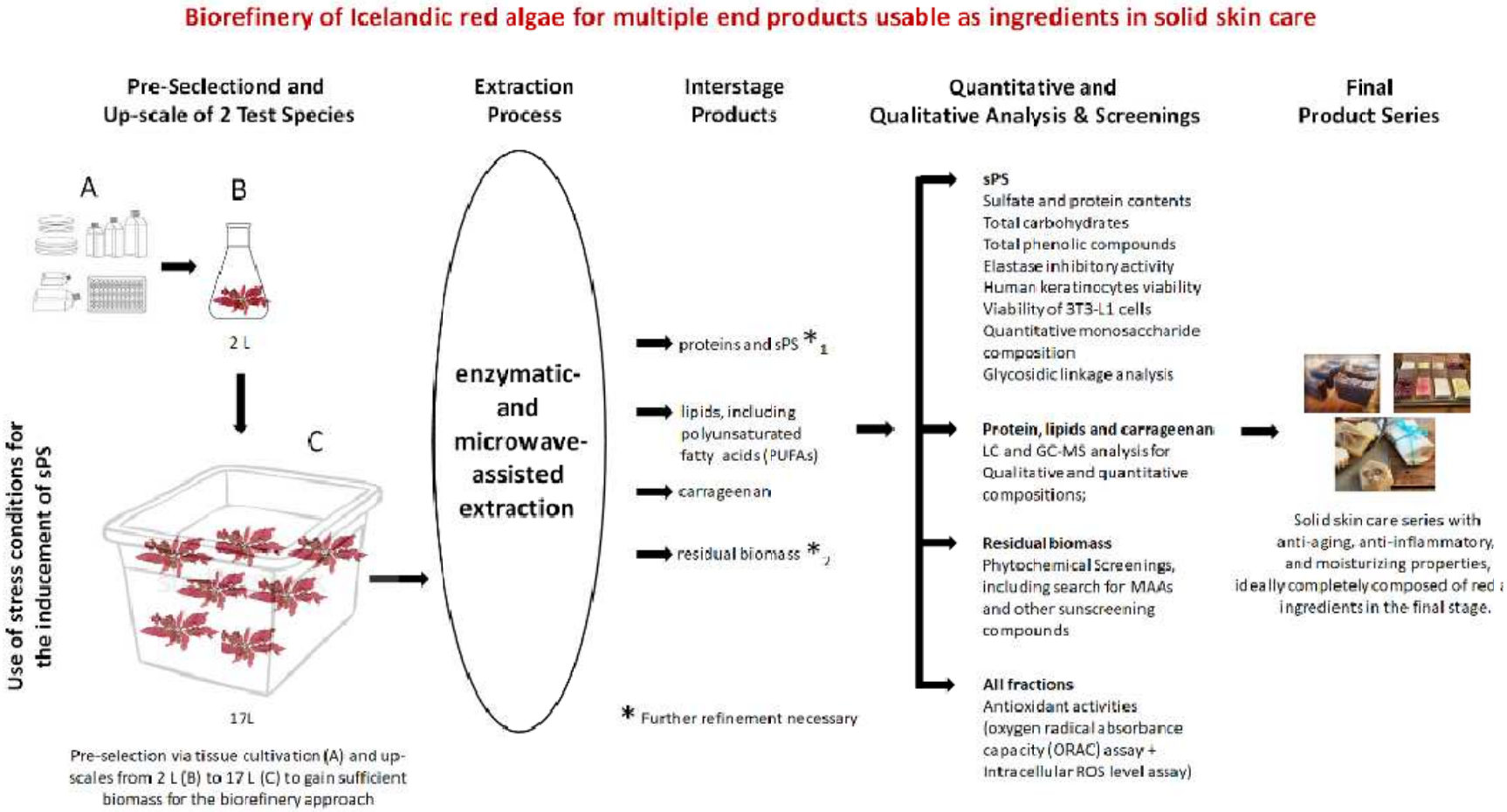

Figure 5. Overview of the present project.

Schematic overview of the different steps for the production of a solid lotion products supplemented with sulfated polysaccharides (sPS) and other fractions from red seaweeds.

3. Material and Methods

If not otherwise mentioned, all chemicals used in this study were of the highest purity from Merck (former Sigma/Aldrich).

3.1 Algal Species, Strain Selection and Tissue Culture Conditions

Figure 6. Long- (A, collected October 2018) and short-term (B, collected February 2022) cultures of *Porphyra umbilicalis tissues* and *Corallina officinalis* fronds (C).

Overall, two red northern Icelandic seaweed species (Rhodophyceae) were chosen for the present project, namely *Porphyra umbilicalis* and *Corallina officinalis* (Fig. 6).

Corallina officinalis specimens were collected from a rocky shore pool near Skagaströnd (north-west Iceland) in October 2020 and stored in bottles containing natural seawater until they were transferred into culture at the algae culture collection facility of BioPol ehf. To facilitate the development and propagation of genotypes of commercial importance, frond cultures from different specimens were maintained at 12°C, 33 PSU, 50 µmol photons m^{-2} s^{-1} light intensity (Phillips, Germany, Master TL-D 18W/840), and a 12:12 h light:dark cycle in artificial seawater (Tropic Marin Reef Salt® Germany) containing ¼ strength Provasoli enrichment medium (Provasoli 1968) in liquid and on agar (10 g · l^{-1}). Calcium chloride ($CaCl_2 \cdot 2H_2O$) was added to the enrichment medium to a final concentration of 10 mM to ensure the presence of calcium for the calcifying algae. *Porphyra umbilicalis* specimens were collected from rocks at the harbour in Skagaströnd in October 2018. Tissue cultures from different specimens were maintained at 15°C, 30 PSU, 30 µmol photons m^{-2} s^{-1} light intensity (Phillips, Germany, Master TL-D 18W/840), and a 12:12 h light:dark cycle in artificial seawater (Tropic Marin Reef Salt® Germany) containing full strength Provasoli enrichment medium (Provasoli 1968) in liquid and on agar (12 g · l^{-1}). Tissue culture of *P. umbilicalis* seed was obtained from natural breeding that

has been done for 2 years, but due to the restricted time frame of the current project, only thalli grown to a size of 10-15 cm were used in the experiments.

The selection process of genotypes with commercial importance was based on a screening for sulfated polysaccharides (cf. 3.6.1.1). Genotypes with the best quality of sPS and were selected for the up-scale and experimental exposure (cf. 3.2). Conductivity, temperature, dissolved oxygen and pH were measured using handheld probes (YK-31SA, YK-200PATC, YK-200PCD and YK-2001PH, SI Model 33, Engineered Systems and Designs-Model 600, Philips W9424). Photosynthetic active radiation (PAR, 400–700 nm) was measured with an underwater spherical quantum sensor LI-193SA connected to a Licor Data Logger LI-250A.

3.2 Exposure Experiments

The inducement of sulfated polysaccharides was conducted by variation of culture conditions. Using the one-factor-at-a-time principle, the two rhodophytes were exposed to a) 40 Practical Salinity Units (=PSU) and b) strong bubbling. The experiments were conducted in 2 L Erlenmeyer flasks in triplicates for 72 h (short-term exposure) and 336 h (long-term exposure), employing *P. umbilicalis* tissues (8-10) and *C. officinalis* fronds (15-25, depending on sizes and wet weights). As control fresh collected specimens were used, resulting in overall 10 samples (without triplicates). Experiments were started by filling in 1000 mL fresh prepared culture medium (cf. 3.1) into the Erlenmeyer flasks with the rhodophytes, which were weighted prior to each experiment. Long-term exposure cultures were supplied with fresh medium in weekly intervals.

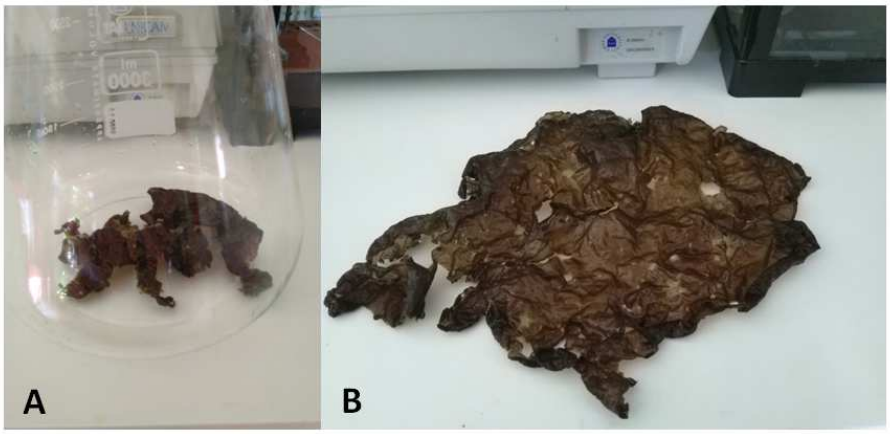

Figure 7. Example of long-term cultured *Porphyra umbilicalis* tissue growth. A) start with 3 thalli in 1 L Erlenmeyer flask, B) one individual thallus after 6 months cultivation (for conditions cf. 3.1).

3.3 Determination of Growth

Growth rates of *Porphyra umbilicalis* and *Corallina officinalis* were calculated by the increase in wet weight of thalli (Fig. 7) and fronds and presented as percentage growth per week using the formula of Penniman et al. (1986):

$$SGR = \% [(Gt/Go)1/t - 1] \times 100$$

SGR = specific growth rate (% in wet weight per week), Gt = weight after t weeks, Go = initial weight,

3.4 Harvest and Processing

After 72 h (short-term assay) and 336 h (long-term assay), the seaweeds were harvested and washed twice with distilled water. Subsequently the specimens were dried, utilizing a horizontal dryer with hot air flow at 60°C (Tray dryer, model no. FDTHQQZ).

3.5 Sequential Extraction Process

Following the biorefinery concept introduced by Peñuela et al. (2018), eco-friendly technologies were combined to achieve an integral utilization of tissue cultivated *Porphyra umbilicalis* and *Corallina officinalis* biomass using a three-step sequential extraction. It resulted in three fractions containing four valuable products (Products 1/2 = sPS/proteins, Product 3 = PUFAs and Product 4 = Carrageenan) (cf. Fig. 8). Products 1/2 were subsequently separated by precipitation of the polysaccharides.

