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Process development for functional food ingredients with gut health benefits from the brown seaweed *Ecklonia radiata*

by

Suvimol Charoensiddhi

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LIST OF ABBREVIATIONS

AOC	Annual operating cost
C. coccoides	*Clostridium coccoides*
C. leptum	*Clostridium leptum*
CF	Crude extract fraction
CFC	Contractor's fee and contingency
DFC	Direct fixed capital
E. coli	*Escherichia coli*
E. radiata	*Ecklonia radiata*
EAE	Enzyme-assisted extraction
F. prausnitzii	*Faecalibacterium prausnitzii*
FF	Fibre-enriched fraction
FJB	Fruit juice-based beverage
FRAP	Ferric reducing ability of plasma
FT-IR	Fourier transform infrared spectroscopy
Fuc	Fucose
Gal	Galactose
GC	Gas chomatography
Glc	Glucose
GlcA	Glucuronic acid
GulA	Guluronic acid
HPF	High MW polysaccharide-enriched fraction
HPGPC	High performance gel permeation chomatography
HPLC	High-performance liquid chomatography
HPPF	High MW polysaccharide and phlorotannin-enriched fraction
HPSEC	High-performance size-exclusion chomatography
ICP-OES	Inductively coupled plasma optical emission spectroscopy
LPF	Low MW polysaccharide-enriched fraction
LPPF	Low MW polysaccharide and phlorotannin-enriched fraction
MAE	Microwave-assisted extraction
Man	Mannose
ManA	Mannuronic acid
MW	Molecular weight
NMR	Nuclear magnetic resonance spectroscopy
NNSP	Non-digestible non-starch polysaccharide
NPV	Net present value
ORAC	Oxygen radical absorbance capacity

PGE	Phloroglucinol equivalents
PF	Phlorotannin-enriched fraction (Chapter 5 and 7)
	Polysaccharide fraction (Chapter 6)
Q-PCR	Quantitative real-time polymerase chain reaction
R. bromii	*Ruminococcus bromii*
ROI	Return on investment
SCFA	Short chain fatty acids
SD	Standard deviation
SEM	Standard error of the mean
TCI	Total capital investment
TE	Trolox equivalents
TPC	Total phlorotannin content
TPDC	Total plant direct costs
TPIC	Total plant indirect costs
WS	Whole seaweed
Xyl	Xylose

EXECUTIVE SUMMARY

South Australia has among the world's highest diversity of seaweeds, with up to 1,500 described species, of which approximately 62% are endemic to the region. There is a growing recognition that seaweeds are important sources of bioactive compounds with a variety of biological activities. The aim of this study was to develop efficient seaweed processing technology and assess the potential of using South Australian brown seaweed as higher-value functional foods and nutraceuticals. Enzyme- and microwave-assisted processing techniques were developed to assist with the extraction of bioactive compounds from seaweed, as conventional techniques are impeded by the high degree of structural complexity in seaweed cell walls. In this study, *Ecklonia radiata* was selected as the experimental species due to its abundance in the region and possessing chemical constituents of commercial interest.

Using microwave-assisted enzymatic extraction, phlorotannin content and total antioxidant activity of the extracts were increased by approximately 23% and 30%, respectively, compared with the conventional acidic extraction. These phenolic compounds are of interest as their functional properties have been widely demonstrated.

Polysaccharides (alginate, fucoidan, and laminarin) represent the major functional components in this brown seaweed, which may provide health benefits to humans though a prebiotic effect. The critical processing parameters (enzyme, pH, and buffer) for the oligo- and polysaccharide production were investigated. Enzyme type and pH had minor impacts on the total sugar yield, but each affected the sugar composition and MW profile of the carbohydrate extracts differently. Acidic extraction yielded lower MW components compared to neutral and alkali extraction, while the inclusion of hydrolytic enzymes further reduced the MW of the extracted polysaccharides by 20–50%, compared with extraction using pH-adjusted water-only. High concentrations of buffer salts were found to inhibit polysaccharide extraction.

The prebiotic effects of the seaweed extracts were enhanced when the enzyme-assisted extraction was used. When added to an *in vitro* anaerobic fermentation system containing human faecal inocula, the extracts underwent fermentation and stimulated the production of SCFA. Furthermore, the extracts demonstrated the capacity to promote the growth of beneficial microbes. The key potential fermentable components were further fractionated in order to investigate their specific prebiotic potential. The high MW polysaccharide-enriched fraction showed greater potential for improving gut health as this fraction was not digestible by enzymes present in the small intestine, and induced significantly higher butyric acid production compared with the positive control, inulin. During *in vivo* studies, rats were fed with a polysaccharide-supplemented diet, raw seaweed-supplemented diet, and basal feed control diet. Significant improvements in cecum digesta weight, total SCFA, and the abundance of the key butyrate producer *F. prausnitzii*, and a decrease in

potentially toxic phenol and *p*-cresol were observed for rats fed with the polysaccharide-supplemented diet.

Industrial process modelling and economic feasibility analyses were performed to assess the commercial feasibility and profitability of four production processes. The results showed that the fractionation of seaweed crude extract and the production of value-added product improved the overall economic performance of crude extract production alone.

The key findings achieved from this work contribute to develop and expand new platform of seaweed utilisation for higher-value products, particularly to functional food and nutraceutical industries in order to serve the social demand for health awareness and support economic development.

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signed....Suvimol Charoensiddhi...

Date.......15th May 2017..

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1. INTRODUCTION

This introductory chapter introduces the research background of the development of seaweed-derived bioactive compounds for use as prebiotics and nutraceuticals using enzyme technologies. The prospects for using South Australia's harvestable beach-cast seaweed biomass for the production of functional food products is also discussed, and *Ecklonia radiata*, which is abundant in that resource, has been chemically analysed and assessed for use as a model species thoughout this PhD project.

A large proportion of this chapter will be submitted to "Trends in Food Science and Technology" for publication.

Author contributions: SC performed the literature search, data summary and analysis, and wrote all primary contents. WZ provided advice on research directions. WZ, MC, and CF provided advice on the review directions to improve the quality of the manuscript contents. All of the co-authors assisted with the revision of the manuscript prior to inclusion in the thesis and journal submission.

The development of seaweed-derived bioactive compounds for use as prebiotics and nutraceuticals using enzyme technologies

Suvimol Charoensiddhi [a,b], Michael A. Conlon [c], Chistopher M.M. Franco [a,b], Wei Zhang [a,b]

[a] Centre for Marine Bioproducts Development, School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia
[b] Department of Medical Biotechnology, School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia
[c] CSIRO Health and Biosecurity, Kintore Avenue, Adelaide, SA 5000, Australia

1.1 Abstract

Background: Seaweeds are a large and diverse group of photosynthetic macro-algae found across the world's oceans. There is a growing recognition that they are important sources of bioactive compounds with a variety of biological activities that could potentially contribute to functional food and nutraceutical industries.

Scope and approach: The complex structure and distinctive components of seaweed cell walls, which differ significantly from terrestrial plants, presents a major challenge for the effective extraction of bioactive compounds from inside the cells. Enzyme technologies have been used to improve the extraction, hydrolysis, and structure modification efficiently with a high degree of environmental sustainability. This review critically analysed the advances, challenges, and future directions in applying enzyme technologies to assist the extraction and processing of bioactive compounds from seaweeds and their potential applications in functional foods and nutraceuticals. In addition, a suitable source of seaweed biomass in South Australia was identified in order to develop an industry based on the extraction and application of bioactive compounds from seaweeds.

Key findings and conclusions: Compounds from seaweeds, in particular polysaccharides, phenolic compounds, and proteins, have been the subject of many studies. Different enzymatic processes have been demonstrated to (1) assist the extraction by breaking down the seaweed cell walls, and (2) degrade or hydrolyse macromolecules including polysaccharides and proteins. These enzymatic processes improve the yield and recovery of bioactive compounds and enhance their biological properties with regard to prebiotic, antioxidant, ACE inhibitory, anti-inflammatory, and antiviral effects. Seaweed-derived bioactive compounds from these processes present significant new opportunities in developing novel food applications. The current food regulations and safety requirements for seaweeds and their products are addressed for commercial product development. The brown seaweed *Ecklonia radiata* was selected as a case study in this project due to its abundance in the

South Australia region and its chemical constituents including bioactive compounds of commercial interest.

Key words Biological properties, Enzymatic process; Food safety; Functional food; Macroalgae; Prebiotic activity

1.2 Background

Marine macroalgae or seaweeds constitute approximately 25,000-30,000 species (Santos et al., 2015), with a great diversity of forms and sizes. They can be categorized into different taxonomic groups reflecting their pigmentation, including brown algae (Phaeophyceae), red algae (Rhodophyceae), and green algae (Chlorophyceae) (Mohamed et al., 2012). Seaweeds have fast growth rates and do not require arable land, fresh water, and fertilizer, so they become an appealing source for commercialisation (Lorbeer et al., 2013). The cultivation of seaweeds has been growing rapidly and is now practiced in about 50 countries, and 28.5 million tonnes of seaweeds and other algae were harvested in 2014 to be used for direct consumption, or as a starting material for the production of food, hydrocolloids, fertilizers, and other purposes (FAO, 2016). Recently, the annual global production of alginate, agar, and carrageenan which are the most important seaweed hydrocolloids for various applications across the food, pharmaceutical, and biotechnology industries has reached 100,000 tonnes and a gross market value just above USD 1.1 billion (Rhein-Knudsen et al., 2015).

Seaweeds also contain a great variety of structurally diverse bioactive metabolites not produced by terrestrial plants (Gupta and Abu-Ghannam, 2011b). Seaweeds are rich in carbohydrates, proteins, polyunsaturated fatty acids (PUFAs) including omega-3 fatty acids, and minerals as well as polyphenols, pigments (chlorophylls, fucoxanthins, phycobilins), and mycosporine-like amino acids (MAAs). These compounds possess various biological functions including antioxidant, anti-HIV, anticancer, antidiabetic, antimicrobial, anticoagulant, antivirus, anti-tumor, anti-inflammatory, immunomodulatory, prebiotic and cholesterol lowering effects (Holdt and Kraan, 2011). Although seaweed bioactive compounds are attractive for commercialisation in different functional food and nutraceutical products, the use of seaweed for this purpose is still not extensive.

The industrial use of enzymes to extract natural compounds from terrestrial plants for food and nutraceutical purposes has been developed and reported as a promising technology with a number of benefits such as saving process time and energy and improving the reproducible extraction process at the commercial scale (Puri et al. 2012). However, the efficiency of enzymatic extraction procedures for the retrieval of active compounds from seaweeds may be inhibited by the more complex and heterogeneous structure and composition of seaweed cell walls in comparison to plants, which have a cell wall mostly consisting of cellulose and hemicellulose.

The main cell wall and storage polysaccharides of seaweeds vary with taxonomy. The structural polysaccharides of green seaweeds are sulphated polysaccharides, such as ulvans and sulphated galactans, xylans, and mannans, while the main storage polysaccharide is starch. Brown seaweeds contain laminarins as the storage polysaccharide, and the main cell walls are composed of alginic acids, fucoidans, and sargassans. On the other hand, red seaweed cell walls consist of agars, carrageenans, xylans, water-soluble sulphated galactans, and porphyrins (mucopolysaccharides), and the main storage polysaccharide is floridean starch (amylopectin-like glucan) (Kraan, 2012; Mišurcová, 2012). This is further complicated by a tightly-integrated network of biopolymers in seaweed cell walls, mainly polysaccharides, that are associated with proteins, proteoglycans, polymeric phenols, and various bound ions such as calcium and potassium (Jeon et al., 2012; Synytsya et al., 2015). An example of the structure model of a brown seaweed cell wall is shown in Fig. 1.1. In addition, marine algae have adapted to salty environments, unlike land plants. Charoensiddhi et al. (2016b) reported that salt buffers significantly reduced the extraction efficiency of carbohydrates from brown seaweed, compared with pure water. This may be due to the ability of pure water to cause an osmotic shock and the rapid influx of water into seaweed cells, which disrupts the structural integrity of the cell wall and facilitates extraction. The high salt content in seaweeds may inhibit enzyme-assisted extraction processes, particularly those requiring buffer systems.

This review aims to critically analyse the potential role of enzyme technology in assisting with the extraction and digestion of bioactive compounds from seaweeds, and to understand the advantages, limitations, challenges, and future development directions in the application of the seaweed-derived ingredients in functional food products and nutraceuticals. A potential source of seaweed biomass in South Australia was also identified for use as the experimental material for the further development of seaweed-derived bioactive ingredients thoughout this project.

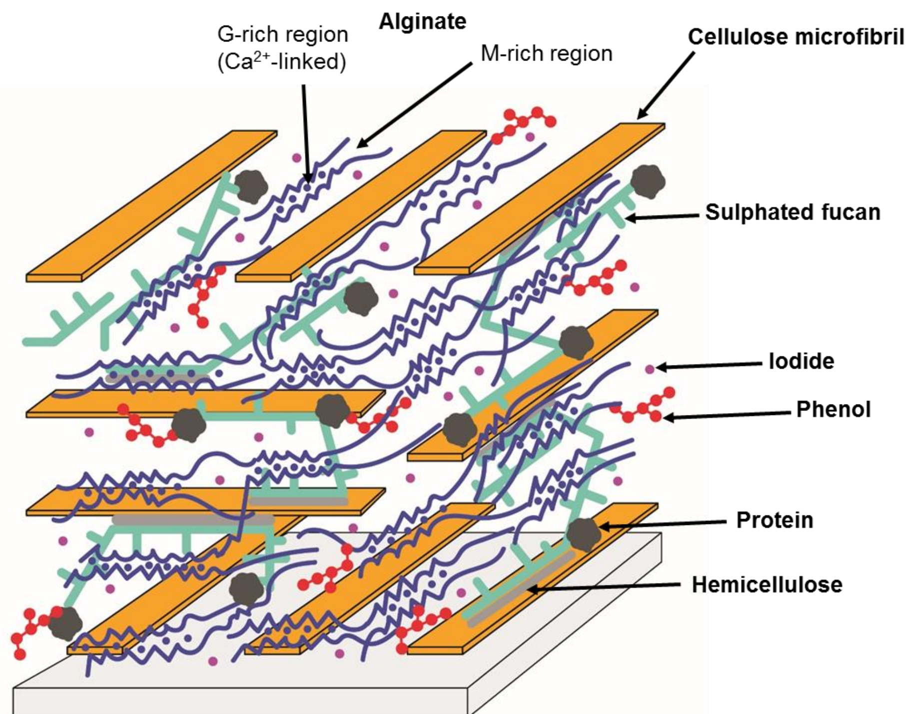

Figure 1.1 Structure model of the brown seaweed cell wall; some sulphated fucans are tightly associated with cellulose microfibrils (flat ribbon-like shape), and they are embedded within the alginate network. Hemicellulose components (short chain form) link with the cellulose by hydrophobic interactions and connect with the sulphated fucans. Alginates and phenolic compounds are associated and can form high molecular weight complexes. Proteins are linked with sulphated fucans and covalently attached to phenolics. (adapted from Kloareg et al., 1988; Michel et al., 2010; Deniaud-Bouët et al., 2014)

1.3 Seaweed bioactive compounds and their potential as functional foods and nutraceuticals

Carbohydrates account for the majority of seaweed biomass. Polysaccharides and oligosaccharides have therefore been the key focus of many studies looking at seaweed-derived compounds. Aside from those, phenolic compounds and proteins from seaweeds have also been widely studied as potential functional ingredients. Therefore, these thee classes will be the main focus of this review.

1.3.1 Polysaccharides

From an economic perspective, seaweed polysaccharides are the most important products produced from seaweeds (Michalak and Chojnacka, 2015). As the major components in seaweeds, polysaccharides account for up to 76% of the dry weight (DW) (Holdt and Kraan, 2011). Seaweeds contain a high total dietary fibre content: 10-75% for brown seaweed, 10-59% for red seaweed, and 29-67% for green seaweed. Seaweeds are particularly rich in soluble dietary fibre, which accounts

for 26-38%, 9-37%, and 17-24% in brown, red, and green seaweed, respectively (de Jesus Raposo et al., 2016). Most of these polysaccharides can be fermented by gut microbiota, which may provide a health benefit to humans though a prebiotic effect (O'Sullivan et al., 2010; Zaporozhets et al., 2014), which will be discussed in more detail later. Additionally, sulphated polysaccharides have shown anti-inflammatory, antioxidant, antibacterial, and immunological activity. These include fucoidans (L-fucose and sulphate ester groups) from brown seaweeds, carrageenans (sulphated galactans) from red seaweeds, and ulvans (sulphated glucuronoxylorhamnans) from green seaweeds (Synytsya et al., 2015).

1.3.2 Phenolic compounds

Phlorotannins are the major phenolic compounds found in brown seaweeds, constituting up to 14% of dry seaweed biomass with lower amounts being found in some red and green seaweeds (Holdt and Kraan, 2011; Machu et al., 2015). Phlorotannins are highly hydrophilic compounds formed by the polymerisation of phloroglucinol (1,3,5-trihydroxybenzene) monomer units with a wide range of molecular weights between 126 Da and 650 kDa. They can be categorized into four groups based on their linkages which are fuhalols and phlorethols (ether linkage), eckols (dibenzodioxin linkage), fucophloroethols (ether and phenyl linkage), and fucols (phenyl linkage) (Li et al., 2011). Phlorotannins have been explored as functional food ingredients with many biological activities such as antioxidant, anti-tumor, anti-inflammatory, antidiabetic, antihypertensive, and antiallergic activities (Freitas et al., 2015).

1.3.3 Proteins

Bioactive proteins and peptides from seaweeds have been demonstrated to have antioxidant, antihypertensive, and anticoagulant activities (Harnedy and FitzGerald, 2011). Generally, a higher content of proteins is found in red and green seaweeds (10-47% of DW) compared to brown seaweeds (3-15% of DW) (Wijesekara and Kim, 2015). Important bioactive proteins from red and green seaweeds include lectin and phycobiliprotein and bioactive peptides from brown seaweeds have also been reported with angiotensin-I-converting enzyme (ACE-I) inhibitory potential (Fitzgerald et al., 2011). Additionally, most seaweed species are a rich source of essential and acidic amino acids (Freitas et al., 2015).

1.4 Seaweed-derived components as prebiotic sources

Interest in prebiotics as functional foods has increased due to their recognised health benefits. The global prebiotics market size was over USD 2.9 billion in 2015, and continued growth is expected due to rising consumer awareness of gut health issues (Grand View Research, 2016). The term 'prebiotic' was introduced by Gibson et al. (2004, 2010), and defined as substrates that (1) are resistant to gastrointestinal digestion and absorption, (2) can be fermented by the microbes in the large intestine, and (3) selectively stimulate the growth and/or activity of the intestinal microbes leading to health benefits of the host.

Dietary habits influence the composition and metabolic activity of the human gut microbiota (Conlon and Bird, 2015; Flint et al., 2015). Several studies have demonstrated that a higher intake of plant dietary fibre affects the makeup of beneficial intestinal microbiota and metabolites, and inhibits the growth of potential pathogens compared with animal-dominated diets richer in fat and protein (Wu et al., 2011; Claesson et al., 2012; Zimmer et al., 2012; David et al., 2014). de Jesus Raposo et al. (2016) suggested that most seaweed polysaccharides such as alginates, fucoidans, laminarins, porphyrins, ulvans, and carrageenans can be regarded as dietary fibre, as they are resistant to digestion by enzymes present in the human gastrointestinal tract, and selectively stimulate the growth of beneficial gut bacteria. Likewise, ingested polyphenols with complex structures can also reach the large intestine where they can be converted into beneficial bioactive metabolites by microbes (Cardona et al., 2013), as shown in Fig. 1.2, and this has been shown to occur for phlorotannins from brown seaweed (Corona et al., 2016). The fermentation of seaweed components by beneficial bacteria has also been shown to generate beneficial metabolites such as short chain fatty acids (SCFA), particularly butyrate, acetate, and propionate (de Jesus Raposo et al., 2016). The beneficial effects of SCFA on host health include protection from obesity, chonic respiratory disease or asthma, cancer, and inflammatory bowel, as well as modulation of immunity, glucose homeostasis, lipid metabolism, and appetite regulation (Bultman, 2016; Dwivedi et al., 2016; Koh et al., 2016; Morrison et al., 2016).

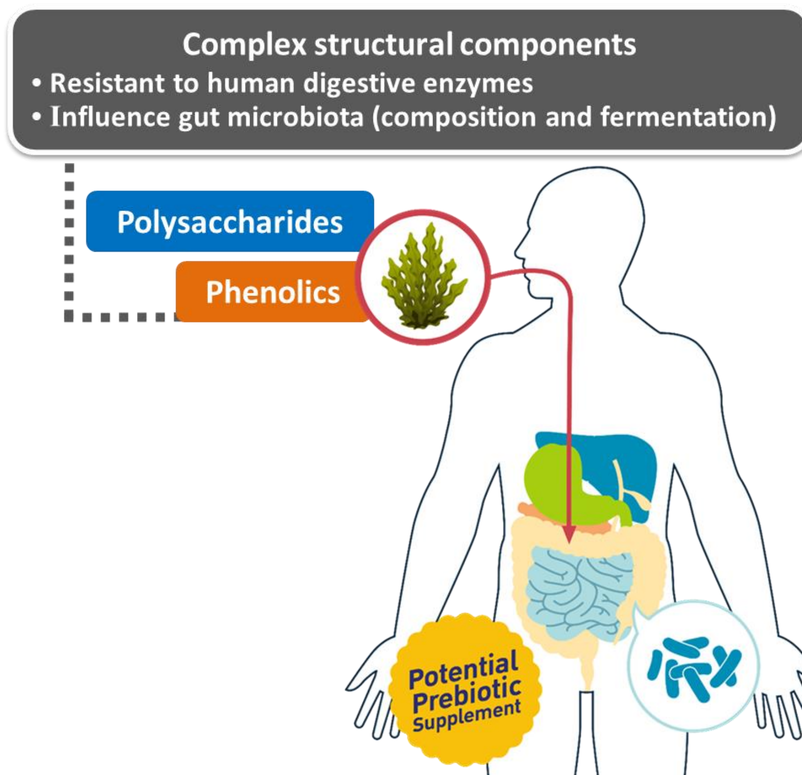

Figure 1.2 Seaweed components and their potential health benefits though prebiotic effects

1.4.1 *In vitro* studies

In vitro studies commonly use anaerobic human fecal fermentation for 24 h as a model for gut fermentation processes. Using this approach, Deville et al. (2007) reported that laminarin could stimulate the production of SCFA; especially butyrate and propionate. Low molecular weight (MW) polysaccharides from the red seaweed *Gelidium sesquipidale* caused a significant increase in populations of *Bifidobacterium*, as well as an increase in acetate and propionate (Ramnani et al., 2012). Rodrigues et al. (2016) obtained a similar result using an extract from the brown seaweed *Osmundea pinnatifida*. Charoensiddhi et al. (2016a, 2017) also demonstrated that extracts from the brown seaweed *Ecklonia radiata* stimulated the production of SCFA and the growth of beneficial microbes such as *Bifidobacterium* and *Lactobacillus*. In addition, Kuda et al. (2015) demonstrated that sodium alginate and laminaran from brown seaweeds inhibited the adhesion and invasion of pathogens (*Salmonella* Typhimurium, *Listeria monocytogenes*, and *Vibrio parahaemolyticus*) in human enterocyte-like HT-29-Luc cells. Also, the effect of intact brown seaweed *Ascophyllum nodosum* on piglet gut flora was studied by simulating small intestinal and caecal conditions, with antibacterial effects, especially on *E.coli*, and a decrease in fermentative activity being clearly observed (Dierick et al., 2010).

The digestibility of seaweed components can be determined *in vitro* to verify whether they are decomposed by human digestive enzymes, and thus their likelihood of reaching the large bowel and its resident microbiota. Neoagaro-oligosaccharides and glycerol galactoside from red seaweeds, for

instance, have shown that they were not digestible by enzymes typically present in the small intestine (Hu et al., 2006; Muraoka et al., 2008).

1.4.2 *In vivo* studies

Apart from *in vitro* studies, health benefits attributed to oligosaccharides and polysaccharides derived from seaweeds have been demonstrated by *in vivo* animal models. Most such studies have investigated prebiotic effects in rats or mice being fed a seaweed-supplemented diet. Results from Liu et al. (2015) showed an increase in the abundance of beneficial gut microbes such as *Bifidobacterium breve* and a decrease in pathogenic bacteria such as *Streptococcus pneumonia* and *Clostridium septicum* in rats supplemented with the red seaweed *Chondrus crispus*. Furthermore, an increase in SCFA production and colonic growth was observed, as well as an improvement of host immunity modulation though elevation of the plasma immunoglobulin levels. Supplementation of diet with the brown seaweeds *Undaria pinnatifida* and *Laminaria japonica* has resulted in suppressed weight gain of rats, influenced the composition of gut microbial communities associated with obesity by reduction in the ratio of *Firmicutes* to *Bacteroidetes*, and reduced populations of pathogenic bacteria including *Clostridium*, *Escherichia*, and *Enterobacter* genera (Kim et al., 2016). The oral administration of fucoidan extracts has also been shown to reduce the inflammatory pathology associated with DSS-induced colitis in mice, indicating its potential for treating inflammatory bowel disease (Lean et al., 2015). In addition, Kuda et al. (2005) reported that rats fed a diet containing laminaran and low MW alginate suppressed the production of indole, *p*-cresol, and sulphide which are the putative risk markers for colon cancer. Neoagaro-oligosaccharides derived from the hydrolysis of agarose by β-agarase resulted in an increase in the numbers of *Lactobacillus* and *Bifidobacterium* in the feces or cecal content of mice, along with a decrease in putrefactive bacteria (Hu et al., 2006).

1.5 Current enzymatic processing of seaweed-derived bioactive compounds

The selection of appropriate enzymatic processes to best suit the desired applications of the final products is a key factor to achieve an effective output. According to the present research in this area, two major enzyme-enhanced processes are normally implemented and designed in order to develop bioactive compounds from seaweeds. They are enzyme-assisted extraction and the enzymatic hydrolysis of macromolecules.

1.5.1 Enzyme-assisted extraction

The disintegration of cell wall structures is an elementary step to facilitate the extraction of bioactive components. Various commercial enzymes, not specific to seaweeds, have been used on seaweed cell walls for the extraction of bioactive compounds from seaweeds. For instance, polysaccharides such as fucoidans are tightly associated with cellulose and proteins (Deniaud-Bouët et al., 2014),

which limits their extractability from brown seaweeds. Therefore, hydrolysis of the cellulose and protein network by commercially available cellulases and proteases may enable the disintegration of the cell wall complex, thus releasing the fucoidans (or other target components).

Enzymes trialled for this purpose to date include carbohydrate hydrolytic enzymes (AMG, Celluclast, Termamyl, Ultraflo, Viscozyme, etc.) and proteases (Alcalase, Flavourzyme, Kojizyme, Nutrease, Protamex, etc.) (Hardouin et al., 2014a). The results from Hardouin et al. (2014b) suggested that enzyme-assisted extraction may be a promising technique for the recovery of neutral sugars, proteins, sulphate groups, and uronic acids from green (*Ulva* sp.), red (*Solieria chordalis*), and brown (*Sargassum muticum*) seaweeds. The extract obtained from red seaweed using carbohydrase also showed antiviral activity. Their approach appeared to have several advantages over conventional extraction methods, including a high extraction rate, no special solvent requirements, and savings in time and costs. Similar results were observed by Lee et al. (2010b). Extracts from the brown seaweed *Ecklonia cava*, obtained using enzyme-assisted extraction with the commercial carbohydrase Celluclast, showed potential for use as a therapeutic agent for diabetes to reduce the damage caused by hyperglycaemia-induced oxidative stress, with several potential benefits for commercialisation including its water solubility, multiple biological activities, high extraction efficiency, and non-toxicity.