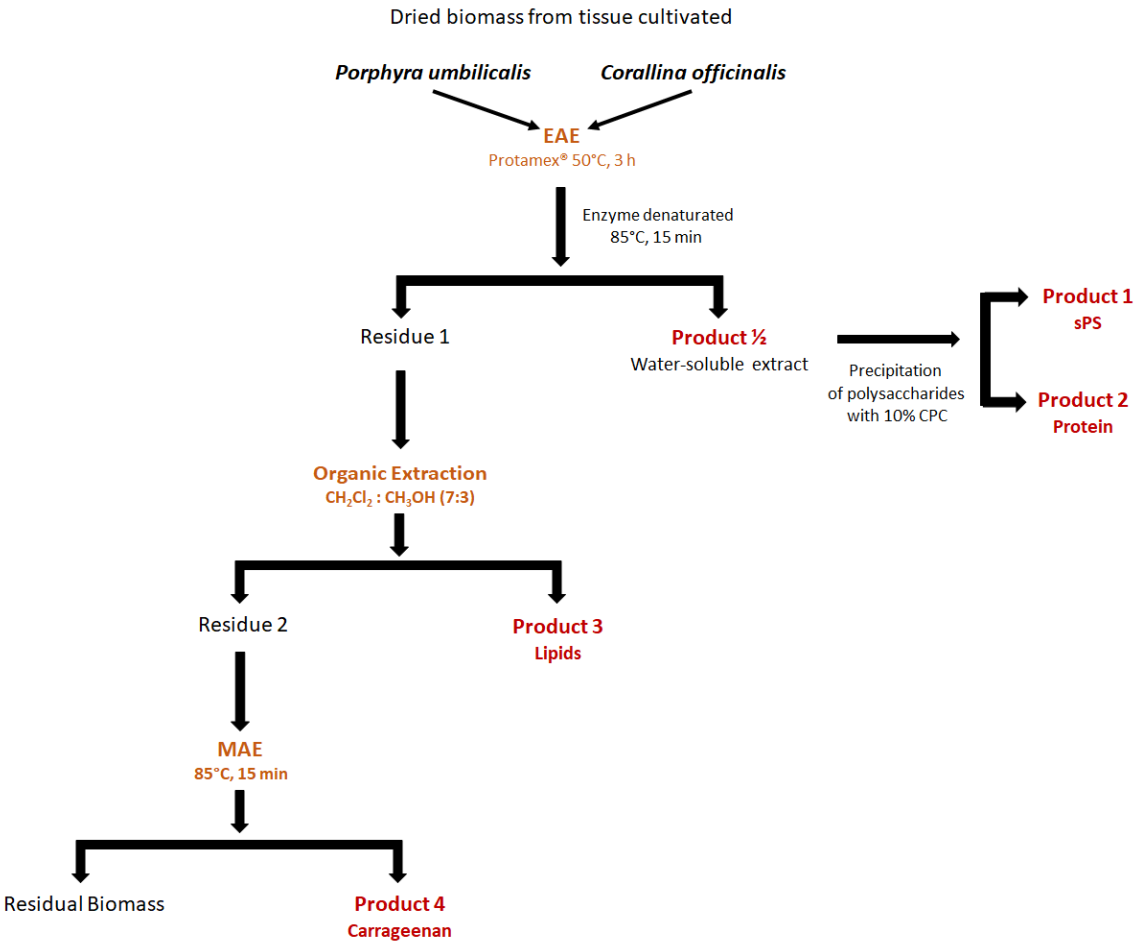

Figure 8. Schematic representation of the sequential extractions used in the present project during the multiproduct biorefinery approach.

In the first extraction step an enzymatic assisted extraction (EAE) of *Porphyra umbilicalis* and *Corallina officinalis* biomass with protease enzyme (Protamex®) was used. For the hydrolysis of seaweed, 200

mL of distilled water were added to 10 g of dried algal biomass, mixed with 5% w/w of protease and placed in a 50 °C water bath for 3 h. The enzyme was then denatured at 85 °C for 15 min. Blanks extracted under the same conditions but without enzymes served as controls (50 °C for 3 h + 85 °C for 15 min; 50 °C for 3 h). After EAE, the extracts obtained in the different conditions were filtered and two fractions were obtained: a water-soluble extract (Products 1/2), and insoluble residues (Residue 1). The polysaccharides in solution were then precipitated with 16 mL of 10% cetylpyridinium chloride (CPC) solution. After 24 h at 25°C, the mixture was centrifuged at 2,560 × g for 20 min at 20°C. The polysaccharides were washed with 500 mL of 0.05% CPC solution, dissolved with 100 mL of a 2 $mol \cdot L^{-1}$ NaCl-ethanol (100:15, v/v) mixture, and the excess of salts was removed by precipitation and wash with 200 mL of absolute ethanol. After 24 h at 4°C, the precipitate was collected by centrifugation (2,560 × g for 20 min at 20 °C), washed extensively with ethanol-80%, then absolute ethanol. After this, the polysaccharides were washed with acetone, which was followed by hot air drying (60°C) until all the acetone was removed. Subsequently, both Products 1 and 2 were freeze dried for further biochemical analyses (protein, carbohydrate, total lipid, and sulfate content, cf. 3.6.1.1 and 3.6.1.2), and for activity tests.

The insoluble residues were used as raw material for an organic extraction to extract lipids and fatty acids (Product 3). Lipids were extracted with dichloromethane/methanol (7:3 v/v) for 24 h, simultaneously with maceration of the tissues assisted by mechanical agitation (cf. 3.3.1.3).

The dried residual mass after lipid extraction (Residue 3) was finally used to obtain carrageenan (Product 4). For comparison, direct extractions (water soluble extract, fatty acid and carrageenan) of the initial biomass were also performed, besides extractions of fresh collected specimens.

Carrageenan extraction was conducted using a microwave at a frequency of 2450 MHz. Briefly, 1 g of the dried residue (Residue 3) was rehydrated overnight in 50 mL of distilled water. The sample was placed in a closed-vessel system (OMNI/XP-1500) to prevent solvent and analyte loss. By using closed vessels, the extraction can be performed at elevated temperatures accelerating the mass transfer of target compounds from the sample matrix. The carrageenan extraction was performed at 85°C for 15 min. As control, a conventional carrageenan extraction was performed using the method described by Freile-Pelegrín et al. (2006). Briefly, the aqueous mixture was extracted at 85 °C for 3.5 h, mixed with diatomaceous earth (Celite), pressure filtered, and the filtrate neutralized to pH 8.9 with 5 M HCl prior to the recovery of the carrageenan from the solution. After the extraction, carrageenan was precipitated with 250 mL of Cetavlon (hexadecyl-tri-methylammonium bromide) in 9:1 (v/v) distilled water/acetone and recovered over filter paper under vacuum. The fibrous carrageenan was carefully washed three times with 63 mL of 95% ethanol saturated with sodium acetate to remove Cetavlon residues. Sodium acetate was removed by three final washes with 95% ethanol. Carrageenan was dried at 60 °C for 24 h, weighed to calculate the percent yield, and milled to powder for structural analyses. The extraction yield was calculated as follows:

$$Carrageenan\ yield\ (\%) = \frac{Wc}{Ws}(100)$$

where *Wc* is the weight (g) of dried carrageenan and *Ws* is the initial weight (g) of dried seaweed used before extraction.

The residual biomass was extracted in different solvents (each 1 h) in an all-glass filtration chamber, using 50 mL ethanol (99.5 %; for flavonoids, alkaloids, phenols, tannins, saponins), 50 mL methanol (80 %; for aldehydes), following different methods referenced in Table 1. The residues from all extractions were evaporated and re-dissolved in 100 ml aqueous EtOH (40%), filtered (Whatman GF/C, 47 mm), and used for the phytochemical screening.

3.6 Product Characterisations

3.6.1 Biochemical Analysis

3.6.1.1 Sulfated Polysaccharide Analysis

Polysaccharides derived from red seaweed are usually composed of repeating disaccharide units of alternating 1,3-linked galactose (Gal) and 1,4-linked 3,6-anhydro-galactose (AnGal) residues. The biological properties of red seaweed polysaccharides are highly dependent on the amount of AnGal, which is a key bioactive monomeric sugar of red algae (e.g., Zheng et al. 2020, Xie et al. 2020). Thus, in the present project, the determination of total carbohydrate content as well as the content of AnGal were used as pre-selection tool. The total carbohydrate content was determined by the anthrone-sulfuric acid method with minor modification described by Xie et al. (2020). Each sample solution (1 mL, 1 mg/mL) was placed in tubes and 0.2 mL distilled water added. After adding 1 mL of anthrone reagents (0.2 g anthrone was mixed with 100 mL of 80% (v/v) sulfuric acid before colorimetric) followed by homogeneous mixing, samples were allowed to react at 80°C for 20 min. Next, tubes were cooled for 5 min in an ice water bath, before the total absorbance was measured three times at 640 nm. Total sugar level was quantified using a calibration curve plotted with Gal standards.

The content of AnGal was determined by resorcinol method with a minor modification (Yaphe & Arsenault 1965). Resorcinol reagent was prepared within 3 h before the colorimetric assay. Briefly, resorcinol reagent was prepared with 9 mL resorcinol solution (1.5 mg/mL), 1 mL acetaldehyde solution (0.04%, v/v) and 100 mL concentrated hydrochloric acid. Next, 0.03 mL aliquot of the sample solution (1 mg/mL) was added to a centrifuge tube followed by the addition of 0.2 mL distilled water. After placing in an ice bath for 5 min, 1 mL of resorcinol reagent was added, mixed homogenously in ice bath, and then placed at room temperature for 2 min. The mixture was incubated for 10 min at 80°C followed by cooling for 5 min in an ice bath. The absorbance of AnGal was measured at 555 nm and the concentration of AnGal calculated using a calibration curve with Gal standards. All samples were analysed in triplicates, with Gal content in samples calculated by the total content of carbohydrate minus the content of AnGal.

For monosaccharide compositions, samples of the polysaccharides extracted from the rhodophytes (5 mg) were hydrolysed with 5 mol·L^{-1} trifluoroacetic acid for 4 h at 100°C, reduced with borohydride, and the alditols were acetylated with acetic anhydride:pyridine (1:1, v/v). The alditols acetates were dissolved in chloroform and analysed in a gas–liquid chromatograph/mass spectrometer (GCMS-QP2010 Shimadzu, Japan) with a DB-5ms column (Agilent) (Kirchner 1960, de Castro et al. 2018).

The total phenolic content (TPC) of each sample was determined according to the method of Gutfinger (1981). Each sample (1.0 mL) was mixed with 1.0 mL of 10% Na_2CO_3 and allowed to stand for 3 min. Then, 1.0 mL of 50% Folin-Ciocalteu reagent was added to each mixture. After incubation at room

temperature, the resulting mixtures were centrifuged at 13,400 *g* for 5 min. Absorbances were measured with a spectrophotometer (TI Unicam 5625 UV/VIS Spectrometer, Richmond Scientific Ltd., Chorley, United Kingdom) at 750 nm, and the total phenolic contents were expressed as gallic acid equivalents.

The presence of sulfate groups in algal polysaccharides have been attributed to their biological activities (Barahonaa et al. 2014, Wang et al. 2016). The sulfate contents of the sPS were determined according to the method given by Terho & Hartiala (1971) utilizing standard operation protocols developed by our partner GlycoMar. The method is based on the use of sodium rhodizonate which forms a coloured compound in the presence of barium ions. This colour is reduced when sulfate is present, due to the formation of barium sulfate. Briefly, samples were hydrolysed using HCl, dried and resuspended in water. For the assay, to 50 µL of each sample or control (blanks, heparin and chondroitin sulfate), 50 µL de-ionised water and 400 µL ethanol were added and mixed thoroughly. 125 µL of each ethanol-added sample, standards (final concentration 0.048 to 0.48 µg Na_2SO_4), and controls were pipetted into a 96-well microplate in triplicate. $BaCl_2$ buffer and sodium rhodizonate solution were added, mixed and incubated at room temperature (18°C) in the dark for 10 min. The colour intensity was then measured spectrophotometrically at 520 nm. The % of sulfate in each sample was then calculated from the standard curve.