1.5.2 Enzymatic hydrolysis of macromolecules

The principle motives for the structural modification of seaweed components are the enhancement of their biological activities to produce novel bioactive compounds, as well as their structural determination. Specific enzymes are generally employed in order to (1) degrade or hydrolyse high MW polysaccharides and proteins; or (2) act specifically on target molecules to modify the structure of specific functional groups.

de Borba Gurpilhares et al. (2016) reported that hydrolases or lyases (alginate lyase, fucoidanase, agarase, carrageenase, etc.) and sulfotransferases or sulfatases are important seaweed-specific enzymes for preparing tailored oligosaccharides and deducing the native structure of sulphated polysaccharides. The study of Sun et al. (2014) showed that a novel *κ*-carrageenase isolated from *Pedobacter hainanensis* offered an alternative approach to prepare *κ*-carrageenan oligosaccharides by cleaving the internal β-1,4 linkage of *κ*-carrageenan. The carrageenan oligosaccharides produced were reported as potential pharmaceutical products with various biological properties including anti-inflammatory, anticoagulant, antioxidant, and antiviral activities. In addition, alginate modifying enzymes (e.g. alginate acetylases, alginate deacetylases, alginate lyases, and mannuronan C-5-epimerases) have also been used for modification of M- and G-block and quantification purposes, for instance (Ertesvåg, 2015).

Food grade hydrolytic enzymes, which are capable of catalyzing seaweed-specific structures, remain uncommon in the commercial market at present. The development of more specific polysaccharide hydrolytic enzymes is required for digesting the parent backbone of seaweeds and to aid in the production of lower MW oligosaccharides with a variety of biological activities (Vavilala and D'Souza, 2015). Most applications of enzymatic modification are currently focused on polysaccharide degradation, but other types of enzymes such as transferase and synthase should be further developed in order to fulfill the application of enzyme technology for this purpose (Li et al., 2016b).

1.5.3 The effects of using enzymes for the extraction and hydrolysis of bioactive products from seaweed

Recent publications related to the use of enzyme-assisted extraction and enzymatic hydrolysis on seaweed substrates are summarised in Tables 1.1 and 1.2. The benefits of using commercially-available enzymes (Table 1.1) and seaweed-specific enzymes (Table 1.2) on the processes, as well as on the biological activities of the seaweed extracts or hydrolysates, are also presented in order to evaluate their potential for use as functional and nutraceutical sources.

Table 1.1 Studies reporting the use of *commercially-available enzymes* in the development of seaweed bioactive compounds;

B, R, and G denote brown, red, and green seaweed, respectively. ↑ and ↓ means increase and decrease, respectively, compared to the control.

Substrates	Enzymes	Processes	Active components or activities	Advantages	Reference
Enzyme-assisted extraction					
Ecklonia radiata (B)	Celluclast (Novozyme)	E/S ratio 100 µL: 1 g, water, pH 4.5, 50°C, 24 h	Prebiotic	***Improved biological properties*** ↑ Total SCFA and butyric acid production ↑ *Bifidobacterium* and *Lactobacillus* (*In vitro* human fecal fermentation 24 h)	Charoensiddhi et al. (2016a)
Ulva armoricana (G)	- Endo-protease - Multiple-mix of glycosyl-hydrolases - Exo-β-1,3(4)-glucanase (Novozymes)	E/S ratio 60 mg: 1 g, water, pH 5.9-6.2, 50°C, 3 h	Antiviral Antioxidant	***High yield, improved biological properties*** ↑ Yield; Endo-protease (2-fold) ↑ Organic matter, neutral sugar, and protein; All enzymatic extracts (up to 2-fold, 2.7-fold, and 1.75-fold, respectively) ↑ Activities against *Herpes simplex* virus type-1 - At EC_{50}; Multiple-mix of glycosyl-hydrolases (373 µg/mL) and Exo-β-1,3(4)-glucanase (321µg/mL), Control (>500 µg/mL) ↑ Free radical scavenging capacity - At IC_{50}; Endo-protease (1.8 and 12.5 mg/mL) and Multiple-mix of glycosyl-hydrolases (6 mg/mL), Control (>500 µg/mL)	Hardouin et al. (2016)
Lessonia nigrescens (B)	α-amylase (Sigma)	E/S ratio 100 mg: 1 g, 0.2 N phosphate buffer, pH 6, 60°C, 17 h	ACE inhibitor	***High yield, improved biological properties*** ↑ Yield (2.3-fold) ↑ ACE inhibition (1.04-fold) ↓ IC_{50} (4.8-fold)	Olivares-Molina and Fernández (2016)
Osmundea pinnatifida (R)	Viscozyme (Sigma-Aldrich)	E/S ratio 50 mg: 1 g, water, pH 4.5, 50°C, 24 h	Prebiotic	***Improved biological properties*** ↑ Total SCFA production ↑ *Bifidobacterium* spp. after 6 h fermentation (*In vitro* human fecal fermentation 24 h)	Rodrigues et al. (2016)

Substrates	Enzymes	Processes	Active components or activities	Advantages	Reference
Chondrus crispus (R) *Codium fragile* (G)	- Cellulase - β-glucanase - Ultraflo - Neutrase (Novozyme)	Enzyme 0.5%, water, 50°C, 3 h	Antiviral	***High yield, improved biological properties*** ↑ Yield (2-3-fold) ↑ Recovery of protein, neutral sugars, uronic acids, and sulphates; *C. crispus* (up to 3-4-fold) ↑ Activity against *Herpes simplex* virus Type 1 - EC_{50} at MOI 0.001 and 0.01 ID_{50}/cells; *C. fragile* (1.4-folds and 2.3-fold, respectively)	Kulsheshtha et al. (2015)
Osmundea pinnatifida (R) *Sargassum muticum* (B) *Codium tomentosum* (G)	- Alcalase - Flavourzyme - Cellulase - Viscozyme L (Sigma-Aldrich)	E/S ratio 50 mg: 1 g, water, pH 8 (Alcalase), pH 7 (Flavourzyme), and pH 4.5 (Cellulase, Viscozyme), 50°C, 24 h	Antioxidant Prebiotic α-Glucosidase Inhibitor	***High yield, improved biological properties*** ↑ Yield; *C. tomentosum* (up to 1.4-fold) ↑ Antioxidant (ABTS, DPPH, Hydroxyl radical Superoxide radical); *S. muticum* ↑ *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* BB-12; *S. muticum* and *C. tomentosum* (*In vitro* enumeration of viable cells) ↑ % Inhibition of α-glucosidase; *C. tomentosum* (1.4-fold)	Rodrigues et al. (2015)
Palmaria palmata (R)	Xylanase (Sigma-Aldrich)	E/S ratio 17.8 g/kg, 50 mM acetate buffer pH 5, 24°C, 320 min	R-phycoerythin	***High yield and selectivity*** ↑ Yield 62-fold ↑ Purity index up to 16-fold	Dumay et al. (2013)
Enteromorpha prolifera (G)	- Viscozyme L - Promozyme - Flavourzyme 500 MG - Protamex (Novozyme)	E/S ratio 20 mg: 1 g, Optimum pH and temperature, 8 h	Antioxidant Anti-acetylcholinesterase (AChE) Anti-inflammatory	***Reported biological properties*** - High antioxidant activity (DPPH, H_2O_2 scavenging, ferrous ion chelating, and reducing power); Viscozyme and Protamex extracts - High AChE inhibitory activities at 1 mg/mL; Promozyme extract (93.6%) and Flavourzyme extract (89.9%)	Ahn et al. (2012)

Substrates	Enzymes	Processes	Active components or activities	Advantages	Reference
Sargassum coreanum (B)	Neutrase 0.8 L (Novozyme)	E/S ratio 0.1 g: 1 g, 0.2 M phosphate buffer, pH 6, 50°C, 12 h	Anticancer	***Reported biological properties*** - DNA fragmentation, apoptoic body and cells; Crude polysaccharide >30 kDa fraction	Ko et al. (2012)
Ecklonia cava (B)	Celluclast (Novozyme)	E/S ratio 100 μL: 1 g, water, pH 4.5, 50°C, 24 h	Anti-diabetic	***Reported biological properties*** - IC_{50}: α-glucosidase 0.62 and α-amylase 0.59 mg/mL, Acarbose (0.68 and 0.71 mg/mL, respectively)	Lee et al. (2012b)
Ecklonia cava (B)	Celluclast (Novozyme)	E/S ratio 10 μL: 1 g, water, 50°C, 24 h	Fucoidan Anti-inflammatory	***High yield, reported biological properties*** ↑ Yield (1.3-fold) ↑ Total carbohydrate (1.4-fold) ↑ Sulphate content (1.7-fold) - Inhibit nitric oxide production from macrophages (RAW 264.7)	Lee et al. (2012a)
Caulerpa microphysa (G)	Pepsin (Sigma)	E/S ratio 100 mg: 1 g, 0.2 M phosphate buffer, pH 3, 37°C, 12 h	Angiotensin I-converting enzyme (ACE) inhibitor Anti-tumor	***Reported biological properties*** - ACE Inhibitory activity IC_{50} 0.2 mg/mL - Inhibit the growth of transplanted myelomonocytic leukaemia (WEHI-3) and human promyelocytic leukaemia (HL-60) cell lines - Increase DNA damage (concentration >100 μg/mL)	Lin et al. (2012)
Laminaria japonica (B)	- AMG 300L - Celluclast 1.5L FG - Dextrozyme - Alcalase 2.4L FG - Flavourzyme 500 MG - Protamex (Novozyme)	E/S ratio 20 mg: 1 g, Optimum pH and temperature, 8 h	Antioxidant Anti-Alzheimer's Anti-inflammatory	***Reported biological properties*** - High total phenolic; Alcalase extract (14.4 mg Gallic acid equivalent/g) - High total flavonoid; Celluclast extract (5.1 mg Quercetin equivalent/g) - High antioxidant activity (DPPH, H_2O_2 scavenging, metal chelating, and reducing power); Flavourzyme and Dextrozyme extracts - High AChE inhibitory activity at 0.25 mg/mL; Flavourzyme extract (90%) and Celluclast extract (60%) - High activity in nitrite scavenging; Protamex and AMG extracts	Sevevirathne et al. (2012)

Substrates	Enzymes	Processes	Active components or activities	Advantages	Reference
Ecklonia cava (B)	Kojizyme (Novozyme)	E/S ratio 100 µg: 1 g, water, pH 6, 40°C, 12 h	Immune regulation	***Reported biological properties*** Increase the production of IL-2 though the activation of NF-κB, and induce the proliferation of lymphocytes with the coordinated stimulation of IL-2	Ahn et al. (2011)
Ecklonia cava (B)	AMG 300 L (Novozyme)	E/S ratio 10 µL: 1 g, Optimum pH and temperature, 12 h	Anticoagulant Sulphated polysaccharides	***Improved biological properties*** ↑ Prolong bleeding time at dosage of 300 µg/kg (>1800 s), Control (900 s)	Wijesinghe et al. (2011)
Porphyra tenera (R)	- Viscozyme L - Maltogenase - Alcalase 2.4L FG - Flavourzyme 500 MG (Novozyme)	E/S ratio 20 mg: 1 g, Optimum pH and temperature, 8 h	Antioxidant Anti-Alzheimer's Anti-inflammatory	***Reported biological properties*** - High antioxidant activity (DPPH, H_2O_2 scavenging, ferrous ion chelating, and reducing power); Alcalase and Maltogenase extracts - High AChE inhibitory activities at 1 mg/mL; Flavourzyme extract (99.3%) and Viscozyme extract (82.7%)	Senevirathne et al. (2010)
Palmaria palmata (R)	Umamizyme (Amano Enzyme Inc.)	E/S ratio 50 mg: 1 g, water, pH 7, 50°C, 24 h	Antioxidant	***High yield, improved biological properties*** ↑ Yield (2-fold) ↑ Total phenolic (3-fold) ↑ Antiradical power (3.5-fold) ↑ ORAC value (4-fold)	Wang et al. (2010b)
Enzyme-assisted extraction intensified with other processes					
Grateloupia turuturu (R)	Mixture enzyme: Sumizyme TG (Shin Nihon Chemical), Sumizyme MC (Takabio), Multifect CX 15L (DuPont), Ultraflo XL (Novozymes)	**Ultrasound-assisted enzymatic extraction** E/S ratio 10 mg: 1 g, water, pH 5.5, 40°C, 6 h Sonitube® 35 kHz, 400 W	Carbohydrates Amino acids	***High yield*** ↑ Liquefaction (1.7-fold) ↑ Carbohydrates (3.3-fold) ↑ Amino acids (2.1-fold) ↑ Nitrogen (1.8-fold) ↑ Carbon (2.2-fold)	Le Guillard et al. (2016)

Substrates	Enzymes	Processes	Active components or activities	Advantages	Reference
Ecklonia radiata (B)	Viscozyme L (Novozyme)	**Microwave-assisted enzymatic extraction** E/S ratio 100 μL: 1 g, 0.1 M sodium acetate buffer, pH 4.5, 50°C, 30 min under open-vessel microwave intensification	Phlorotannins Antioxidant	***Improved extraction time, cost-savings, and extraction efficiency*** ↑ Phlorotannins (1.3-fold) ↑ Antioxidant FRAP and ORAC (1.4-fold) - Short extraction time 5-30 min	Charoensiddhi et al. (2015)
Gracilaria birdiae (R)	Proteolytic enzyme	**Ultrasound-assisted enzymatic extraction** 2 step: 0.1 M sodium hydroxide, 22°C; Sonication: 60°C, 30 min, 60 W Enzyme: pH 8.0, 60°C, 12 h	Anticoagulant Antioxidant Sulphated Polysaccharides	***High yield, improved biological properties*** ↑ Total yield (15.9-fold) ↑ Sulphated polysaccharide: sugar/sulphate (5.1), Control (8.5) ↑ Anticoagulant and antioxidant (~2-fold)	Fidelis et al. (2014)
- *Palmaria palmate* - *Porphyra umbilicalis* (R) - *Ulva rigida* (G) - *Laminaria ocholeuca* - *Undaria pinnatifida* (B)	Pancreatin, (Sigma-Aldrich)	**Ultrasound-assisted enzymatic extraction** E/S ratio 0.2 g: 1 g, 0.2 M dihydrogen phosphate/sodium hydroxide buffer, pH 8.0 under ultrasound irradiation 45 kHz, 50°C, 12 h	Iodine (minerals)	***High yield*** ↑ Large amount of extracted iodine 76-96% of total iodine.	Romaris-Hortas et al. (2013)

Substrates	Enzymes	Processes	Active components or activities	Advantages	Reference
Enzymatic hydrolysis of target molecules					
Saccharina longicruris (B) *(Protein extracts)*	Trypsin (Sigma-Aldrich)	E/S ratio 50 mg: 1 g, 20 mM phosphate buffer, pH 7, 30°C, 24 h	Antibacterial	***Selectivity, improved biological properties*** - Degree of hydrolysis (DH); Fraction >10 kDa (45.4% w/w) ↑ Antibacterial activity against *Staphylococcus aureus* ↓ Maximum specific growth rate at 2.5 mg/mL from 0.19 to 0.02 μ_{max}/h	Beaulieu et al. (2015)
Pyropia columbina (R) *(Protein extracts)*	- Alkaline protease (Danisco) - Flavourzyme (Sigma)	<u>*Simple hydrolysis (A)*</u> *Alkaline protease:* E/S ratio 4 mg: 1 g, water, pH 9.5, 55°C, 2 h <u>*Sequential hydrolysis (AF)*</u> Start with Alkaline protease hydrolysis 2 h, following to Flavourzyme hydrolysis E/S ratio 5 mg: 1 g, water, pH 7, 55°C, 4 h (total 6 h)	ACE inhibitor Antioxidant Antiplatelet aggregation	***Selectivity, improved biological properties*** - Peptide A (MW 2.3 kDa) Peptide AF (MW 2.3 kDa and 287 Da) Control (MW >175 kDa and 52.1 kDa) ↑ ACE inhibition IC_{50}; Peptide A (4.5-fold) ↑ Antioxidant ABTS and DPPH radical Inhibition IC_{50}; Peptide AF (3.7-fold and 2.1-fold, respectively) ↑ Copper-chelating activity and Antiplatelet aggregation; Peptide AF (4.2-fold and 3-fold, respectively)	Cian et al. (2015)
Pterocladia capillacea (R) *(Sulphated polysaccharide extracts)*	Viscozyme L (Sigma-Aldrich)	Enzyme 30 units, 0.1 M sodium acetate buffer, pH 4.5, 37°C, 30 min	Phenolics Antioxidant Antibacterial	***Selectivity, high yield, improved biological properties*** ↑ >50% of phenolic content and DPPH scavenging ↑ Anti-bacterial activity, Control = antibiotics (tetracycline and cefuroxime)	Fleita et al. (2015)

Substrates	Enzymes	Processes	Active components or activities	Advantages	Reference
Agarose (Sigma)	Celluclast 1.5L (Novozyme)	E/S ratio 20 mL: 1 g, 0.05 M acetate buffer, pH 5, 50°C, 24 h	Antioxidant	***Selectivity, reported biological properties*** - Hydrolysis yield 20.5% and low viscosity - High solubility up to 86.8% - High water and oil adsorption capacities (3.0 and 4.7 g/g, respectively) - High antioxidant activity (DPPH 24.6%, ABTS 83.8%, and FRAP 1.6)	Kang et al. (2014)
Porphyra columbina (R) *(Residue cake)*	- Flavourzyme (Sigma) - Fungal protease (Genencor)	*Subsequent hydrolysis* *Protease:* E/S ratio 50 mg: 1 g, pH 4.3, 55°C, 3 h *Flavourzyme:* E/S ratio 20 mg: 1 g, pH 7, 55°C, 4 h	ACE inhibitor Antioxidant	***Selectivity, Improved functional properties*** ↓ MW of peptides (Asp, Glu, Ala, Leu) ↑ Protein solubility - ACE inhibition ~45% - IC_{50}: ABTS 1.01 and DPPH 0.91 g/L - High copper chelating activity ~97.5%	Cian et al. (2013)
Palmaria palmata (R) *(Protein extracts)*	- Corolase PP (AB Enzymes) - Alcalase 2.4L (Novozyme)	E/S ratio 10 µL or mg: 1 g, water, pH 7, 50°C, 4 h	Anti-diabetic Antioxidant ACE inhibitor	***Selectivity, improved biological properties*** ↑ Amino acid concentration; Alcalase (3-fold) and Corolase (5-fold), Low MW peptide ↑ ACE and DPP IV inhibitory activity: IC_{50}; Alcalase (0.2 and 2.5 mg/mL, respectively) and Corolase (0.3 and 1.7 mg/mL, respectively), Control (>2 and >5 mg/mL, respectively) ↑ ~50% Renin inhibition; Corolase ↑ Antioxidant FRAP and ORAC; Alcalase (8-9-fold) and Corolase (10-13-fold)	Harnedy and FitzGerald (2013)
Enzymatic hydrolysis of target molecules, intensified with other processes					
Undaria pinnatifida sporophylls (B) *(Crude fucoidan)*	Tunicase (β-glucanase) (Daiwakasei)	**Ultra high pressure (UHP)-assisted enzymatic extraction** Enzyme 60 mg: 1g, pH 8, 40°C, 24 h in UHP unit 100 MPa	Anticoagulant	***Selectivity, Improved biological properties*** ↓ Average MW 687 kDa, Control 877 kDa ↑ Anticoagulant activity, APTT (1.3-fold) and TT (1.6-fold)	Park et al. (2012)

Substrates	Enzymes	Processes	Active components or activities	Advantages	Reference
Pyropia yezoensis (R)	AMG (Novozyme)	**Microwave-assisted enzymatic extraction** E/S ratio 1:10 pH 4.5, 60°C, 2 h under microwave intensification power 400 W	Antioxidant	***Selectivity, improved biological properties*** ↑ Degree of hydrolysis (DH 25%) ↑ Antioxidant IC_{50}; Alkyl radical (196 μg/mL) and H_2O_2 (96 μg/mL), Control (554 and >180 μg/mL, respectively)	Lee et al. (2016)

*E/S: Enzyme and substrate ratio; EC_{50}: Half-maximal effective concentration; IC_{50}: Half-maximal inhibitory concentration; MOI: Multiplicity of infection ID_{50}/cells, ratio (virus titer)/(cell number/mL); ABTS: 2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; DPPH: 2,2-diphenyl-1-picrylhydrazyl; FRAP: Ferric reducing antioxidant power; DPP: Dipeptidyl peptidase; ORAC: Oxygen radical antioxidant capacity; APTT: Activated partial thomboplastin time; TT: Thombin time

Table 1.2 Studies reporting the use of *seaweed-specific enzymes* in the development of seaweed bioactive compounds;

B, R, and G denote brown, red, and green seaweed, respectively. ↑ and ↓ means increase and decrease compared to control.

Substrates	Enzymes	Processes	Active components or activities	Advantages	Reference
Enzymatic hydrolysis of target molecules					
Ulva sp. (G) *(Ulvan extract)*	β-lyase (*Alteromonas* species)	0.01 mg/mL of crude enzyme mix, pH 6-8, 20-35°C, 0-50 h	Monosaccharides Oligosaccharides	***Selectivity*** ↓ Ulvan MW to 5 kDa, Control 674 kDa	Coste et al. (2015)
Gracilaria sp. (R) *Monostroma nitidum* (G) *(Polysaccharide extract)*	Agarase AS-II (*Aeromonas salmonicida* MAEF 108)	Appropriate amount of agarase, water, 40°C, 2 h	Prebiotic Oligosaccharides Antiviral	***Selectivity, improved biological properties*** ↑ >3 log cfu/mL *Bifidobacterium pseudolongum* BCRC 14673; *Gracilaria* oligosaccharide - Exhibit the decrease of Japanese encephalitis virus (JEV, Beijing-1 strain) infection	Wu et al. (2012)
Gelidium sp. *Gracilaria* sp. *Porphyra dentate* (R) *Monostroma nitidum* (G) *(Polysaccharide extract)*	Agarases (*Aeromonas salmonicida* MAEF108 and *Pseudomonas vesicularis* MA103)	E/S ratio 250 or 500 AU: 0.5 g, buffer solution (0.5 M tris-HCl buffer (pH 6.2), 1.0 M NaCl, 0.1 M $CaCl_2$), 40°C, 24 h	Prebiotic Oligosaccharides	***Selectivity, improved biological properties*** ↑ *E. faecalis* BCRC13076 and *L. rhamnosus* BCRC14068 (*In vitro* enumeration of viable cells)	Wu et al. (2007)
Agarose (Biowest)	Agarase (*E. coli* BL21 (DE3))	Appropriate amount of agarase, water, 37°C, 12 h	Prebiotic Neoagaro-oligosaccharides (NAOS)	***Selectivity, improved biological properties*** ↑ Bifidobacteria and Lactobacilli (*In vitro* enumeration of viable cells) - Resistant to enzymes of the upper gastrointestinal tract ↑ Bifidobacteria and Lactobacilli (*In vivo* mice model) ↓ Putrefactive microorganisms ↑ Degrees of polymerization ↑ Prebiotic activity	Hu et al. (2006)

Substrates	Enzymes	Processes	Active components or activities	Advantages	Reference
Alginate (Qingdao Yijia Huayi Import and Export)	Alginate lyase (*Vibrio* sp. QY102)	E/S ratio 10 U: 0.5 g, water, 37°C, 6 h	Prebiotic Alginate oligosaccharides (AOS)	***Selectivity, improved biological properties*** ↑ *Bifidobacterium bifidum* ATCC 29521 and *Bifidobacterium longum* SMU 27001 (*In vitro* enumeration of viable cells) - Resistant to enzymes of the upper gastrointestinal tract ↑ Bifidobacteria; 13-fold for control and 4.7-fold for FOS, Lactobacilli; 5-fold for control (*In vivo* mice model) ↓ Enterobacteriaceae and Enterococci	Wang et al. (2006)
Enzyme-assisted extraction intensified with other processes					
Undaria pinnatifida (B)	Alginate lyase (Sigma-Aldrich)	**Enzyme-assisted supercritical fluid extraction** *Enzyme pre-treatment:* Enzyme 0.05% (w/w of dried seaweed), water pH 6.2, 37°C, 2 h *Supercritical fluid extraction:* Dimethyl ether extraction with ethanol co-solvent, 60°C, 40 bar	Fucoxanthin Lipids (ω-3 and ω-6) polyunsaturated fatty acids (PUFAs)	***High yield, cost-saving*** ↑ Fucoxanthin 1.1-fold ↑ Lipids rich in PUFAs 1.3-fold	Billakanti et al. (2013)

Most of the research thus far has focused on the use of commercially-available carbohydrate hydrolytic enzymes and proteases. Although they might not hold as much promise as the development of seaweed-specific enzymes, they are convenient for use in commercial applications and are cost effective at present for industry. Also, the use of enzymes to hydrolyse cellulose and protein networks in the cell wall, in order to facilitate the liberation of other target components, can avoid excessive structural degradation common with harsh physical and chemical treatments. High MW polysaccharides, such as fucoidans of ~390-2200 kDa, have shown potential to induce anticancer activity (Yang et al., 2008).

On the other hand, commercial enzymes have been used to reduce the MW of target molecules in some instances. Proteases, for example, are commonly used for hydrolysis of proteins extracted from seaweeds. Commercial carbohydrases have also been applied, in a small number of cases, to specifically digest target polysaccharides, but this is not common due to the fact that commercially available carbohydrases with activity toward seaweed-specific polysaccharides remain rather rare (and expensive). Given the current lack of commercial enzymes with selectivity toward the cell wall polysaccharides of seaweeds, Sánchez-Camargo et al. (2016) suggested that pressurized liquids are currently a more effective option compared with enzymatic treatments for the recovery of phlorotannins from the seaweed *Sargassum muticum*. Therefore, the isolation, identification, and purification of enzymes from microorganisms that can degrade the constituents specific to seaweeds needs to be investigated, in order to improve hydrolysis efficiency.

It should be noted that most publications using seaweed-specific enzymes to produce low MW seaweed fractions, such as oligosaccharides, have reported prebiotic properties. The introduction of oligosaccharides as prebiotic ingredients in functional food has increased, and a number of studies on plant polysaccharides have indicated that low MW or hydrolysed oligosaccharides have improved fermentability by gut microbial communities (Hughes et al., 2007, 2008; Mussatto and Mancilha, 2007).

The utilisation of enzymes for polysaccharide degradation may be intensified with physical treatments of seaweed materials before or during enzyme processes. As shown in Tables 1.1, this approach has shown significant improvements in the time and cost of the process, as well as the extraction yield and biological properties of bioactive compounds, as the disaggregation of the cell walls is expected to improve the accessibility of enzymes to their substrates. Michalak and Chojnacka (2014) reported several merits of other novel extraction processes, such as microwave, ultrasound, and ultra-high pressure treatments, so the intensification of enzyme processes with these techniques could potentially yield additional benefits, particularly with regard to process efficiency for the improvement of prebiotic and other nutraceutical functions in the future, but not many studies have focused on this research area to date.

Overall, most of the available literature on the use of enzymes for seaweed extraction and digestion suggests that the use of enzymes has a number of advantages for seaweed extraction and digestion, as they are able to yield target compounds with improved selectivity, high yields, and improved biological properties, while using mild conditions and non-toxic chemicals, and with reduced time, energy and process cost. These capabilities may benefit large-scale operations and improve the prospects for using seaweeds in functional food and nutraceutical industries if some commercial and technical limitations presented on an industry scale, particularly concerning the stability and cost of enzymes (Puri et al., 2012), and the availability of seaweed-specific enzymes, can be overcome.

1.6 Application and legislation of seaweed use in the functional food sector

1.6.1 Seaweed and the functional food industry

Increasing consumer awareness regarding the complex relationship between diet and health is resulting in demand for new functional foods that can specifically contribute to health-promotion or disease prevention, beyond providing basic nutrition (Gul et al., 2016). The global nutraceutical market, including functional foods and beverages as well as dietary supplements, was valued at around USD 250 billion in 2014, and with the rapid increase in consumer demand, is expected to reach around USD 385 billion by 2020 (Suleria et al., 2015).

Seaweeds are commonly used as general foodstuffs, such as flavorings for noodles, soups, and meals, as well as in snacks, salads, wrap-up vegetable, and pickled side-dishes, in Japan, China, Korea, and other coastal populations (Lee, 2008). While the recent studies on seaweed-derived functional food ingredients have shown that seaweeds are a rich source of bioactive compounds with a variety of potential health benefits, the volume of clinical research in humans into the health-promoting properties of seaweeds in food and nutraceutical applications is currently rather limited.