3.6.1.2 Protein Contents and Amino Acid Analysis

Contaminant proteins (CPs) were measured using the Lowry method as described by Herbert et al. (1971) with bovine serum bumin as a standard. In brief, 5 mL 1.0 N NaOH was added to the biomass aliquot and incubated for 5 min in a boiling water bath (95°C, Typ 1083, GFL mbH, Burgwedel, Germany). After cooling, 2.5 mL of the reactive mixture (5% Na_2CO_3 + 0.5% $CuSO_4$ $5H_2O$ in 1.0% Na-K-Tartarate; ratio 25:1 v/v) were added and incubated for 10 min at room temperature. This was followed by the addition of 0.5 mL Folin-phenol reagent (1.0 N) and incubation for another 15 min. After centrifugation (Omnifuge 2.0 RS, Heraeus Sepatch, Osterode, Germany), the intensity of the resulting blue colour was determined at 650 nm.

Free amino acid analysis was conducted according to the method described in Reinholdt et al. (2019). Briefly, ∼50 mg sample was extracted in 500 µL of ice-cold LC-MS/MS buffer [150 µL chloroform, 350 µL methanol, 1 µL of MES as internal standard (1 mg/mL)]. Following addition of 400 µL ice-cold water, samples were vortexed thoroughly and incubated for at least 2 h at −20°C. After centrifugation (10 min, 20,000 *g*, 4°C), the aqueous phase was transferred to a new tube and 400 µL of ice-cold water again added to the extraction tube. Following stirring and centrifugation (5 min, 20,000 *g*, 4°C), supernatants were combined and lyophilized. Next, the dried extracts were dissolved in 400 µL water and filtrated through 0.2 µm filters (Omnifix-F, Braun, Germany).

The cleared supernatants were analysed using the high-performance liquid chromatograph mass spectrometer LCMS-8050 system (Shimadzu) and the incorporated LC-MS/MS method package for primary metabolites (version 2, Shimadzu). In brief, 1 µL of each extract was separated on a pentafluorophenylpropyl column (Supelco Discovery HS FS, 3 µm, 150 × 2.1 mm) with a mobile phase containing 0.1% (v/v) formic acid. The compounds were eluted at 0.25 mL min^{-1} using the following gradient: 1 min 0.1% (v/v) formic acid, 95% *Aqua destillata* (*A. dest*.), 5% acetonitrile, within 15 min linear gradient to 0.1% (v/v) formic acid, 5% *A*. *dest*., 95% acetonitrile, 10 min 0.1% (v/v) formic acid, 5% *A*. *dest*., 95% acetonitrile. Aliquots were continuously injected in the MS/MS part and ionized via

electrospray ionization. The compounds were identified and quantified using the multiple reaction monitoring values given in the LC-MS/MS method package and the LabSolutions software package (Shimadzu). Authentic standard substances (Merck) at varying concentrations were used for calibration and peak areas normalized to signals of the internal standard. Glyoxylate, 3-HP, and glycerate were determined in the negative ion mode using selective ion monitoring for *m/z* 73, 102, and 105 corresponding to the deprotonated glyoxylate, 3-HP, and glycerate ions $[M-H]^-$. Retention time acquisition window (2 min) was verified with coelution experiments using purchased glyxoylate, 3-HP, and glycerate (Sigma-Aldrich). Varying concentrations of the three metabolites were also used for calibration curves. Data were interpreted using the Lab solution software package (Shimadzu).

3.6.1.3 Lipid Contents and Fatty Acid Analysis

The total lipid content was evaluated by the gravimetric method and reported as percentage of the algae dry weight (Bligh & Dyer 1959). The fatty acids from the total lipids were obtained by one-step direct transesterification method (Kumari et al. 2011). Briefly, 5 mg of lipids were treated with 5 mL of acetyl chloride/methanol (1:19 v/v) and esterified at 80 °C for 1 h. After cooling, 1 mL of water and 2 mL of n-hexane were added to the mixture, vortex, and centrifuged. The organic phases were collected, filtered, and dried with anhydrous sodium sulfate. Solvents were removed under nitrogen and the fatty acids methyl esters (FAMEs) solubilized in n-hexane were identified. The FAMEs were analysed by gas chromatography (Agilent, Model 7890B, Palo Alto, CA, USA) coupled with mass spectrometry (Agilent, Model 7000C, Palo Alto, CA, USA). FAMEs were separated on an HP-5MS capillary column (30 m x 0.25 mm, i.d., 0.25 µm film thickness), (Agilent J&W Scientific, Folsom, CA, USA). A sample (5 µL) was injected at temperature of 250 °C. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. The column temperature was programmed as follows: initial temperature at 100 °C for 4 min, increasing with 10 °C min^{-1} to 200 °C (for 5 min), and 10 °C min^{-1} to 300 °C for 15 min. Mass detector conditions were: electron impact mode at 70 eV, transfer line temperature 280 °C, source temperature 230 °C, mass acquisition range 50–500 amu (atomic mass units) and solvent delay 3.75 min. The FAMEs were identified by comparison of standard Supelco 37 Component FAME Mix (SUPELCO, St. Louis, MO, USA) and their mass spectra with those from the NIST/EPA/NIH Mass Spectral Library version NIST 2.2 (Agilent, Palo Alto, CA, USA).

3.6.1.4 Carrageenan Analysis

The functional groups of the gelling and non-gelling fractions of carrageenan were determined with the transmission method (laminated sample) by Fourier Transform Infrared spectrophotometry (FT-IR), using a Nicolet iS50 FT-IR spectrophotometer (Thermo Fisher Scientific, USA) between 4000 to 700 cm^{-1} (Gómez-Ordóñez & Rupérez 2011).

For NMR analysis, 0.5 mg mL^{-1} of carrageenan in deuterium water was used. The spectra were recorded on a Varian/Agilent Premium Compact 600 NMR spectrometer at 70 °C, operating at frequencies of 150.81 MHz for 13C nucleus. The extracts were exchanged twice with 99.8% deuterium oxide (D2O) with intermediate lyophilization and dissolver in D2O (30 mg mL^{-1}). Sodium [3-trimethylsilyl 2,20,3,30-2-H4] propionate (TSP-d4) was used as an internal reference for the baseline (0.00 ppm).

3.6.1.5 Screening for further utilizable Compound Classes

A phytochemical screening of the residual biomass targeting bioactive compound groups such as alkaloids, aldehydes, flavonoids, saponins and tannins, was conducted**,** following different methods referenced in Table 1.

Table 1. Methods used during the phytochemical screenings.

Compound group	Extraction Solvent	Calibration standard	Method	Ref.
Aldehydes	MeOH (80%)	Formaldehyde CH_2O	Schiff's and Fehling's tests*	Turner (1916)
Alkaloids	EtOH (99%)	Piperine $C_{17}H_{19}NO_3$	Mayer´s and Wagner`s reagent*	Scholz & Liebezeit 2006
Flavonoids	EtOH (99%)	Quercetin $C_{15}H_{10}O_7$	Alkaline Reagent Test	Tiwari et al. 2011
Glycosides	EtOH (99%)	Oleandrin $C_{32}H_{48}O_9$	Keller-Killiani Test	Usunobun et al. 2015
Phenols	EtOH (99%)	Hydroquinone $C_6H_6O_2$	Folin-Ciocalteu reagent/$FeCl_3$	LeBlanc et al. 2009
Phyto-sterols	MeOH (80%)	Ergosterol $C_{28}H_{44}O$	Liebermann-Burchardt test	Tiwari et al. 2011
Saponins	EtOH (99%)	Saponin S4521	Frothing test	Scholz & Liebezeit 2006
Tannins	EtOH (99%)	Tannic acid $C_{76}H_{52}O_{46}$	Gelatine-Saltblock test	Scholz & Liebezeit 2006

*Only samples that gave positive reactions to both reagents and tests are assumed to contain alkaloids or aldehydes, respectively

Abbreviations: EtOH: ethanol; MeOH: methanol.

3.7.2 Activity Assays

3.7.2.1 Evaluation of Antioxidant Activity

It has been assumed that an antioxidant with a high potential to defend ROS and deplete oxidative stress, thus, a compound with a strong antioxidant activity additionally facilitates the skin protection against oxidative damages along with delaying the skin aging process (Palmer & Kitchin 2010, Chanda et al. 2015). Therefore, two antioxidative activity tests were conducted in the present project. The first was the DPPH scavenging activity assay which was performed as described by Nanjo et al. (1996). Briefly, DPPH reagent was dissolved in methanol for a solution concentration of 1.5×10^{-4} M. One hundred microliters of DPPH reagent was mixed with 100 µL sample in 96-well plates. After incubation at room temperature for 30 min, the absorbance was measured 517 nm. The second test carried out was the Hydrogen peroxide scavenging activity test which was conducted according to the method given by Müller (1985) with slight modification introduced by Jiratchayamaethasakul et al. (2020). A 100 µL of 0.1 M PBS buffer (pH 5) was added into a 96-well plate. Each 20 µL of sample and 20 mM hydrogen peroxide (H_2O_2) were added to mix with the buffer, and then incubate 37 °C for 5 min. After the incubation, a 30 µL of 1.25 mM ABTS and peroxidase (1 unit/mL) were added to the mixture and then incubated at 37 °C for 10 min. The absorbance was read at 405 nm.

3.7.2.2 MTT Assay

The MTT assay was used to examine the effects of the sPS on human keratinocytes viability. Human keratinocytes were cultured in a 96-well plate (1×10^4 cells/well) for 24 h at 37°C with 5% CO_2. Next,

the cells were treated with the rhodophyte extracts (200 µg/mL) for 48 h and then incubated with 100 µL of MTT reagent (5 mg/mL) for 1 h. Then, the reaction medium was removed and the insoluble formazan remaining in the keratinocytes was dissolved in 100 µL of DMSO at room temperature for 15 min. The absorbance of each well was measured at 540 nm. The viability of the extract-treated cells was expressed as a percentage of the viability of untreated cells.

3.7.2.3 Adipocyte Differentiation (AD)

3T3-L1 cells were maintained at 37°C in a humidified, 5% CO_2 atmosphere for all procedures. The 3T3-L1 preadipocytes were seeded in a 12-well plate and cultured for 3∼4 days, until confluency. Two-day post confluent 3T3-L1 preadipocytes were maintained in DMEM supplemented with 10% FCS and 100 units/mL penicillin-streptomycin, and the medium was replaced every 2 days. Two days after reaching confluency (Day 0∼2), the preadipocytes were treated with DMEM supplemented with 10% FBS and 100 units/mL penicillin streptomycin (FBS-medium) and containing 500 µM IBMX, 5.2 µM dexamethasone, and 167 nM insulin (differentiation medium; DM). The 3T3-L1 cells were treated with 200 µg/mL doses of the rhodophyte extracts until 2 days post-confluence. After Day 2, the medium was changed to FBS-medium with 167 nM insulin for additional 2 days (post-differentiation medium; Post-DM). Thereafter, the 3T3-L1 adipocytes were cultured in FBS-medium. The cells were harvested at Day 7 and stained for matured adipocytes.

After adipocyte differentiation, the cells were stained with Oil Red O staining (Inazawa et al. 2003), an indicator of cell lipid content. Briefly, cells were washed with phosphate-buffered saline, fixed with 10% buffered formalin and stained with Oil Red O solution (0.5 g in 100 ml isopropanol) for 10 min. After removing the staining solution, the dye retained in the cells was eluted into isopropanol, and OD_{540} was determined.

3.7.2.4 Enzyme Assays

Solar radiation, or UV radiation, is the major stimulator that accelerates the overproduction of reactive oxygen species (ROS) which leads the endogenous oxidative stress in the epidermis (Kim et al. 2016). The accumulated ROS after skin exposure to photoaging stressors can indirectly activate dermal enzymes such as collagenase and elastase which basically break down and degrade collagen as well as elastin, respectively (Sahasrabudhe & Deodhar 2010, Popoola et al. 2015, Chatatikun & Chiabchalard 2017). Thereby, the synthesis of elastase and collagenase promotes premature skin aging as evidenced by signs such as wrinkles, freckles, sallowness, deep furrows or severe atrophy, laxity, and leathery appearance (Peres et al. 2011, Ding et al. 2018).

Each test was performed in triplicate using standards reported in the literature, known for their high inhibitory activity, which have been used in this study as positive controls to validate each assay. Negative controls were prepared by adding water instead of inhibitor. The quantification of the inhibitory effect of the SPs, and those of the reference compounds, were calculated using the following equation:

$$\% \ Inhibition = [(A_n - A_s)/A_n] \times 100$$

Where A_n is the absorbance of negative control and A is the absorbance of the sample measured at the wavelength indicated for each test.

3.7.2.4.1 Collagenase inhibitory Assay

The enzyme assay was based on the method reported by Madhan et al. (2007), with some modifications. For the enzymatic hydrolysis an 8 mg/mL solution of collagen was used as substrate. As enzyme, a stock collagenase solution of 3.6 x 103U/mL was prepared in a 0.1 M Tris-HCl buffer (pH 7.4) containing 0.006 mM $CaCl_2$. Inhibitors and enzyme were incubated for 30 minutes at 25°C before adding the substrate. The 1 mL reaction mixture containing 902.83 U of enzyme, 2.4 mg of substrate and 250 µL of the sPS at different final concentrations (2.5, 1.25, 0.63 and 0.25 mg/mL) was incubated for 8 hours at 37°C. Finally, samples were centrifugated at 8500 rpm for 15 minutes at 4°C and hydroxyproline was detected in 500 µL of supernatant by the method of Kolar (1990) adapted for a microplate reading. To validate the method, we compared the effect of the sPS with the caused by a positive control containing EDTA at a concentration of 0.25 mg/mL. The spectrophotometric measurements were made at a wavelength of 558 nm.

3.7.2.4.2 Elastase inhibitory Assay

This test was based on the protocol described by Sachar et al. (1955). The principle of the method is the elastin-orcein hydrolysis, which is initially insoluble in water but when it is digested by elastase, it generates soluble products which can be determined spectrophotometrically at a wavelength of 590 nm. For this assay, an elastase solution of 0.399 U/mL dissolved in Tris-HCl buffer (pH 8.8) and a substrate solution at a final concentration of 4.7 mg/mL were used. The experiment began by adding 500 µL of the sPS extract (2.5, 1.25, 0.63 and 0.25 mg/mL) to 500 µL of enzyme and then incubating for 30 minutes at 25 °C. Then, the reaction was obtained with the addition of the substrate and then the reaction mixture was incubated at 37 °C for 4 hours. Subsequently, all reaction mixtures were centrifugated for 15 minutes at 8500 rpm and 4 °C, and then the absorbance of the supernatant was read at 590 nm, using a solution containing the same amount of substrate dissolved in the buffer as blank. To validate the test, the inhibition caused by the sPS was compared to those caused by EDTA (disodium salt) at 15 mg/mL. At this concentration EDTA exhibits a high inhibition of elastase.

3.8 Statistical Analysis

Measures were carried out in triplicate (n = 3), and the results are given as mean values and standard deviations. The results were statistically analysed using a one-way ANOVA with a statistical difference of 5% and the Tukey TSD test of the IBM SPSS software version for multiple comparisons.

4 Results

4.1 Product Characterisation

4.1.1 Biochemical Compositions

4.2.1.1 Product 1 – sulfated Polysaccharides: Total Polysaccharid concentration, C-Protein, Total Phenolic and Sulfate Contents as well as Monosaccharide Compositions

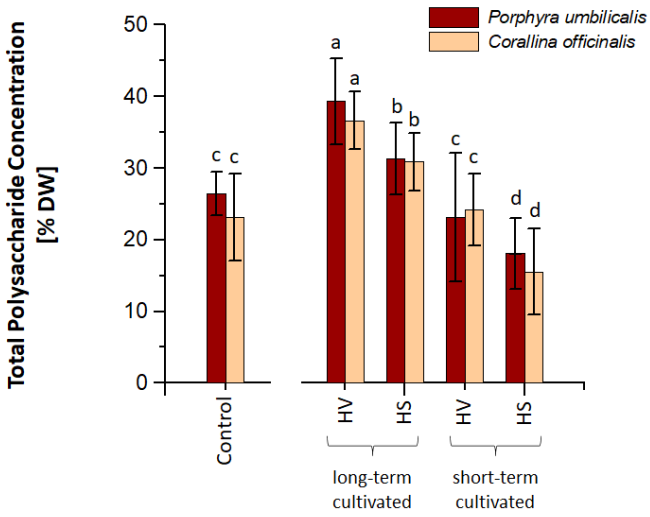

Figure 9. Total polysaccharide concentrations of the first product fraction, after the precipitation step, obtained from tissue cultivated *P. umbilicalis* and *C. officinalis* after long- and short-term exposure to high salinities (HS) and high velocities (HV). As control fresh collected specimens were used. Values correspond to the average of triplicates ± standard deviation. Letters indicate significant differences between assays ($p<0.05$).

The polysaccharide concentration of *Porphyra umbilicalis* (short Pu) and *Corallina officinalis* (Co) samples obtained from the long-term (LT) exposure to high velocity [Pu-LT-HV, Co-LT-HV] were significantly higher compared to the concentrations gained from samples from the short-term (ST) exposure assays as well as fresh collected specimens (control, $p < 0.05$, Fig. 9), being the highest for *P. umbilicalis* with 39.5 ± 1.2% DW (3.27 g/g). The carbohydrate contents varied between 30.1 ± 1.50% DW and 39.5 ± 1.15% DW, being the highest in the sample Co-LT-HV (Table 2). The contaminant proteins (CPs) content varied between 0.02 and 0.11% DW [Pu-LT-HV, Pu-ST-HS]. Furthermore, out of the ten samples tested, only the control assays gave positive results in the TPC assay.

The monosaccharide composition of the polysaccharides was determined based on gas chromatography/ mass spectrometry analysis of the alditol acetates formed after acid hydrolysis. The monosaccharide composition of *C. officinalis* samples was constituted of galactose (Gal, 72.15-73.93% DW) and xylose (Xyl, 25.69-27.23% DW), while *P. umbilicalis* samples were mainly composed of galactose (84.03-88.01% DW) with lower amounts of mannose (Man, 7.18-8.80% DW) and with small amounts (< 2.5% DW) of xylose, arabinose, galactose, glucose and rhamnose (Table 3). Sulfate

contents varied between 9.06 ± 0.05 and 13.93 ± 0.25% DW [Pu-control, Co-LT-HV], being the highest for all *C. officinalis* samples.

Table 2. Carbohydrate, contaminate (=C)-protein and total phenolic contents of the first product fraction obtained from tissue cultivated *P. umbilicalis* and *C. officinalis* after long- and short-term exposure to high salinities (HS) and high velocities (HV). As control fresh collected specimens were used. Values correspond to the average of triplicates ± standard deviation. Letters indicate significant differences between assays ($p<0.05$).

Sample	Carbohydrate [% DW]	C-Protein [% DW]	TPC [% DW]
Pu-control	32.1 ± 0.11^{c}	0.03 ± 0.01^{d}	0.02 ± 0.01
Pu-LT-HV	33.9 ± 0.08^{a}	0.02 ± 0.00^{d}	n.d.
Pu-ST-HV	33.5 ± 1.23^{c}	0.07 ± 0.03^{b}	n.d.
Pu-LT-HS	32.9 ± 0.27^{c}	0.08 ± 0.01^{b}	n.d.
Pu-ST-HS	31.5 ± 1.19	0.11 ± 0.02^{a}	n.d.
Co-control	36.6 ± 1.09^{b}	0.04 ± 0.03^{c}	0.09 ± 0.01
Co-LT-HV	39.5 ± 1.15^{a}	0.05 ± 0.03^{b}	n.d.
Co-ST-HV	35.2 ± 1.00^{b}	0.03 ± 0.01^{d}	n.d.
Co-LT-HS	32.4 ± 0.73^{c}	0.06 ± 0.01^{c}	n.d.
Co-ST-HS	30.1 ± 1.50^{d}	0.05 ± 0.01^{c}	n.d.

Abbreviations: Pu, *Porphyra umbilicalis*; Co, *Corallina officinalis*; LT, long-term cultivated; ST, short-term cultivated; HV, high velocity; HS, high salinity; n.d., below detection limit (0.01).

Table 3. Monosaccharide composition of sPS and their sulfate contents detected in the first product fraction obtained from tissue cultivated *P. umbilicalis* and *C. officinalis* after long- and short-term exposure to high salinities (HS) and high velocities (HS). As control fresh collected specimens were used. Values correspond to the average of triplicates ± standard deviation. Letters indicate significant differences between assays ($p<0.05$).

Sample	Monosaccharides [% DW]								Sulfate content
	Gal	Xyl	Ara	Rha	Glu	Man	GlcA	GalA	[% DW]
Pu-control	84.03^{b}	2.45^{c}	1.54^{b}	1.63^{b}	1.41^{e}	8.80^{a}	0.05	0.09	9.06 ± 0.05^{f}
Pu-LT-HV	88.01^{a}	1.02^{c}	1.32^{c}	1.25^{c}	1.22^{f}	7.18^{b}	n.d.	n.d.	11.23 ± 0.11^{d}
Pu-ST-HV	86.24^{a}	2.04^{c}	1.13^{d}	1.91^{a}	1.18^{f}	7.50^{b}	n.d.	n.d.	11.03 ± 0.09^{d}
Pu-LT-HS	86.41^{a}	2.14^{c}	1.36^{d}	1.19^{d}	1.27^{f}	7.63^{b}	n.d.	n.d.	10.93 ± 0.24^{e}
Pu-ST-HS	84.49^{b}	2.01^{c}	1.76^{a}	1.63^{b}	2.89^{d}	7.22^{b}	n.d.	n.d.	11.07 ± 0.42^{d}
Co-control	72.16^{d}	27.04^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	12.86 ± 0.16^{c}
Co-LT-HV	73.61^{c}	25.57^{b}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	13.93 ± 0.25^{a}
Co-ST-HV	72.61^{d}	27.23^{a}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	13.31 ± 0.11^{b}
Co-LT-HS	73.93^{c}	25.69^{b}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	13.59 ± 0.37^{b}
Co-ST-HS	73.89^{c}	25.72^{b}	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	13.33 ± 0.21^{b}

Abbreviations: Ara = arabinose; Gal = galactose; Glc = glucose; Man = mannose; Rha = rhamnose; Xyl = xylose; GlcA = glucuronic acid; GalA = galacturonic acid; n.d., below detection limit (0.001); Pu, *Porphyra umbilicalis*; Co, *Corallina officinalis*; LT, long-term cultivated; ST, short-term cultivated; HV, high velocity; S, high salinity.