Enzyme-enhanced processing could have potential for developing various functionalities and improving the quality of bioactive compounds from seaweeds (Wijesekara and Kim, 2015), and could expand the breadth of product applications in the functional food market. For instance, Rhein-Knudsn et al. (2015) reviewed the advantages of using enzyme-assisted extraction to enhance the value and potential applications of commercial seaweed hydrocolloids. Enzymes facilitated selective extraction at mild conditions, leading to better preserved chemical structures and functional properties, and the avoidance of contaminants. Lakmal et al. (2015) also reported that enzyme-assisted extraction is an effective method to isolate sulphated polysaccharides from seaweeds for new nutraceutical applications, as these polysaccharides exhibit many bioactivities, such as antioxidant, anti-inflammatory, anticancer, and anticoagulant effects. In addition, highly soluble oligosaccharides, produced using enzymatic hydrolysis, can be incorporated into food systems more easily and with greater functional properties (Kang et al., 2014). The potential value-enhancement of seaweed-

derived food and nutraceutical ingredients using enzyme-enhanced processing is summarised in Fig.1.3.

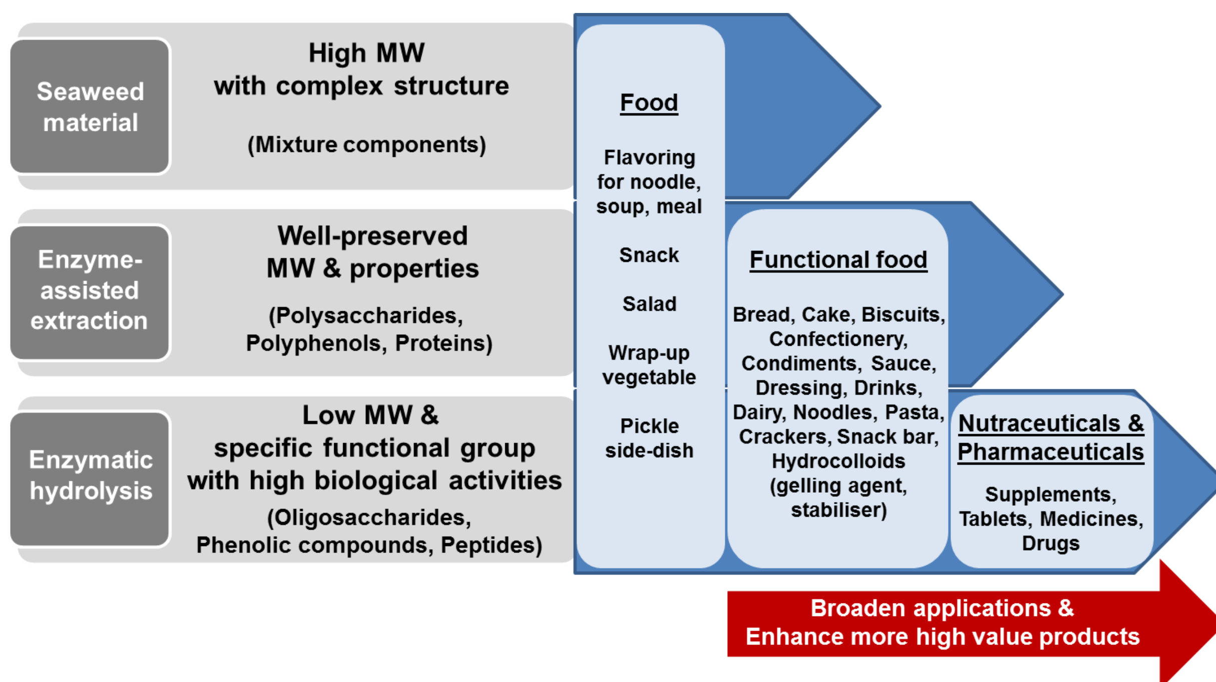

Figure 1.3 Potential of using enzyme-enhanced processes to improve the value and uses of seaweed-derived food and nutraceutical ingredients

1.6.2 Relevant regulations on seaweed functional food supplements

This review summarises the currently available knowledge on the regulations in various countries relevant to seaweed use in food and functional food supplements.

In France, 21 species of macroalgae, including brown seaweeds (*Ascophyllum nodosum*, *Undaria pinnatifida*, *Laminaria japonica*, etc.), red seaweeds (*Palmaria palmata*, *Porphyra umbilicalis*, *Gracilaria verrucosa*, etc.), and green seaweeds (*Ulva* and *Enteromorpha* species), are authorized as vegetables and condiments for food consumption (CEVA, 2014). Some brown seaweed (*Laminaria* sp. and *Nereocystis* sp.), red seaweed (*Porphyra* sp. and *Rhodymenia palmata* (L.) Grev.), and the materials derived from these species are also classified for “Generally Recognized As Safe (GRAS) food substances” in U.S. Food and Drug Administration, SCOGS-Report Number 38 (FDA, 1973). These authorizations present an opportunity for the food industry to include seaweeds as raw materials in food formulations and applications.

However, of course these seaweeds and their products still must meet the requirement of consumer safety regulations. The maximum allowed levels of heavy metals including arsenic, cadmium, lead, mercury, tin, and iodine have been defined for edible seaweeds in most jurisdictions, but the maximum allowed bacterial levels within dried seaweeds have been defined only in France. The

quality criteria and related regulations applied to seaweeds and their products are summarised in Table 1.3. The high levels of iodine in seaweeds appear to be gaining increasing cautionary interest. The Australian Quarantine and Inspection Service (AQIS) has now included brown seaweeds on the imported food 'Risk List' and are monitored at the border to ensure that only products with safe levels of iodine are imported (≤1000 mg iodine/kg dried weight) (Food Standards Australia New Zealand; FSANZ, 2010). Also, pregnant and breastfeeding women as well as young children are suggested to eat no more than one serve a week of brown seaweed (FSANZ, 2011). Bouga et al. (2015) surveyed the iodine content of seaweed and seaweed-containing products in the UK market, with the median content being 110 μg/g and 585 μg per estimated serving. They identified 26 products that may lead to an iodine intake over the upper level of European tolerable adult at 600 μg/day.

Table 1.3 Quality criteria and regulations applied to seaweeds and their products

Risk items	Upper limit			
	Australia	**France**	**USA**	**EU**
Toxic minerals (mg/kg DW, ppm)				
- Inorganic arsenic	≤ 1.0	≤ 3.0	≤ 3.0	
- Lead		≤ 5.0	≤ 10	≤ 3.0
- Cadmium		≤ 0.5		≤ 3.0
- Tin		≤ 5.0		
- Mercury		≤ 0.1		≤ 0.1
- Iodine	≤ 1000	≤ 2000	≤ 5000	
- Heavy metals			≤ 40	
Bacteria *Dried seaweeds only (cfu/g)				
- Aerobes		≤ 100		
- Fecal coliforms		≤ 10		
- *Clostridium perfringens*		≤ 1		
- Anaerobes		≤ 100		
Reference	- FSANZ (2013) - FSANZ (2010)	- CEVA (2014) - Mabeau and Fleurence (1993)	Mabeau and Fleurence (1993)	- Holdt and Kraan (2011) - EU (2008)

The safety of seaweed consumption was also recently evaluated in Belgium and Norway. Belgium's report from the Superior Health Council (2015) conducted a risk assessment of inorganic arsenic though the consumption of edible seaweeds. They recommend that the consumption of *Hizikia fusiforme* should be of concern due to high levels of inorganic arsenic, and the consumption of other seaweeds should be limited to 7 g dried material per day. Norway's report from its National Institute of Nutrition and Seafood Research (NIFES) (2016) suggested that high levels of inorganic arsenic and cadmium which may limit the use of some seaweed species as food ingredients were particularly

found in Norwegian brown seaweeds. However, Smith et al. (2010) compared the heavy metal content of edible seaweeds in New Zealand, including commercially available and wild-harvested seaweeds (*Macrocystis pyrifera*, *Undaria pinnatifida*, *Porphyra*, *Ecklonia radiata*, *Ulva stenophylla*, *Durvillaea antarctica*, and *Hormosira banksii*). None of the seaweeds studied showed significant risks with regard to heavy metals, considering the quantities that they are normally consumed. Moreover, NIFES (2016) also mentioned the risks posed by persistent organic pollutants (dioxin and polychlorinated biphenyls, etc.), toxins (pinnatoxin, aplysiatoxin, and kainic acid, etc.), anti-nutrients (polyphenols), radioactivity (radionuclides), microorganisms (bacteria associated with seaweeds and viral contagious agents), and microplastic (particle size <5 mm) contamination in seaweeds. However, a low risk of food safety hazards was observed in all of these parameters.

Apart from the aforementioned regulations applying to seaweeds and their products, some other general regulations should be considered. For example, Codex General Standard for Contaminants and Toxins in Food and Feed (Codex, 1995) provides the toxicological guidance for tolerable intake levels of contaminant for humans. The Provisional Tolerable Weekly Intake (PTWI) of inorganic arsenic, cadmium, lead, mercury, and tin are defined at 0.015, 0.007, 0.025, 0.005, and 14 mg/kg body weight, respectively. Meanwhile, the FSANZ standard 1.3.4 (FSANZ, 2012) ensures that food additives (novel food and nutritive substances, etc.) added to food meet appropriate specifications for identity and purity. These substances must not contain lead at >2 mg/kg DW and arsenic, cadmium, and mercury, each at >1 mg/kg DW. In addition, the guideline levels for determining the microbiological quality of ready-to-eat foods (FSANZ, 2001) may be applied if seaweeds are used as one ingredient in products specified in this food category.

1.7 Seaweed biodiversity and endemism in South Australia

Aforementioned reviews in the previous section demonstrate that seaweeds are a valuable source of bioactive compounds, which potentially provide a number of health benefits to humans. In order to develop an industry based on the extraction of bioactive compounds from seaweed, it is crucial to identify a suitable source of seaweed biomass.

South Australia has a high diversity of seaweed, with up to 1,500 described species (Waters et al., 2010), of which approximately 62% are endemic to the region (Phillips, 2001). Among all macroalgae, the red seaweeds showed the highest diversity and endemism (over 800 species, 75% of which were considered endemic). A relatively high diversity (231 species) and endemism (57%) was also recorded for brown seaweeds, while the lowest diversity (124 species) and endemism (30%) was observed for the green seaweeds of South Australia (Womersley 1990). Biodiversity aside, South Australia's brown seaweeds may have more potential for industrial utilisation, as they comprise the highest proportion of seaweed biomass in key harvesting sites (Lorbeer et al., 2013). Currently, Rivoli Bay is the main harvesting site for beach-cast seaweed in South Australia, and the brown seaweed

Ecklonia radiata (C. Agardh) J. Agardh is one of the most abundant seaweeds in that resource. However, the harvested seaweed in this region is still limited to use for low-value agricultural products such as fertiliser and animal feed (Lorbeer et al., 2013; PIRSA, 2014). Therefore, the brown seaweed *E. radiata* was selected as a model species in this study.

1.8 Bioactive compounds, biological activity, and potential functional and nutraceutical applications of brown seaweeds

Recent research on seaweed-derived nutraceutical ingredients has shown that brown seaweeds are a rich source of bioactive compounds, particularly sulphated polysaccharides (fucoidan), phenolic compounds (phlorotannin), and carotenoids (fucoxanthin). In addition, they also consist of bioactive peptides and polyunsaturated fatty acids (PUFAs) including omega-3 fatty acids (Holdt and Kraan 2011). These compounds possess several biological properties which may be exploited in functional food and nutraceutical applications (Gupta and Abu-Ghannam, 2011a). In this study, polysaccharides (alginate, fucoidan, and laminarin) and phenolic compounds (mainly phlorotannin) from brown seaweeds are the key components of focus, as they have been widely reported to possess a number of relevant biological properties, as illustrated in Table 1.4.

Table 1.4 Bioactive compounds obtained from different brown seaweed species and their bioactivities

Compound	**Source of seaweed/ Content** (%DW)	Biological activity
Alginate	*Laminaria* 17-46% *Undaria* 24% *Sargassum* 3.3-41% *Fucus* 18-22% *Ascophyllum* 24-29% (Holdt and Kraan, 2011; Zubia et al., 2008)	Antibacterial (Hu et al., 2005; Khan et al., 2012) Anticancer, Anti-tumor (Murata and Nakazoe, 2001) Cholesterol-lowering effect (Paxman et al., 2008; Idota et al., 2016) Anti-hypertention (Holdt and Kraan, 2011) Dietary fibre and prebiotic (Kuda et al., 2005; Ramnani et al., 2012; Zhu et al., 2015; Li et al., 2016a) Anti-diabetes (Holdt and Kraan, 2011) Anti-obesity (Khoury et al., 2015) Antioxidant (Hu et al., 2001; Şen, 2011; Zhao et al., 2012; Kelishomi et al., 2016) Anticoagulant (Ma et al., 2016)

Compound	Source of seaweed/ Content (%DW)	Biological activity
Fucoidan	*Laminaria* 2-5.5% *Undaria* 1.5% *Sargassum* 4.3-26% *Fucus* 16-20% *Ascophyllum* 4-12% (Holdt and Kraan, 2011; García-Ríos et al., 2012)	Anti-inflammatory (Cumashi et al., 2007; Fernando et al., 2016) Prebiotic (Lynch et al., 2010; Kong et al., 2016; Shang et al., 2016) Anticoagulant (Li et al., 2008a; Wang et al., 2010a; Jin et al., 2013; Zhang et al., 2015b) Antibacterial (Shannon and Abu-Ghannam, 2016) Anti-arteriosclerosis (Murata and Nakazoe, 2001) Antioxidant (Ruperez et al., 2002; Xue et al., 2004; de Souza et al., 2007; Wang et al., 2008; Wang et al., 2009; Huang et al., 2016) Anticancer (Murata and Nakazoe, 2001; Choi et al., 2013; Moghadamtousi et al., 2014; Kalimuthu et al., 2015; Anastyuk et al., 2017) Immunomodulator (Li et al., 2008a; Kawashima et al., 2012; Zhang et al., 2015a) Antiviral (Besednova et al., 2016; Lee et al., 2004; Rabanal et al., 2014) Anti-HIV (Thuy et al., 2015; Dinesh et al., 2016) Improve metabolic syndrome (Shang et al., 2017) Neuroprotective effect (Fitton, 2011) Anti-diabetes (Wang et al., 2014, Shan et al., 2016)
Laminarin	*Laminaria* 0-32% *Undaria* 3% *Sargassum* 0.3% *Fucus* 0.04-0.4% *Ascophyllum* 1.2-10% (Holdt and Kraan, 2011; Graiff et al., 2016)	Antibacterial (Shannon and Abu-Ghannam, 2016) Antioxidant (Kadam et al., 2015) Dietary fibre and prebiotic (Deville et al., 2004; Deville et al., 2007) Reduce cholesterol levels (Holdt and Kraan, 2011) Anti-obesity (Nguyen et al., 2016) Immunostimulating (Holdt and Kraan, 2011) Anticancer (Moussavou et al., 2014) Antiviral (Wang et al., 2012b)
Phenolic compounds (mainly phlorotannin)	*Laminaria* 0.2-5.3% *Undaria* <0.4% *Sargassum* 1.1-12.7% *Fucus* <0.4-12.2% *Ascophyllum* 0.5-14% (Holdt and Kraan, 2011)	Antioxidant (Kang et al., 2003; Zou et al., 2008; Zubia et al., 2008; Li et al., 2011; Wang et al., 2012a; Kirke et al., 2017) Anti-diabetic (Lee and Jeon, 2013; Lopes et al., 2017) Neuroprotective effect (Pangestuti and Kim, 2013; Kang et al., 2013) Prevent cardiovascular disease (Murray et al., 2016) Antibacterial (Nagayama et al., 2002; Eom et al., 2012; Lee et al., 2014) Anti-inflammatory (Dutot et al., 2012; Jung et al., 2013; Wijesinghe et al., 2013) Antibiotic (Eom et al., 2013) Anticancer (Li et al., 2011; Namvar et al., 2012; Zenthoefer et al., 2017) Anti-HIV (Ahn et al., 2004; Artan et al., 2008; Vo and Kim, 2010; Karadeniz et al., 2014) Hepatoprotective effect (Kang et al., 2012) Anti-allergic (Li et al., 2008b; Vo et al., 2012) Prevent autoimmune disorder (Holdt and Kraan, 2011)