4.1.1.2 Product 2 – Protein Contents and Amino Acid Compositions

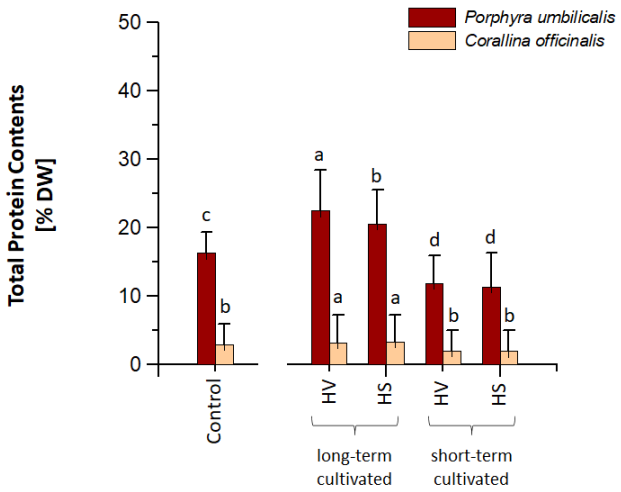

Figure 10. Total protein content of the second product fraction, after the precipitation step, obtained from tissue cultivated *P. umbilicalis* (Pu) and *C. officinalis* (Co) after long- (LT) and short-term (ST) exposure to high salinities (HS) and high velocities (HV). As control fresh collected specimens were used. Values correspond to the average of triplicates ± standard deviation. Letters indicate significant differences between assays ($p<0.05$).

Table 4. Amino acid profiles of the two selected protein fractions of tissue cultivated *P. umbilicalis* and *C. officinalis* obtained from the high-velocity assays (HV). As control fresh collected specimens were used.

	P. umbilicalis		***C. officinalis***	
Amino acid ($mg \cdot g\ DW^{-1}$)	**Control**	**Pu-LT-HV**	**Control**	**Co-LT-HV**
Asparagine	0.72	23.69	1.35	3.13
Serine	3.85	10.81	1.14	2.53
Alanine	7.28	21.86	0.11	1.76
Glycine	13.60	13.94	0.11	1.61
Glutamine	5.21	21.43	0.25	1.01
Threonine	6.91	12.60	0.13	1.21
Cysteine	1.59	2.31	n.d.	0.43
Proline	4.32	8.31	0.08	0.61
Lysine	6.15	9.55	1.18	2.93
Histidine	1.42	1.93	0.09	0.63
Arginine	8.61	14.13	1.18	3.20
Valine	6.53	12.79	1.14	1.99
Methionine	1.09	2.43	0.05	0.18
Tyrosine	4.27	5.62	0.12	0.93
Isoleucine	4.13	8.50	0.12	1.01
Leucine	7.06	16.62	0.13	0.81
Phenylalanine	4.38	8.56	0.13	0.51
Tryptophan	4.25	0.77	n.d.	0.13
Ornithine	0.32	1.14	n.d.	n.d.
Total	**91.69**	**196.99**	**7.31**	**24.61**

The total protein content of *P. umbilicalis* varied between 11.41 ± 2.13% and 22.57 ± 2.37% DW, being the highest in the long-term exposed high velocity assay [Pu-LT-HV] (3.49 g/g DW; Fig. 10). In contrast, the protein content of *C. officinalis* was in most cases characterized by a narrow range of variation (2.21-2.95%, 0.17-0.18 g/g DW), and it was relatively higher in the long-term cultivated assays (3.24%, 0.27 g/g DW, [Co-LT-HV, Co-ST-HV]; Fig. 10).

The protein fractions obtained from the samples Pu-LT-HV and Co-LT-HV were subjected to further amino acid analysis (cf. Table 4). As control fresh collected specimens were used for comparison. The most abundant amino acids in fresh collected *P. umbilicalis* (control) were glycine, arginine, leucine, valine and threonine, with concentrations ranging from 6.53-13.60 mg/g DW. In the protein fraction of the sample Pu-LT-HV this dominance shifted to the amino acids asparagine, serine, alanine and glutamine with values ranging from 10.81 to 23.69 mg/g DW.

In contrast, the amino acid profile of fresh collected specimens of *C. officinalis* was dominated by asparagine, serine, lysine, arginine and valine, ranging from 1.14-1.35 mg/g DW. The protein fraction of the sample Co-LT-HV showed the dominance of asparagine, serine, lysine and arginine with values ranging from 2.53 to 3.13 mg/g DW.

4.1.1.3 Product 3 – Lipid Contents and Fatty Acid Compositions

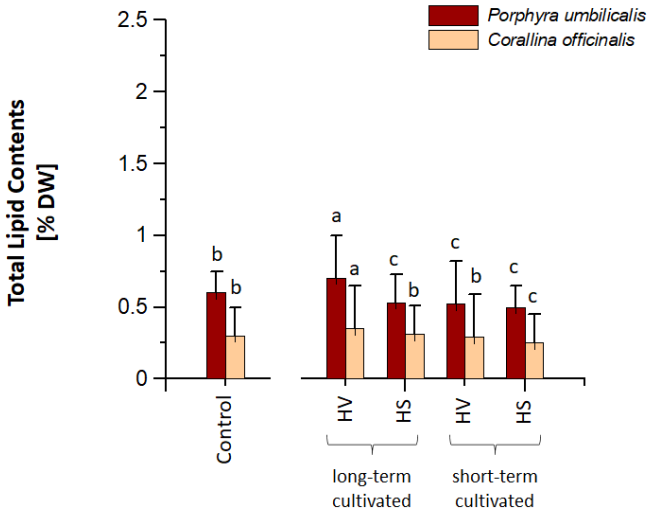

Figure 11. Total lipid content of the third product fraction obtained from tissue cultivated *P. umbilicalis* (Pu) and *C. officinalis* (Co) after long- (LT) and short-term (ST) exposure to high salinities (HS) and high velocities (HV). As control fresh collected specimens were used. Values correspond to the average of triplicates ± standard deviation. Letters indicate significant differences between assays ($p<0.05$).

The total lipid content of *P. umbilicalis* varied between 0.51 ± 0.15% and 0.73 ± 0.13% DW, being the highest in the in the long-term exposed high velocity assay [Pu-LT-HV], while contents measured for *C. officinalis* were almost 50% lower in all assays (0.25-0.35% DW; Fig. 11).

The lipid profile of *P. umbilicalis* was dominated by high values of polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA, 20:5n-3) and arachidonic acid (AA, 20:4n-6) in the control as well as experimental assay with concentrations ranging from 982.45-1397.06 µg/g DW and 1029.08-

2945.37 µg/g DW, respectively (Table 5). In contrast, the lipid profile of *C. officinalis* was dominated by unsaturated fatty acids such as palmitic acid (16:0, 81.23-238.51 µg/g DW) and saturated fatty acids such as myristic acid (14:0, 5.43-25.33 µg/g DW) and stearic acid (18:0, 10.22-22.61 µg/g DW). In addition, significant concentrations of docosadienoic acid (22:2n-6) and docosahexaenoic acid (DHA, 22:6n-3) were detected in the high velocity assay of *C. officinalis*, ranging from 13.61 to 17.24 µg/g DW (Table 5).

Table 5. Fatty acid profiles of the two selected lipid fractions of *P. umbilicalis* and *C. officinalis* obtained from the high-velocity assays (HV). As control fresh collected specimens were used.

	P. umbilicalis		***C. officinalis***	
Fatty acid ($\mu g \cdot g\ DW^{-1}$)	**Control**	**Pu-LT-HV**	**Control**	**Co-LT-HV**
13:0	n.d.	n.d.	0.22	1.36
14:0	68.05	72.11	5.43	25.33
15:0	n.d.	n.d.	2.54	10.06
16:0	108.43	211.05	81.23	238.51
16:1n-7	40.82	52.39	3.84	4.58
16:1n-9	54.41	50.06	n.d.	1.05
16:2n-6	n.d.	2.23	0.27	1.41
16:3n-3	n.d.	0.85	n.d.	n.d.
16:3n-4	n.d.	0.99	n.d.	n.d.
16:4n-3	n.d.	0.52	n.d.	n.d.
18:0	108.33	111.94	10.22	22.61
18:1n-7	258.41	369.35	n.d.	n.d.
18:1n-9	340.16	428.22	0.41	2.23
18:2n-6	349.05	402.31	0.67	2.19
18:3n-6	57.23	59.05	0.49	1.05
18:3n-3	27.54	34.89	n.d.	0.08
18:4n-3	55.68	63.15	n.d.	0.03
20:0	n.d.	n.d.	3.05	7.36
20:1n-9	n.d.	0.31	n.d.	n.d.
20:2n-6	176.83	231.13	n.d.	5.91
20:3n-6	381.90	394.52	n.d.	4.88
20:4n-3	53.92	55.79	n.d.	n.d.
20:4n-6	982.45	1397.06	n.d.	n.d.
20:5n-3	1029.08	2945.37	n.d.	n.d.
22:1n-7	n.d.	n.d.	n.d.	6.08
22:2n-6	n.d.	n.d.	0.34	13.61
22:5n-3	n.d.	0.11	n.d.	n.d.
22:6n-3	n.d.	n.d.	n.d.	17.24
Total	**4092.29**	**6883.4**	**108.19**	**364.21**

Abbreviations: n.d., not detected; Pu, *Porphyra umbilicalis*; Co, *Corallina officinalis*; LT, long-term exposed; HV, high velocities.

4.1.1.4 Product 4 – Carrageenan Yields and Structural Analysis

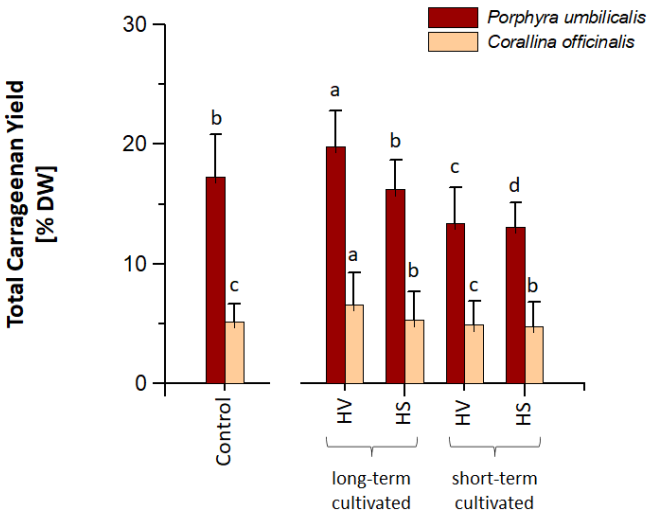

Figure 12. Total carrageenan yield in the fourth product fraction obtained from tissue cultivated *P. umbilicalis* (Pu) and *C. officinalis* (Co) after long- (LT) and short-term (ST) exposure to high salinities (HS) and high velocities (HV). As control fresh collected specimens were used. Values correspond to the average of triplicates ± standard deviation. Letters indicate significant differences between assays ($p<0.05$).

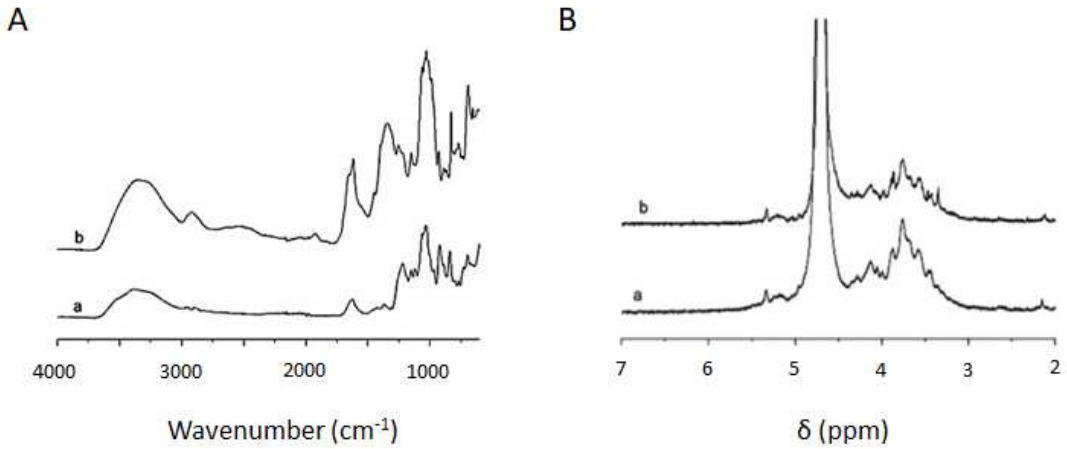

Figure 13. FTIR spectrum (A) of (a) the reference carrageenan and (b) the carrageenan-rich fraction extracted from *Porphyra umbilicalis* [Pu-LT-HV] and the ^{1}H NMR spectrum (B) of (a) the reference carrageenan and (b) the carrageenan-rich fraction.

The carrageenan yield of *P. umbilicalis* varied between 13.15 ± 2.01% and 19.82 ± 2.71% DW, being again the highest in the in the long-term exposed high velocity assay [Pu-LT-HV] (Fig. 12). In contrast, the carrageenan yield of *C. officinalis* ranged between 4.82 ± 1.52% and 6.65 ± 1.03% DW.

The FTIR spectrum depicted in Fig. 13A confirmed the presence of the characteristic peaks of carrageenan in the recovered fraction of sample Pu-LT-HV from *P. umbilicalis*. In addition, a small peak at 806 cm^{-1} was observable, which indicates the presence of ι-carrageenan. Furthermore, the FTIR spectrum also revealed that there is no peak present at ~1550 cm^{-1}, which would corresponding to

N−H bending indicating that no contaminant protein is left in the carrageenan fraction. In addition, no peak at ∼1700 cm^{-1} was observed in the FTIR spectrum for carrageenan, which indicates that there are no C-O groups in the carrageenan. This means that no uronic acid is present in this fraction. Based on FTIR analysis, the extracted carrageenan is a mixture of κ-, ι-, and λ-carrageenan.

The signals between 4.5 and 3.0 ppm in the NMR spectrum (Fig. 13B) are characteristic of the ring hydrogens of polysaccharides. The weak signals at 5.17 ppm and 5.33 ppm are characteristic of κ-carrageenan and ι-carrageenan, respectively. No peak for λ-carrageenan was observed probably due to the low resolution caused by the high viscosity of the sample.

4.1.1.5 Residual Biomass

Table 6. Results of the phytochemical screening of the two selected residual biomass fractions of *P. umbilicalis* and *C. officinalis* obtained from the high-velocity assays (HV). As control standard cultivated specimens were used (cf. 3.1).

	P. umbilicalis		***C. officinalis***	
Compound	**Control**	**Pu-LT-HV**	**Control**	**Co-LT-HV**
Aldehydes	+++	+	++	n.d.
Alkaloids	+++	++	+++	+
Flavonoids	+++	+++	+++	++
Glycosides	++	++	+	n.d.
Phenols	++++	+	++++	+
Phytosterols	++	n.d.	+	n.d.
Saponins	++	+	+++	+
Tannins	+++	++	+++	+

Abbreviations: n.d., not detected; Pu, *Porphyra umbilicalis*; Co, *Corallina officinalis*; LT, long-term exposed; HV, high velocities; +, weak colorimetric response; ++, medium colorimetric response; +++ strong colorimetric response; ++++ very strong colorimetric response.

The phytochemical screening of the residual biomass obtained after the sequential extraction procedure of tissue cultivated *P. umbilicalis* and *C. officinalis* considered overall eight compound groups (Table 6). While in the control assays (specimens cultivated under standard conditions and directly used for compound extraction) all compounds were present in fronds and tissues of both species, the colorimetric responses of samples from the residual biomass showed only the presence of seven in the case of *P. umbilicalis* and five compounds for *C. officinalis*. Out of these, only flavonoids gave a strong colorimetric response in the residual biomass of *P. umbilicalis* using the Alkaline Reagent Test (cf. Table 1), whereas for alkaloids, glycosides and tannins medium colorimetric responses were recorded. For aldehydes, phenols and saponins only weak colorimetric responses were found. In the high velocity assay of *C. officinalis* [Co-LT-HV], a medium colorimetric response was detected for flavonoids, whereas alkaloids, phenols, saponins and tannins were only present in minor concentrations (weak colorimetric response).

4.1.2 Activity Assays

Table 7. Results of the activity screenings of the sPS and carrageenan extracts (Products 1 and 4). As control standard cultivated specimens were used.

Sample	Antioxidant Capacities		MTT	AD	Enzyme Assays	
	DPPH radical [%[	Hydrogen peroxide [%[	Limit capacity of keratino-cytes to reduce MTT [%]	Reduction of lipid droplets [%]	Collagenase inhibition [%[	Elastase inhibition [%[
sPS extracts						
Pu-Control	70.49 ± 0.31^{d}	18.15 ± 0.05^{d}	n.e.	10.5 ± 1.3^{c}	15.23 ± 0.09^{d}	10.75 ± 0.54^{d}
Pu-LT-HV	83.80 ± 0.21^{b}	31.06 ± 0.23^{b}	n.e.	13.4 ± 1.0^{b}	30.16 ± 0.34^{b}	17.33 ± 2.01^{c}
Co-Control	73.09 ± 0.12^{c}	23.43 ± 0.18^{c}	n.e.	12.0 ± 1.0^{b}	26.07 ± 0.26^{c}	23.91 ± 1.65^{b}
Co-LT-HV	85.26 ± 0.40^{a}	34.55 ± 1.03^{a}	n.e.	22.3 ± 1.2^{a}	66.53 ± 2.52^{a}	69.71 ± 2.13^{a}
Carrageenan extracts						
Pu-Control	40.68 ± 0.29^{d}	9.27 ± 0.08^{d}	n.e.	7.1 ± 0.5^{b}	n.d.	n.d.
Pu-LT-HV	52.07 ± 0.13^{b}	26.33 ± 0.54^{b}	n.e.	9.2 ± 0.8^{b}	18.02 ± 0.07^{b}	12.53 ± 2.11^{b}
Co-Control	49.22 ± 0.32^{c}	12.73 ± 0.11^{c}	n.e.	10.9 ± 1.0^{a}	n.d.	n.d.
Co-LT-HV	57.14 ± 0.17^{a}	31.81 ± 1.23^{a}	n.e.	11.3 ± 0.7^{a}	22.09 ± 1.33^{a}	21.41 ± 2.05^{a}

Values correspond to the average of triplicates ± standard deviation. Percent calculated compared to untreated controls. Letters indicate significant differences between stress conditions ($p<0.05$). The final concentration of tested samples was 1 mg/ml. Abbreviations: n.e., no effect; n.d., not detected; Pu, *Porphyra umbilicalis*; Co, *Corallina officinalis*; LT, long-term exposed; HV, high velocities.

4.1.2.1 Antioxidant Capacities

In this study, there were two methods used to assess antioxidant activities, namely the DPPH free radical and the hydrogen peroxide scavenging assay. As depicted in Table 7, all extracts exhibited antioxidant activities, although to a varying degree, ranging from 9.27% and 40.68% to 34.55% and 85.26% [both Co-LT-HV] in the hydrogen peroxide and DPPH free radical assay, respectively.

4.1.2.2 MTT Assay and Adipocyte Differentiation (AD)

To investigate the cytotoxic effect of the selected extracts, human keratinocytes were treated with a concentration of 200 µg/mL of the tested extracts and cellular viability was assessed via MTT assay. Treatment for 48 hours did not affect the capacity of keratinocytes to reduce MTT (Table 7). As shown in Table 7, incubation of 3T3-L1 cells with 200 µg/ml of extracts significantly decreased the lipid droplets by 22.3% compared to the adipocyte control, suggesting that the sPS sample from *C. officinalis* [Co-LT-HV] can reduce adipogenesis in 3T3-L1 cells ($p < 0.05$).

4.1.2.3 Anti-Collagenase and Anti-Elastase Activities

The collagenase and elastase inhibition effects of all four sPS and carrageenan extracts at a final concentration of 1 mg/ml were determined and elucidated as shown in Table 7. It was notable that the highest collagenase inhibitory effect was exhibited by the Co-LT-HV extract (66.53 ± 2.52) and it also showed the highest effect in the anti-elastase activity (69.71 ± 2.13).

4.2 Integrating new Components into the Solid Skin Care Product Series

Based on the results presented in 4.1, in particular the activities given in 4.1.2, selected fractions (extracts) of *P. umbilicalis* and *C. officinalis* were integrated into the solid skin care products, thereby replacing or in some cases reducing the concentrations of standard components in the receipes. Specifically, the sPS and carrageenan extracts from the samples Pu-LT-HV and Co-LT-HV as well as lipid and hydrolysed protein fractions from *P. umbilicalis* [Pu-LT-HV] were utilized. In the following the final receipes of the products are presented.