Considering that data on the functional properties of brown seaweed components is now available, the potential applications of brown seaweeds and their extracts as functional ingredients in food products were reviewed (Table 1.5). As shown in Table 1.5, most of the potential applications for brown seaweed ingredients have focused on improving the nutritional, textural, and sensorial properties of food products, particularly meat, bakery, and dairy products. The reported effects of brown seaweed food additives for specific health-promoting applications are still not extensive. In addition, the specific taste and aroma of seaweeds may affect consumer acceptance and limit the use of seaweeds as functional ingredients. However, it is interesting that the sensorial characteristics of seaweed were reported to be acceptable when formulated into most food applications.

Table 1.5 The effects associated with the incorporation of brown seaweeds and their extracts into food products, with regard to food properties and health

Product	Source	Seaweed-derived additive	Effect on food and health properties	Reference
Yoghurt	*Ascophyllum nodosum* *Fucus vesiculosus*	Seaweed extract: 0.25 and 0.5%	- Increased yellowness and reduced levels of lipid oxidation - No negative effect on shelf life characteristics and sensory perspectives (colour, flavour, and texture) - Did not alter cellular antioxidant status or protect against DNA damage	O'Sullivan et al. (2016)
	Himanthalia elongata *Saccharina latissima* *Undaria pinnatifida*	Seaweed powder: 0.5%	- *S. latissima* showed the lowest seaweed flavour and the highest flavour quality.	Nunez and Picon (2017)
Milk	*Ascophyllum nodosum* *Fucus vesiculosus*	Seaweed extract: 0.25 and 0.5%	- Extracts were stable in milk, and showed antioxidant activities before and after *in vitro* digestion. - Improved milk quality and shelf life characteristics	O'Sullivan et al. (2014)
Bread	*Myagropsis myagroides*	Seaweed extract: 0.5, 1, and 2%	- Improved sensory acceptance with 0.5% supplementation and shelf life relative to the control - Decreased total microbial count with 2% supplementation	Lee et al. (2010a)
	Himanthalia elongata	Seaweed powder: 5-15%	- Predicted values from response surface methodology, 17.07% seaweed with 21.89% white flour provided the maximum total dietary fibre, phenolic, and antioxidant activity, while retaining acceptable sensory evaluation in the optimised sample.	Cox and Abu-Ghannam (2013a)

Product	Source	Seaweed-derived additive	Effect on food and health properties	Reference
Pasta	*Undaria pinnatifida*	Seaweed extract: 5, 10, 20, and 30%	- 20% supplementation retained sensory acceptance, while improving bio-functional properties. - Extract-enriched pasta had an improved amino acid and fatty acid profile, and contained fucoxanthin and fucosterol. - Fucoxanthin was not degraded by the pasta making and cooking process.	Prabhasankar et al. (2009)
Pork product	*Himanthalia elongata* *Undaria pinnatifida*	Seaweed powder: 2.5 and 5%	- Improved the water and fat binding properties - Chewiness and hardness of the cooked products was higher, while springiness and cohesiveness were lower. - Dietary fibre, antioxidant, polyphenol, and carotenoid content were higher.	Cofrades et al. (2008)
	Himanthalia elongata	Seaweed powder: 3.4%	- Water/oil retention capacity, elastic modulus, and hardness were reinforced. - The presence of alginates prevented the thermal denaturation of proteins.	Fernández-Martín et al. (2009)
	Himanthalia elongata *Undaria pinnatifida*	Seaweed powder: 5.6%	- Increased n-3 PUFA and reduced n-6/n-3 PUFA ratio - The thombogenic index decreased in *U. pinnatifida* added meat. - The concentrations of K, Ca, Mg, and Mn were increased, while Na was decreased. - *H. elongata* supplemented samples showed the greatest increase in polyphenol and antioxidant.	López-López et al. (2009a)
	Laminaria digitata	Seaweed extract (containing 9.3% Laminarin & 7.8% Fucoidan): 0.01, 0.1, and 0.5%	- The laminarin/fucoidan extract at 0.5% showed the highest lipid pro-oxidant activity in fresh patties, but significantly reduced lipid oxidation in cooked patties. - 0.01% supplementation showed no adverse effect on texture, colour, lipid oxidation, and sensorial acceptance of pork patties.	Moroney et al. (2013)

Product	Source	Seaweed-derived additive	Effect on food and health properties	Reference
		Seaweed extract: 3 and 6 mg/mL	- The laminarin/fucoidan supplemented product had higher antioxidant activity than the one supplemented only with fucoidan and control, after cooking and post digestion.	Moroney et al. (2015)
Beef product	*Undaria pinnatifida*	Seaweed powder: 3%	- Addition of seaweed and olive oil in water emulsion improved the binding properties and the cooking retention values of moisture, fat, fatty acid, and ash with acceptable sensory and good nutritional properties.	López-López et al. (2010) López-López et al. (2011)
	Himanthalia elongata	Seaweed powder: 10-40%	- Reduced cooking losses, microbiological counts, and lipid oxidation, and increased the tenderness (~50%), the dietary fibre and phenolic content, and the antioxidant activity - Patties supplemented with 40% seaweed had the highest overall acceptability (texture and mouthfeel).	Cox and Abu-Ghannam (2013b)
Chicken product	*Undaria pinnatifida*	Seaweed extract (fucoxanthin): 0.02%	- Enhanced colour in ground chicken breast meat, and inhibited lipid peroxidation in chilling storage after cooking	Sasaki et al. (2008)
	Himanthalia elongata	Seaweed powder: 3%	- Reduced the cooking loss, while retaining sensory acceptance in low-salt restructured poultry steaks	Cofrades et al. (2011)
Seafood product	*Fucus vesiculosus*	Seaweed extract (oligomeric phlorotannin-rich subfraction): 0.03%	- Demonstrated high potential for use as natural antioxidants in fish and fish products, with effectiveness comparable to 100 mg/kg of the positive control propyl gallate	Wang et al. (2010c)
		Seaweed extract (phloroglucinol): 0.03%	Demonstrated potential as natural antioxidants against lipid oxidation in fish muscle foods	Jónsdóttir et al. (2016)
Sausage	*Himanthalia elongata*	Seaweed powder: 5.5%	- Improved Na/K ratios, increased calcium, and increased fibre content	López-López (2009c)
		Seaweed powder: 5%	- Improved fat and water binding properties, decreased lightness and redness, increased the hardness and chewiness - Sensory acceptance was reduced by seaweed flavour.	López-López (2009b)

Product	Source	Seaweed-derived additive	Effect on food and health properties	Reference
	Laminaria japonica	Seaweed powder: 1, 2, 3, and 4%	- 1% supplementation had the highest overall acceptability (physicochemical and sensory). - Improved cooking loss and emulsion stability, and increased hardness, gumminess, and chewiness	Kim et al. (2010)

1.9 The chemical composition of the South Australian seaweed, *E. radiata,* and its potential for utilisation in health foods

Given the abundance of *E. radiata* in the harvestable biomass of South Australia, as well as the content of micro- and macronutrients reported in brown seaweeds and their potential applications in functional food and nutraceutical products, the composition of *E. radiata* was analysed in order to assess its suitability as a model species. Materials and methods used for composition analyses are presented in the appendix of this chapter. The results of the compositional analyses are shown in Table 1.6. The seaweed materials used in this study were collected at the same period in different years on March 2013 and 2016 in an attempt to provide consistency in sample composition.

Carbohydrates and minerals were the main components in both batches accounting for approximately 70 and 20%, respectively. Some chemical variation between the two samples was observed, with the sample collected in March 2016 containing 2.3-fold higher fat, 1.5-fold higher protein, and 1.4-fold lower phlorotannin content. Total carbohydrate calculated by subtracting the moisture, fat, protein, and ash from the total dry weight was relatively higher compared with other brown seaweed species, *Ascophyllum nodosum*, *Saccharina latissimi*, *Fucus vesiculosus*, and *Alaria esculenta* (53.7-59.8%; calculated with the same method), but levels of mineral as ash content were similar to other species (17.6-24.9%), as reported by Tibbetts et al. (2016). The phlorotannin content of *E. radiata* was similar to, or slightly higher than, other brown seaweed sources (<0.4-14%) (Holdt and Kraan 2011).

As carbohydrate was a major component in this brown seaweed, the sugar profile was analysed in order to deduce its polysaccharide compositions. Results showed that glucose was the major sugar in both seaweed samples, but the free-sugar fraction of the sample collected in March 2013 was 2-fold higher in glucose compared to that of the March 2016 sample. Alginate, fucoidan, and laminarin are typically the main polysaccharides in brown seaweeds, which provide a number of benefits to human health (Holdt and Kraan, 2011). In order to determine the polysaccharide composition in seaweed, sugar composition was analysed after sulphuric acid hydrolysis as it is still difficult to separate the individual polysaccharide components for quantification purposes. Therefore, the alginate content was inferred from the guluronic and mannuronic acid quantitation. Fucose was likely

to indicate the presence of fucoidan, and the glucose was likely from laminarin and cellulose, and potentially also a small amount from fucoidan (O'Sullivan et al., 2010; Ale and Meyer, 2013). These sugars, detected in both seaweed samples, indicated the presence of alginate, fucoidan, and laminarin in *E. radiata*. The glucose (12.09 and 11.31%) and fucose (1.62 and 1.97%) in the bound-sugar fractions of both samples were similar, while the proportions of guluronic acid (6.86 and 8.67%) and mannuronic acid (9.73 and 11.73%) were slightly higher in the sample collected in March 2016.

In terms of mineral content, the *E. radiata* sample collected in March 2016 showed higher Ca, Fe, Mg, and Zn, with lower Na and I compared with the sample collected in 2013. Compared with the mineral contents of other brown seaweeds *Ascophyllum nodosum*, *Fucus vesiculosus*, and *Alaria esculenta* reported in the literature (Ca 0.9-1.1%, P 0.1-0.4%, Fe 26.0-59.1 mg/kg, Na 3.6-3.9%, and Mn 3.5-52.9 mg/kg DW) (Tibbetts et al., 2016), the *E. radiata* samples analysed here appeared to have higher contents of Ca, P, and Fe, and lower contents of Na and Mn. In addition, relatively high amounts of Ca, Mg, and K were detected in the seaweed samples, compared with the typical values for other grains, vegetables, and fruits (Ca 8-510; Mg 20-370; and K 70-2490 mg/100g DW, but with K being as high as (4833 mg/1000g DW in tomato) reported by Marles (2017). Although relatively high Na content was detected in *E. radiata*, it was noticed that Na/K ratios of this brown seaweed (0.5-0.6) was lower than spinach (0.8) (Bhattacharjee et al., 1998) and olives (43.6) (Rupérez, 2002) but higher than banana (0.02) (Leterme et al., 2006). A high dietary Na/K ratio has been correlated with higher incidence of hypertension (Rupérez, 2002).

More importantly, the selected seaweed species in this study must meet the requirements of consumer safety regulations. The safety assessment for seaweed consumption is generally focused on potential critical parameters, particularly heavy metals. Similar heavy metal contents were observed in both seaweed samples, with all items complying with international regulations shown in Table 1.6. However, it was noted that the iodine content (6645 mg/kg) of the sample collected in March 2013 was higher than the maximum quality criteria (≤5000 mg/kg).

Table 1.6 Compositions of brown seaweed *E. radiata* collected in March 2013 and 2016

Analysis items		*E. radiata*	
		March 2013	**March 2016**
Proximates (g/100g)			
Fat		0.4	0.9
Protein		5.1	7.8
Ash		20.7	21.1
Carbohydrates		74	70
Energy (kJ/100g)		1362	1361
Phlorotannin (g PGE/100g)		6.5	4.5
Sugar composition (g/100g)			
Free-sugar			
Glucose		4.55	2.11
Bound-sugar			
Guluronic acid		6.86	8.67
Mannuronic acid		9.73	11.73
Mannose		0.80	0.83
Glucuronic acid		0.56	0.68
Glucose		12.09	11.31
Galactose		0.39	0.50
Xylose		0.31	0.44
Fucose		1.62	1.97
Minerals (mg/kg)			
Calcium (Ca)		10675	20761
Chomium (Cr)		0.45	0.52
Copper (Cu)		1.2	1.0
Iron (Fe)		23	115
Magnesium (Mg)		5773	7843
Manganese (Mn)		1.3	2.3
Phosphorus (P)		6209	4614
Potassium (K)		41394	40369
Selenium (Se)		0.032	0.037
Sodium (Na)		25054	20761
Zinc (Zn)		16	25
Heavy metals and Iodine (mg/kg)	**Maximum upper limit** (Table 1.3)		
Inorganic arsenic	≤ 3.0	0.7	0.4
Cadmium (Cd)	≤ 3.0	1.4	1.5
Lead (Pb)	≤ 10	0.031	0.277
Mercury (Hg)	≤ 0.1	<0.01	0.014
Tin (Sn)	≤ 5.0	0.027	0.085
Iodine (I)	≤ 5000	6645	3230

1.10 Conclusion and future perspectives

Seaweeds are a valuable source of bioactive compounds. Advanced enzymatic processing is a key trend for the efficient extraction and digestion of value-added bioactive compounds from seaweeds. The potential of these compounds for a number of functional food applications is being increasingly recognised, particularly with regard to prebiotic supplements. Despite the opportunities, challenges remain in improving this technique in terms of its productivity and profitability. Enzyme intensification using other physical inputs, and enzymes with activities specific to seaweed structures, should be further investigated. Furthermore, the scaled up industrial process should be designed to support commercial implementation. The adherence of products to safety legislation are also critical and complex subjects requiring attention in order to push forward with the commercial development of seaweed ingredients for functional food and nutraceutical products. Brown seaweed *E. radiata* is a potential source to further develop seaweed-derived bioactive ingredients, given its abundance in the South Australia and chemical constituents including compounds of commercial interest, especially with regard to polysaccharides, polyphenols, and minerals.