4.2.1 Moisturising Lotion Bar

- 50 g raw organic shea butter
- 10 g unrefined coconut oil
- 3 mL hydrolysed protein fraction from *P. umbilicalis*
- 3 mL concentrated lipid fraction of *P. umbilicalis*
- 5 mg olive wax
- 1 mL aqueous sPS extract Pu-LT-HV (1.5 mg/mL)
- 1 mL aqueous sPS extract Co-LT-HV (2.5 mg/mL)
- 200 µL of essential oils (100 µL chamomile, 75 µL geranium and 25 µL orange)

All ingredients, except the essential oils, lipid and sPS extracts, were slowly melted down in a sturdy glass bowl over a small pan of boiling water. After melting, the bowl was removed from the heat and the sPS, lipid fraction and essential oils were added. The mixture was then poured into silicone molds and left to cool at room temperature. In this formula the olive oil was replaced by the lipid fraction obtained from *P. umbilicalis* and beeswax with olive wax. In addition, the unrefined coconut oil was partially replaced by the hydrolysed protein fraction and the concentrated lipid fraction.

4.3.2 Anti-Inflammatory Lotion Bar

- 70 g olive wax pastilles
- 20 g unrefined cocoa butter
- 5 g virgin coconut oil
- 1.5 mL carrageenan extract from *P. umbilicalis*
- 1.5 mL carrageenan extract from *C. officinalis*
- 15 mL concentrated lipid fraction of *P. umbilicalis*
- 20 mg dried calendula flowers
- 30 mg dried rose petals
- 30 mg dried lavender buds
- 2 mL aqueous sPS extract Co-LT-HV (1 mg/mL)
- 200 µL essential oils (lavender, geranium)

Calendula, rose petals, lavender, cocoa butter, and coconut oil were placed together into a small pan and heated (medium heat). The temperature was kept at around 75°C for 5 h. Next, the mixture was strained out through a nut milk bag into a clean saucepan. Then olive wax was added and warmed

over medium heat until the wax was melted. The mixture was allowed to cool briefly before the lipid fraction, carrageenan and sPS extracts as well as essential oils were added and mixed. After pouring the mixture into silicone molds the lotion bars were left to cool and harden at room temperature. Also, in this formula the olive oil was replaced by the lipid fraction obtained from *P. umbilicalis*. In addition, the unrefined coconut oil was partially replaced by the carrageenan extracts, the hydrolysed protein fraction and the concentrated lipid fraction.

4.3.3 Anti-Aging Lotion Bar

- 70 g raw organic shea butter
- 28 g olive wax pellets
- 112 g Macadamia nut seed oil
- 42 g cocoa butter
- 3 mL concentrated lipid fraction of *P. umbilicalis*
- 3 mL Sea Buckthorn Oil
- 3 mL aqueous sPS extract Co-LT-HV (1.5 mg/mL)
- 15 drops essential oil

All ingredients, except for the Sea Buckthorn oil, sPS and the essential oils, were slowly melted down in a sturdy glass bowl over a small pan of boiling water. As soon as the wax, oils and butters were melted, the bowl was removed from the heat and the concentrated lipid fraction, Sea Buckthorn oil, sPS and the essential oils were quickly added and mixed. The mixture was poured into silicone molds the lotion bars were left to cool and harden at room temperature.

5 Discussion

The application of algae, based on their valuable bioactive chemical constituents, has gained considerable attention in recent years, with applications in areas such as food, medicine, cosmetics, aquaculture and horticulture. Basically, the abundance and diversity of these valuable bioactive compounds within a seaweed species is determined by their geographical distribution (Hagen Rødde et al. 2004, Takeshi et al. 2005, Young et al. 2013), seasonal patterns (Hagen Rødde et al. 2004, Afonso et al. 2021, Cavaco et al. 2021, Kumar et al 2021) and processing methodologies (Nisizawa et al. 1987). Furthermore, there have been increasing efforts to find novel, biologically active compounds from red seaweeds with cost-efficient and economically viable nutraceutical, cosmeceutical and pharmaceutical applicability (Cotas et al. 2020, Freitas et al. 2022).

In the present study, two Icelandic seaweed rhodophytes, *Porphyra umbilicalis* and *Corallina officinalis*, were grown in tissue (frond) cultures under controlled conditions. While the growth of *C. officinalis* fronds was rather slow and still needs optimizations regarding the optimum culture conditions, the tissue cultivation of *P. umbilicalis* was a success, pointing to future possibilities for large-scale tank and photobioreactor applications. The biomass produced was used as a feedstock to develop a valorisation strategy to obtain valuable products using a cascading biorefinery approach by the assistance of green technologies such as enzyme-assisted extraction (EAE) and microwave-assisted extraction (MAE). These eco-friendly but energy intensive techniques led to high extraction yields that represent an advantage for sustainable development. However, one of the technological targets to consider in the future is to optimize conditions during for instance the EAE (i.e., testing different enzymes and biomass ratios) to extract higher quantity and quality of valuable products. The replacement of conventional solvents such as dichloromethane and methanol used in the lipid and fatty acid extractions in the present study by alternative solvents such as ionic liquids and dimethyl carbonate (DMC) as environmentally friendlier solvents should be also targeted in future approaches.

Although the focus was to obtain sulfated polysaccharides as the main product from *P. umbilicalis* and *C. officinalis*, our results contribute towards an integrated biorefinery approach for maximizing the whole biomass value. For instance, the global carrageenan industries process 202,500 dry tons of carrageenophytes annually to produce 65,000 tons of carrageenan (Bixler & Porse 2011), while the residual biomass is lost as waste. However, processing of this biomass using a biorefinery model can lead to the recovery of several products along with tons of carrageenan. In the present study, the application of the process developed by Peñuela et al. (2018) successfully recovered four products of commercial value i) a sulphated polysaccharide (sPS) and ii) protein containing fraction which were separated by precipitation, iii) a PUFAs fraction and iv) a carrageenan-rich fraction.

In our study, specifically, *P. umbilicalis* stood out in several analyses, presenting for instance the highest protein, lipid and carbohydrate contents when compared to *C. officinalis*. *Porphyra* is a Bangiophyceae and is an ancient red alga with a unique biochemical profile likely shaped by millions of years of thriving in environments that suffer daily fluctuations in temperature, salinity and irradiance. *P. umbilicalis* is well known for its nutritional value (e.g., Wells et al. 2017, Freitas et al. 2022), and the present study supports that fact. Coralline algae such as *C. officinalis* are particularly important from an ecological standpoint, known as ecosystem engineers, and their strong physical structure provides habitat and shelter for numerous forms of aquatic life (Nelson 2009, Williamson et

al. 2014). As stated, coralline algae are one of the most important vegetable sources of calcium and have the ability to accumulate carbonate salts from seawater (Freitas et al. 2022). For instance, *C. officinalis* extracts are utilized as skin's oil production regulator when applied on the skin (Pereira 2018). In fact, since several years this alga has been particularly used in the cosmetics industry although it is known that coralline algae are generally poor in the metabolites that commonly occur in other seaweed species (e.g., Aslam et al. 2010). Indeed, due to its anti-inflammatory, slimming, firming and moisturizing properties, and its content in minerals, it is often used as an active ingredient in many products like toners, moisturizers, cleansers, emulsions, astringents, eye creams, wash gels, shower gels, shave balms, hydration sprays and creams, slimming products, masks, etc (www.codif-recherche-et-nature.com). Therefore, the biorefinery process needs to be adapted in the future to target for instance photosynthetic pigments, bioactive compounds and minerals.

In seaweeds, the abundance and composition of carbohydrates varies across species; within the Rhodophyta floridean starch, cellulose, xylan and mannan are typically to find (Pereira 2018). The soluble fibre fraction is, in turn, rich in sulphur-containing galactans such as agar and carrageenan, both utilized in global food industries (Pereira 2017, 2018). Specifically, in the order Bangiales genus Porphyra/Pyropia, the sulphated polysaccharide porphyran is found. It is a highly substituted complex carbohydrate related to agarose with a linear backbone consisting of 3-linked β-D-galactosyl units alternating with either 4-linked α-L-galactosyl 6-sulfate or 3,6-anhydro-α-L-galactosyl units. The composition includes 6-O-sulfated L-galactose, 6-O-methylated D-galactose, L-galactose, 3,6-anhydro-L-galactose, 6-O-methyl D-galactose and ester sulfate. For porphyran there has been well-documented research on its bioactivity potential (such as anti-tumoral and anti-viral activity) and its possibilities as a promoter of human health (Pereira 2018). In the present study, the extracts from *C. officinalis* (Co-LT-HV) showed the highest antioxidant property in both tests conducted. Similar high activities were found by Costa et al. (2010) and Yang et al. (2011), while other authors reported that, for instance, the DPPH free-radical scavenging efficiency of the isolated crude polysaccharides obtained from *C. officinalis* had only a moderate impact on preventing the formation of these radicals (e.g., Ismail & Amer 2020). Yang et al. (2011) identified two sPS fractions rich in galactose and xylose in *C. officinalis*, whereas Ismail & Amer (2020) identified a signal which was assigned to H-1 of β-d-galactose linked to α-l-galactose-6-sulfate in the ^{1}H NMR analysis conducted on the sPS fraction of *C. officinalis* and concluded that the fraction is composed of carrageenan. Cases et al. (1994) characterized the structure of the main polysaccharides extracted from *C. officinalis* and named the sulfated xylogalactans they identified 'corallinan'. Beside others, the authors found that the backbone has an alternating →4)-α-1-Gal-(1→3)-β-d-Gal-(1→ structure and that the C6 position of 3-linked units is substituted mainly by β-d-xylosyl side stubs but also by sulfate ester groups and minor amounts of 4-*O*-methylgalactosyl side stubs (Cases et al. 1994, Navarro et al. 2008, Martone et al. 2010). Although there were no further structural analysis of the sPS conducted in the present study, the results of the monosaccharide composition analysis showed for *C. officinalis* samples that they were principally constituted of galactose, xylose and small amounts of glucuronic acid, while *P. umbilicalis* samples were mainly composed of galactose and lower amounts of mannose as well as small amounts (< 2.5% DW) of xylose, arabinose, galactose, glucose and rhamnose (cf. Table 3). Similar monosaccharide compositions as detected in the tested species in the present study were reported by Yang et al. (2011) (*C. officinalis*) and Wahlström et al. (2018) (*P. umbilicalis*) and it seems highly likely that the detected sPS in *C. officinalis* is in fact corallinan. But still the chosen method, missing for instance the detection of side stubs, seems to be insufficient for an impeccable identification of the polysaccharides from *C.*

officinalis and *P. umbilicalis*. Thus, further structural analysis of the sPS fractions will be necessary in the future.