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1.12 References

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Appendix 1.1: Materials and methods used for chemical composition analyses of brown seaweed *E. radiata*

1.1.1 Seaweed

E. radiata (identification confirmed by the State Herbarium of South Australia) were collected from freshly deposited beach-cast seaweed in Rivoli Bay, Beachport, South Australia (Latitude 37° 28' 36.24" S and Longitude 140° 2' 29.53" E) in March 2013 and 2016. They were rinsed in fresh water to remove any visible surface contaminants, and placed on mesh racks to dry. They were blended (Blendtec, Orem, UT, USA), then passed though a 0.25-mm sieve, and dried in an oven at 45°C to achieve a moisture content of approximately 10%. The powder was then stored at -20°C prior to analyses.

1.1.2 Chemicals and substrates

All chemicals used are of analytical or chomatography grade from Merck and Sigma.

1.1.3 Composition analyses of dried *E. radiata*

The compositions of dried seaweed (expressed as g/100g or mg/kg of DW) were investigated according to the methods described in Charoensiddhi et al. (2015, 2016, 2017) with some modifications.

Moisture, protein (Kjeldahl; N×6.25), total fat (Mojonnier or Soxhlet extraction), ash (ignition at 550°C), carbohydrate (by subtracting moisture, fat, protein, and ash), and essential mineral and heavy metal contents (Inductively coupled plasma; ICP method) of the dried seaweed samples were analysed by standard methods of the National Measurement Institute.

Total phlorotannins of dried seaweed samples were extracted using 70% acetone, and then analysed by Folin Ciocalteu's phenol reagent (results expressed as g phloroglucinol equivalent; PGE) (Koivikko et al., 2005; Wang et al., 2012).

Sugar contents of dried seaweed samples were separated into two parts (1) free-sugar and (2) bound-sugar by washing with 70% ethanol. Both free-sugar (soluble fractions) and bound-sugar (insoluble fractions) were dried in a centrifugal evaporator (Labconco, Kansas City, MO, USA), and then the monomeric composition of both fractions was determined using HPLC, after sulphuric acid hydrolysis and 1-phenyl-3-methyl-5-pyrazolone derivatisation (Comino et al., 2013).

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2. IMPROVED ANTIOXIDANT ACTIVITIES OF BROWN SEAWEED *ECKLONIA RADIATA* EXTRACTS PREPARED BY MICROWAVE-ASSISTED ENZYMATIC EXTRACTION

Phenolic compounds in brown seaweeds are considered products of interest due to their widely demonstrated bioactivities and potential applications as functional food ingredients. However, the complex structure of the seaweed cell wall can act as a major barrier for efficient extraction of intracellular bioactive compounds, including phenolics.

While the literature review in the previous chapter showed that enzyme-assisted extraction is becoming a key trend for the recovery of value-added bioactive compounds from seaweeds, few studies have explored the potential intensification of enzyme processes with physical treatments, which may yield additional benefits. Therefore, here we investigated the use of a microwave-assisted enzymatic extraction process for producing antioxidant extracts from the brown seaweed, *Ecklonia radiata*.

This study was orally presented at the 5th Congress of the International Society for Applied Phycology in Sydney on June 2014, and published in the conference special issue of the "Journal of Applied Phycology" (Vol. 27; 2015, pp. 2049-2058). The first page of the publication is attached in Appendix 2.1.

Author contributions: SC designed and performed all experiments as well as analysed the data and wrote all primary contents. WZ helped decide on the research directions and plan. WZ and CF provided advice regarding the experimental results and scope of the manuscript. PS supported the setup of extraction processes and training in the use of the equipment. All of the co-authors assisted with the revision of the manuscript before and during the journal peer-review process.

Improved antioxidant activities of brown seaweed *Ecklonia radiata* extracts prepared by microwave-assisted enzymatic extraction

Suvimol Charoensiddhi[1,2], Chis Franco[1,2], Peng Su[1,2], Wei Zhang[1,2]

[1] Centre for Marine Bioproducts Development, and [2] Department of Medical Biotechnology, School of Medicine, Flinders University, Adelaide, South Australia 5042, Australia

2.1 Abstract

Seaweeds are important sources of bioactive compounds with potential use in functional foods and nutraceutical products. This study aims to investigate the extraction efficiency of phlorotannins and antioxidant compounds of a South Australian brown seaweed *Ecklonia radiata* by enzymatic and microwave-assisted enzymatic extraction in order to evaluate their uses as potential functional food ingredients. A selected group of carbohydrases (Viscozyme, Celluclast, and Ultraflo) and proteases (Alcalase, Neutrase, and Flavourzyme) has been applied to improve the extraction efficiency, alone and intensified with microwave heating, using conventional acid-base and water extractions as controls. The antioxidant activities of the extracts were evaluated using both ferric reducing ability of plasma (FRAP) and oxygen radical absorbance capacity (ORAC) assays. Significantly higher yields in total phlorotannin content (TPC) and antioxidant activities of the extracts were achieved by enzymatic and microwave-assisted enzymatic extraction. Microwave-assisted Viscozyme extraction for 5 to 30 min was the most effective process with an extraction yield achieved of 52%. The extract had a TPC of 4.4 g phloroglucinol equivalents (PGE).100 g^{-1} dry weight (DW) and antioxidant activities of 29.7 mmol $FeSO_4$ equivalents.100 g^{-1} DW and 740.1 μmol Trolox equivalents (TE).g^{-1} DW. In contrast, the conventional acidic extraction for 24 h resulted in a TPC of 3.4 g PGE.100 g^{-1} DW, and antioxidant activities of 21.1 mmol $FeSO_4$ equivalents.100 g^{-1} DW and 512.4 μmol TE.g^{-1} DW. Extracts of brown seaweed *E. radiata* have potential for use in value-added products for nutritional purposes, using the microwave-assisted enzymatic extraction techniques.

Keywords: Carbohydrase; FRAP; Macroalga; ORAC; Phlorotannins; Protease

2.2 Introduction

The relationship between diet, health, and disease prevention is being widely recognized by consumers. There has been an increasing interest in research, development, and commercialization of functional foods, nutraceuticals, and dietary supplements around the world (Bernal et al., 2011; Freitas et al., 2012). Sources of functional ingredients from many different fruits, vegetables, cereals and mushooms have been explored in preference to marine macroalgae or seaweeds (Rasmussen and Morrissey, 2007). As a new potential source of functional ingredients, a number of studies reported that marine macroalgae produce a large variety of bioactive metabolites which are not produced by terrestrial plants (Plaza et al., 2008).

Among all macroalgae, brown seaweeds (Phaeophyceae) have relatively high diversity and endemism of the 231 species reported in Southern Australia, 57% are considered endemic (Womersley, 1990). Despite this natural abundance, Australia remains a net importer of seaweed products, with 5000 T valued at over AUS$17 million imported during the 2008-2009 financial year (Lee, 2010). Furthermore, much of what is harvested locally is underutilized and processed primarily into low-value commodities such as fertilizers and animal feeds (Lorbeer et al., 2013). Recent research on seaweed-derived functional food ingredients has shown that brown seaweeds are a rich source of nutraceuticals with a variety of bioactive compounds, mainly sulfated polysaccharides (fucoidan), phenolic compounds (phlorotannin), and carotenoids (fucoxanthin). In addition, they also contain polyunsaturated fatty acids (PUFAs) including omega-3 fatty acids, and bioactive peptides (Holdt and Kraan, 2011). These compounds possess various biological functions such as antioxidant, antiHIV, anticancer, antidiabetic, antimicrobial, anticoagulant, antivirus, antitumor, anti-inflammatory, immunomodulatory, dietary fibre and gastric protective effects, and blood lipid and cholesterol reduction (Mohamed et al., 2012). However, the main uses of brown seaweed are primarily as a raw material for the extraction of the hydrocolloid, alginate, for the food industry (McHugh, 2003).

The major obstacle to efficiently extract the intracellular bioactive compounds is the high degree of structural complexity and rigidity of the seaweed cell wall (Deniaud-Bouët et al., 2014). Conventional water and solvent extraction have several disadvantages as they are time-consuming and energy intensive, feature low selectivity and low extraction efficiency, and are also toxic to human health. As an alternative technology, enzyme-assisted extraction (EAE) and microwave-assisted extraction (MAE) have attracted considerable interest (Azmir et al., 2013; Gil-Chávez et al., 2013; Hahn et al., 2012; Jeon et al., 2012; Wijesinghe and Jeon, 2012). The application of these extraction technologies have shown a great potential to improve extraction efficiency in terms of yield, time, cost, environmental impact, and safety for food application. Enzyme treatments assist in disrupting or weakening the cell wall structure (Kadam et al., 2013). Similarly, positive results have been found for microwave extraction technique. Kadam et al. (2013) and Tatke and Jaiswal (2011) reported that

the penetration of microwave energy with non-ionizing electromagnetic radiation (300 MHz-300 GHz) to the material structure produced a heat source due to molecular friction by ionic conduction and dipole rotation. This may increase the penetration of solvent into the cell wall structure and facilitate the extraction of target compounds because of the disruption of hydrogen bonds and migration of dissolved ions. Therefore, both technologies can enhance the release of plant secondary metabolites and preserve their bioactive properties.

Ecklonia radiata (C. Agardh) J. Agardh is one of the most abundant brown seaweed species in southern Australia, constituting the largest fraction of biomass productivity, but this seaweed has not been studied and explored as a source of bioactive ingredients. In addition, only a few studies reported the use of enzyme or microwave-based extraction techniques alone for the extraction of bioactive compounds from seaweeds (He et al., 2013; Heo et al., 2003a,b; Heo et al., 2005a,b; Heo et al., 2008; Rodriguez-Jasso et al., 2011; Siriwardhana et al., 2008; Wang et al. 2010). Therefore, the aim of the present study was to investigate the efficiency of integration of enzymatic and microwave-assisted extraction of phlorotannins and antioxidant activities from *E. radiata*. The potential of enzyme and microwave treatments to improve the antioxidant activities of the extraction were assessed by two *in vitro* antioxidant assays based upon different reaction mechanisms- ferric reducing ability of plasma (FRAP) assay and oxygen radical absorbance capacity (ORAC) assay.

2.3 Materials and methods

2.3.1 Seaweed

Brown seaweed (*Ecklonia radiata*- identification confirmed by the State Herbarium of South Australia) was collected from freshly deposited beach-cast seaweed in Rivoli Bay, Beachport, South Australia in March 2013. They were rinsed in fresh water to remove any visible surface contaminants, and placed on mesh racks to dry. The whole plants were blended (Blendtec, USA), then passed though a 0.25 mm sieve, and dried in an oven at 45°C. The ground powder was stored at -20°C prior to extraction.

2.3.2 Chemicals

Folin Ciocalteu's phenol reagent, Phloroglucinol, 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ), 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), 6-Hydroxy-2,5,7,8-tetramethylchomane-2-carboxylic acid (Trolox), and Fluorescein sodium salt were purchased from Sigma Aldrich (St. Louis, MO, USA), and all other reagents were of analytical grade.

2.3.3 Enzymes

Six commercial enzymes which were used for the preparation of extracts including thee carbohydrases (Viscozyme® L, Celluclast® 1.5 L, Ultraflo® L) and thee proteases (Alacalase® 2.4 L FG, Neutrase® 0.8 L, and Flavourzyme® 1000 L) were kindly provided by Novozyme (Bagsvaerd, Denmark). The optimum hydrolysis conditions (Heo et al., 2005a; Sato et al., 2002) and characteristics of these enzymes are summarized in Table 2.1.

Table 2.1 Optimum hydrolysis conditions, activities, and sources of selected enzymes (Heo et al., 2005a; Sato et al., 2002)

Enzyme		Optimum conditions		Buffer used	Activities		Sources
		pH	Temperature (°C)		Key	Side	
Carbohydrase	Viscozyme	4.5	50	0.1 N AB[b]	Endo-β-glucanase: Hydrolysis (1,3)- or (1,4)-linkages in β-D-glucans	Xylanase Cellulase Hemicellulase	*Aspergillus aculeatus*
	Celluclast	4.5	50		Cellulase: Hydrolysis (1,4)-β-D-glucosidic linkages in cellulose and other β-D-glucans	-	*Trichoderma reesei*
	Ultraflo	7.0	60[a]	0.2 M PB[c]	Endo-β-glucanase: Hydrolysis (1,3)- or (1,4)-linkages in β-D-glucans	Cellulase Xylanase	*Humicola insolens*
Protease	Alcalase	8.0	50	0.2 M PB	Endoprotease: Hydrolysis internal peptide bonds	-	*Bacillus licheniformis*
	Neutrase	6.0	50				*Bacillus amyloliquefaciens*
	Flavourzyme	7.0	50		Exopeptidase: Hydrolysis N-terminal peptide bonds	Protease	*Aspergillus oryzae*

[a] Ultraflo was performed at temperature 50°C
[b] Acetate buffer
[c] Phosphate buffer

2.3.4 Proximate composition analyses of dried *E. radiata*

Moisture, protein (Kjeldahl), total fat (Mojonnier or Soxhlet extraction), ash (ignition at 550°C), and carbohydrate contents of the prepared seaweed powders were analyzed by standard methods of the National Measurement Institute.