The protein found in seaweeds is an excellent source of essential amino acids, which represent almost half of the total amino acids they accumulate (Freitas et al. 2022). Therefore, seaweeds are particularly interesting from a nutritional standpoint and can potentially minimize the issue of protein malnutrition in the human diet (Pereira 2018). Above this, protein derivatives are used in a variety of skin care formulations. Protein values can reportedly vary between taxa, although red seaweeds are known to have the highest protein content, which can reach up to 47% DW, of all phyla (Fleurence 1999); in turn, the lowest values are found for coralline species according to the literature (e.g., Freitas et al. 2022). In our study, the highest value obtained was 22.57% DW for *P. umbilicalis* but several factors play into calculating the metabolite content and diversity even within species, namely, geographic location, season, environment and even methodologies utilized (Patarra et al. 2011) as well, as in our case, culture and experimental conditions. For example, *P. umbilicalis* protein extracts with 22.6% yield were reported by Harrysson et al. (2018), while dry biomass had highly variable protein values among the literature considering field collected samples, with authors reporting 24.11% DW (Sánchez-Machado et al. 2004), 24.82% DW (Paiva et al. 2014), 25.80% DW (Patarra et al. 2011), 28.29% DW (Rupérez et al. 2001), 31.4% DW (all for *Porphyra* sp.) (Dawczynski et al. 2007), 40% DW (for *P. umbilicalis*) (Cofrades et al. 2010) and 44% DW (for *Porphyra* sp.) (Marsham et al. 2007). In this context, the values found for *P. umbilicalis* in the present study were low in comparison, but again, this can be explained not only by the stress conditions they were exposed to as well as adaptation to long-term culture conditions or seasonal circumstances (valid for the control specimens) but also by the different extraction methodologies used by each author.

In red seaweeds, lipid content is known to be low, with values that are generally nutritionally adequate for a healthy diet (Afonso et al. 2021), although they poorly contribute as energy providers (Lordan et al. 2011). Similar to what was observed for protein content, all *C. officinalis* showed lower values than *P. umbilicalis* in each of the assays analysed. Nevertheless, all values found were below 1.00% DW, thus being within the range of what is usually reported for red seaweeds (Dawczynski et al. 2007, Francavilla et al. 2013, Rodrigues et al. 2015, Gamero-Vega et al. 2020, Afonso et al. 2021, Carpena et al. 2021). In the present study, the lipid profile of *C. officinalis* was dominated by unsaturated fatty acids such as palmitic acid (16:0) and saturated fatty acids such as myristic acid (14:0) and stearic acid, whereas in *P. umbilicalis* eicosapentaenoic acid (EPA; 20:5(n-3)) and arachidonic acid (AA; 20:4(ω-6)) were the most common fatty acids. These results complied to a high degree with the works of Fleurence et al. (1994), Blouin et al. (2006), Paterra et al. (2013) and Shaltout & El-Din (2015). Basically, fatty acid profiles are influenced by environmental factors such as temperature, light, pH, salinity and nutrients (Aknin et al. 1990, Dembitsky et al. 1991), whereas for high velocities such an effect is not considered. The temperature is one of the most important environmental factors that influence algal growth rate, cell size, biochemical composition and nutrient requirements (Juneja et al. 2013). Kalacheva et al. (2002) stated that, lipid composition is one of the most commonly observed changes with temperature shift. Moreover, Thompson et al. (1992) reported that Myristic acids (14:0) increased with higher temperature while PUFA were consistently higher at lower temperature. The reported data for the low-temperature adapted *P. umbilicalis* in the present study followed in principle Thompson et al. (1992), where both analysed assays (Pu-LT-HV and the control) showed the highest PUFA concentrations (cf. Table 5). Another important factor is pH, since it determines the solubility and availability of CO_2 and essential nutrients and because it can have a significant impact

on algal metabolism (Chen & Durbin 1994). Alkaline pH increases the flexibility of the cell wall, resulting in an increase in triglyceride accumulation but a decrease in membrane-associated polar lipids (Guckert & Cooksey 1990). On the other hand, acidic conditions can alter nutrient uptake (Gensemer et al. 1993). Tatsuzawa et al. (1996) observed that acidic conditions cause an increase in saturated fatty acid content, which reduces membrane fluidity and inhibits high proton concentrations. The two latter effects are some of the problems which will *C. officinalis* and *P. umbilicalis* have to adapt to in the near future when ocean acidification rises due to elevated CO_2 concentrations (e.g., Yue et al. 2019). As much more important it will be that tissue-based tank and photobioreactor cultivation replaces wild harvests.

Marine algae are reported to produce a wide variety of bioactive secondary metabolites as antimicrobial, cytotoxic agents and the bioactive substances included alkaloids, polyketides, cyclic peptide, polysaccharide, phlorotannins, diterpenoids, sterols, quinones, lipids and glycerols (Cabrita et al. 2010). Marine seaweeds are even considered as the actual producers of some bioactive compounds with high activity (Shimizu 1996) and have thus the potential to serve as source of bioactive compounds for the pharmaceutical industry in drug development (Morsy et al. 2018). Specifically, algal phenolic compounds which are derived from polymerised phloroglucinol (1,3,5-trihydroxybenzene) units, represent important constituents considering their bioactive properties (Cotas et al. 2020). Despite their well-known antioxidant-promoting potential (Yuan et al. 2018, Kumar et al. 2020), seaweed phenolics are also linked to other protective bioactivities, such as anti-bacterial (Lavoie et al. 2019), anti-diabetic (Paudel et al. 2019), anti-inflammatory (Abdelhamid et al. 2018), anti-cancer and anti-proliferative (Wang et al. 2019) and neuroprotective (Seong et al. 2019) activities. Regarding phenolic compounds, there is a considerable number of studies focusing on seaweed total phenol content, nevertheless, data concerning *P. umbilicalis* and *C. officinalis* phenolic content are still scarce (Ferreira et al. 2021). In the present study the residual biomass of *C. officinalis* and *P. umbilicalis* after the extraction of the carrageenan as well as directly extracted long-term standard cultivated specimens (control) were screened for the presence of certain phytochemicals (cf. Table 1). Out of the eight tested compound groups, only flavonoids gave a strong colorimetric response in the residual biomass of *P. umbilicalis* [Pu-LT-HV] using the Alkaline Reagent Test, whereas for alkaloids, glycosides and tannins medium colorimetric responses were recorded. For aldehydes, phenols and saponins only weak colorimetric responses were found. In the high velocity assay of *C. officinalis* [Co-LT-HV], a medium colorimetric response was detected for flavonoids, whereas alkaloids, phenols, saponins and tannins were only present in minor concentrations (weak colorimetric response) (Table 6). Concerning flavonoids, several studies have focused on flavonoid content in terrestrial plants, due to their wide distribution, abundance and relevant antioxidant potential. However, few studies have focussed on the flavonoids content of seaweeds, and for red seaweeds, Monteiro et al. (2020) reported a low flavonoid content of 0.52 mg CE g^{-1} DW for *Gracilaria* sp. ethanolic extracts. In the present study, the Folin-Ciocalteau method was used to screen for the presence of phenols. It detects reducing compounds, however as phenolic compounds are the main constituents of plant extracts, the reducing power of plant extracts is an estimate of total phenolic compounds. Nevertheless, Folin-Ciocalteu also reacts with other reducing compounds, such as proteins, amino acids, thiols and vitamin derivatives (Everette et al. 2010) and can overestimate the phenolic compounds when used for its determination. Therefore, if mycosporine-like amino acids (MAAs) would have been present in these extracts as found in the study conducted by Ferreira et al. (2021), they may contribute to the overestimation of 'total phenols' measured by this method. Thus, as defended by many authors, Folin-Ciocalteu reagent

should be used as a measure of total reducing substances/antioxidant activity, in samples that unlike aromatic plants are not rich in polyphenols (Everette et al. 2010, Schouten et al. 2021, Ferreira et al. 2021). As the present study constitutes only a first screening of the residual biomasses without any quantification, structural analysis and bioactivity trials, further investigations are necessary to estimate and elucidate the compounds behind the detected colorimetric responses and determine their biological activities.

Although most of the valuable fractions obtained in the present study from *C. officinalis* and *P. umbilicalis* were successfully integrated into our solid skin care product series, it has to be questioned if these compounds are not better used as food ingredients due to recent geopolitical and environmental developments. Red seaweeds represent an economically valuable natural resource and their potential as food-grade feedstock cannot be ignored, particularly in the context of forecasted protein shortages owing the global population growth and the Russian war against the Ukraine. Climate change driven reduction of nutritional values in traditional crops (accelerated CO_2 levels resulting in shifts of the biochemical composition of certain agricultural plants, e.g., Dong et al. 2018, Dusenge et al. 2019) as well as harvest failures (e.g., due to droughts, heatwaves, floodings, storms, etc.) are increasing the shortage problem additionally and will not be resolved until the CO_2 problem is addressed.

6 Conclusions and Perspectives

The present study focussed on the use of a sequential multiproduct biorefinery, utilizing biomass from tissue cultivated northern Icelandic *C. officinalis* and *P. umbilicalis* after exposure to high velocities and salinities. By the use of tissue cultivation losses in the yield of valuable bioactive compounds due to alternating environmental conditions in wild harvested specimens can be omitted. While the growth of *C. officinalis* fronds was rather slow and still needs optimizations regarding the optimum culture conditions, the tissue cultivation of *P. umbilicalis* was a success, pointing to future possibilities for large-scale tank and photobioreactor applications. Thus, tissue cultivated seaweeds have the potential to supply environmentally sustainable bioactive and nutritional important compounds, ensuring that the United Nations Sustainable Development Goals and Paris Agreement are achieved, making them widely accessible to different layers of society.

Biorefinery approaches are necessary for solving economic and environmental drawbacks, enabling less residue production close to the much recommended zero waste system. The applied biorefinery approach used in the present study, resulted in the recovery of four products of commercial value i) a sulphated polysaccharide (sPS) and ii) protein containing fraction which were separated by precipitation, iii) a PUFAs fraction and iv) a carrageenan-rich fraction. However, one of the technological targets to consider in the future is to optimize conditions during for instance the enzyme-assisted extraction (i.e., testing different enzymes and biomass ratios) to extract higher quantity and quality of valuable products. Moreover, the replacement of conventional solvents such as dichloromethane and methanol used for instance in the lipid and fatty acid extractions by

alternative solvents such as ionic liquids and dimethyl carbonate (DMC) as environmentally friendlier solvents should be also targeted in future approaches.

P. umbilicalis high velocity assays stood out in several analyses, presenting for instance the highest protein, lipid and carbohydrate contents when compared to the ones of *C. officinalis*, but still *C. officinalis* sPS and carrageenan extracts showed the highest activities in the antioxidant and enzyme assays. While the carrageenan fraction of *P. umbilicalis* was successfully identified utilizing FTIR and the ^{1}H NMR spectroscopy, the results of the monosaccharide compositions of the sPS fractions were somewhat insufficient in identifying impeccable the polysaccharides from *C. officinalis* and *P. umbilicalis*. Thus, further structural analysis of the sPS fractions will be necessary in the future. Due to the low product yields in the samples from *C. officinalis* it would be reasonable to adapt the biorefinery process in the future to target for instance rather photosynthetic pigments, bioactive compounds and minerals. Finally, the residual biomass was tested in a phytochemical screening, showing that flavonoids gave a strong and medium colorimetric response in the Icelandic *P. umbilicalis* and *C. officinalis*, respectively. As the screening in the present study is only a first test of the residual biomasses without any quantification structural analysis and bioactivity trials, further investigations are necessary to estimate and elucidate the compounds behind the detected colorimetric responses and determine their biological activities.

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