2.3.5 Preparation of seaweed extracts

2.3.5.1 Enzymatic extraction

The enzymatic extracts were prepared according to the method of Heo et al. (2003b) with slight modifications. Briefly, 1 g of dried seaweed was dispersed in 100 mL of buffer solution (details as in Table 2.1), and 100 μL of enzyme was added. The enzymatic hydrolysis was performed under optimal conditions of the particular enzyme at 50°C for 24 h in an orbital mixer incubator (Ratek Instruments, Australia) with stirring set to a speed equal to that used for microwave-assisted enzymatic extraction. The enzyme was inactivated by boiling the sample at 100°C for 10 min and cooled immediately in an ice bath. The extract was centrifuged at 8000×g for 20 min at 4°C. The supernatant was freeze dried, weighed, and stored at -20°C until analyzed. The extraction yield (amount of extractable substance) was determined by subtracting the dried weight of the residue from 1 gram of dried seaweed sample, and was expressed as a percentage.

2.3.5.2 Open-vessel microwave-assisted enzymatic extraction

A StartSYNTH Microwave Synthesis Labstation (Milestone Inc., USA) was used, with stirring speed set to 70%, and the temperature controlled using an infrared sensor and automatic power adjustment. For each experiment, the microwave intensification was performed under optimum temperature of the particular enzyme at 50°C for 3 h.

2.3.5.3 Conventional extraction

Conventional methods typically involve reducing the materials into small particles and then extracting in solvent overnight by using a shaker bath (Laroze et al., 2010; Proestos and Komaitis, 2006; Torti et al., 1995). Therefore, in this study, conventional water, basic and acidic extractions were prepared as follows: 1 g of dried seaweed powder was extracted with 100 mL of MilliQ water or buffer solution pH 4.5, 6.0, 7.0, and 8.0 for 24 h under optimum temperature of the particular enzyme at 50°C in an orbital mixer incubator with stirring set to a speed equal to that used for microwave-assisted enzymatic extraction.

2.3.6 Optimization of Viscozyme extracts with and without microwave intensification

As Viscozyme was the most efficient enzyme in terms of the highest TPC and antioxidant activities achieved among all the enzymes tested, extraction time using Viscozyme was further optimized and investigated with different duration varying from 5 min to 24 h (5, 15, 30 min and 1, 3, 6, 9, 12, 18, 24 h). After that the microwave intensification was examined at short duration 5, 15, and 30 min in order to improve extraction yield, TPC, and antioxidant activities. Both tests were performed under optimum temperature of this enzyme at 50°C.

2.3.7 Determination of total phlorotannin content (TPC)

Total phlorotannin content of dried seaweed materials and all seaweed extracts prepared by enzymatic, microwave-assisted enzymatic, and conventional extraction were quantified according to the method of Koivikko et al. (2005) and Wang et al. (2012) with minor modifications. Extract solution, 0.1 mL, was mixed with 0.5 mL of Folin Ciocalteu's phenol reagent (10% in MilliQ water). After 5 min, 0.4 mL of sodium carbonate (7.5% in MilliQ water) was added. The sample was incubated for 2 h at room temperature in the dark. The absorbance was measured at 725 nm in a Microplate reader (μQuant™, Biotek® Instruments, Inc., USA). A standard curve with phloroglucinol standards (0-100 $\mu g.mL^{-1}$) was used for calibration. The results were expressed as g phloroglucinol equivalents (PGE).100 g^{-1} DW.

For analysis of the maximum TPC in dried seaweed materials, dried seaweed was prepared by acetone extraction. Briefly, 2 g of dried seaweed powder was extracted with 50 ml of 70% acetone by incubating in an orbital mixer incubator, 200 rpm for 24 h at room temperature. The extracted sample was centrifuged at 8000×g for 20 min at 4°C. The residue was re-extracted three times under the same conditions as mentioned above. The combined supernatant was evaporated and kept at -20°C until analyzed.

2.3.8 Antioxidant activities assay

2.3.8.1 Ferric reducing ability of plasma (FRAP)

The antioxidant power measured by FRAP assay was estimated according to Benzie and Strain (1996) with minor modifications. Briefly, 900 μL of freshly prepared FRAP reagent (25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3.6H_2O$ solution) was warmed to 37°C. After that 90 μL of MilliQ water and 30 μL of extract solution were added, and sample was incubated for another 4 min. The absorbance was measured at 593 nm with Microplate reader (μQuant™, Biotek® Instruments, Inc., USA). The standard curve was linear between 0 and 1.0 mM $FeSO_4$. Results were expressed in mmol $FeSO_4$ equivalents.100 g^{-1} DW.

2.3.8.2 Oxygen radical absorbance capacity (ORAC)

The ORAC assay was performed according to Huang et al. (2002a,b) and Wang et al. 2009 with slight modifications. AAPH (0.414 g) was dissolved in 10 mL of 75 mM phosphate buffer (pH 7.4) to a final concentration of 153 mM and was kept in an ice bath. Fluorescein stock solution (4.19×10^{-3} mM) was prepared in 75 mM phosphate buffer (pH 7.4) and was kept at 4°C in dark condition. The 8.16×10^{-5} mM fresh fluorescein working solution was made daily by further diluting the stock solution in 75 mM phosphate buffer (pH 7.4). Microplate Reader DTX 880 (Beckman Coulter Inc., CA) was used for the fluorescence measurements. 30 μL of extract solution was mixed with 180 μL of fresh fluorescein working solution and pre-incubated at 37°C for 10 min. The reaction was initiated by addition of 30 μL of 153 mM AAPH solution. The fluorescence was recorded every 1.5 min for 50 cycles. Excitation and emission filter wavelengths were set at 485 nm and 535 nm, respectively. The ORAC value was calculated and expressed as μmol Trolox equivalents (TE).g^{-1} DW using the calibration curve of Trolox standards (concentration 0-100 μM).

2.3.9 Statistical analysis

Results were expressed as mean±SD in triplicate. One-way analysis of variance (ANOVA) was used to compare the means. Differences in extraction yield, TPC, and antioxidant activities were considered significant at $p<0.05$ by Duncan's test, and the correlation between TPC and antioxidant activities was defined by the Pearson's correlation coefficients (r) at $p<0.01$ in the IBM SPSS Statistics 22 (IBM Corporation Software Group, NY).

2.4 Results

2.4.1 Proximate composition of *E. radiata*

Analysis of the starting material used in this investigation indicated that *E. radiata* contained approximately 69.5% carbohydrate, 9.6% protein, 1.2% fat, and 20.2% ash (w/w). A bioactive component identified with various bioactivities in brown seaweeds is phlorotannins which contributed 6.5±0.2 g PGE.100 g^{-1} DW.

2.4.2 Effect of enzymatic and microwave-assisted enzymatic processes on *E. radiata* extracts

2.4.2.1 Extraction yield

The use of commercial enzymes intensified with microwave heating in this study increased the extraction yield by 5-20% compared to conventional methods (Fig. 2.1). The conventional acid-base and water extraction did not show much improvement. However, significantly higher yields of almost 70% was achieved using proteases (Alcalase and Flavourzyme), carbohydrase (Ultraflo), and a mixture of two enzymes (Flavourzyme and Ultraflo) intensified with microwave.

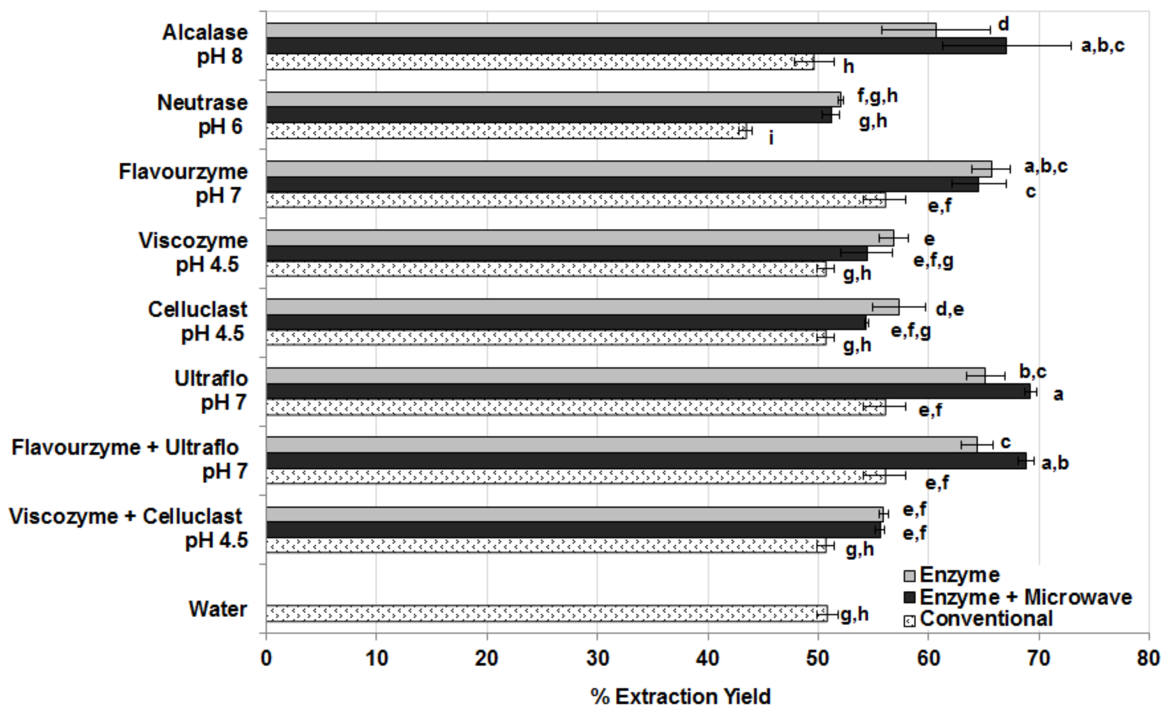

Figure 2.1 Comparison of extract yield of different extraction processes: enzyme-assisted extraction (24 h), microwave-assisted enzymatic extraction (3 h), and conventional acid-base at different pH and water extractions (24 h); the extract yield was expressed as g dried extract.100 g^{-1} dried seaweed (%). Values with different letters are significantly different ($p<0.05$).

2.4.2.2 Total phlorotannin content (TPC)

Considerable differences were observed in total phlorotannin content (TPC) between the enzymatic extraction and microwave-assisted enzymatic extraction (Fig. 2.2). Extracts prepared by carbohydrases (Viscozyme, Celluclast, and the mixture of both enzymes) with and without microwave intensification had significantly higher ($p<0.05$) TPC (3.7 to 4.2 g PGE.100 g^{-1} DW) than water extraction (2.6 g PGE.100 g^{-1} DW), approximately 1.5-fold and higher than other processes. By contrast, the TPC of extracts by protease (Alcalase, Neutrase, and Flavourzyme), carbohydrase (Ultraflo), and a mixture of Flavourzyme and Ultraflo were generally lower. It was noticed that the microwave-assisted enzymatic extraction significantly improved ($p<0.05$) TPC content of all extracts compared to the enzymatic extraction only. From these results, not only the extracts using Viscozyme and Celluclast could enhance TPC extraction, acidic extraction (pH 4.5) also provided high TPC (3.4 g PGE.100 g^{-1} DW).

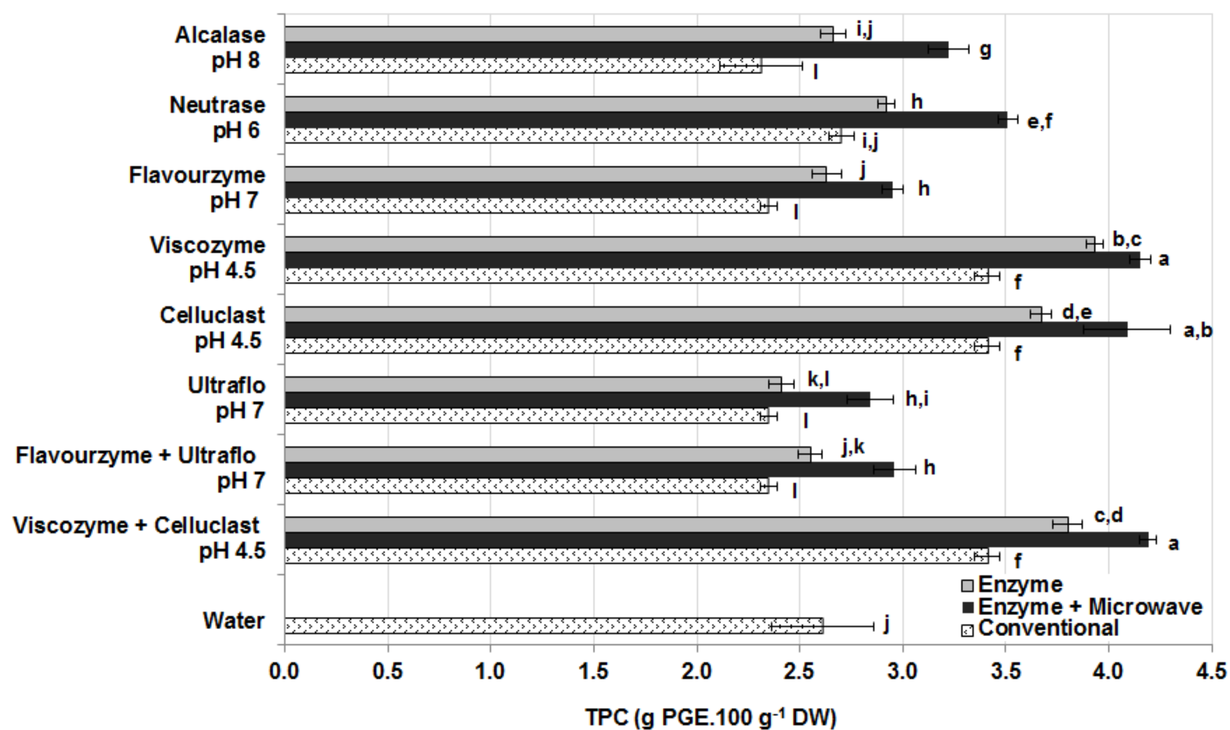

Figure 2.2 Total phlorotannin content (TPC) of different extraction processes: enzyme-assisted extraction (24 h), microwave-assisted enzymatic extraction (3 h), and conventional acid-base at different pH and water extractions (24 h); values with different letters are significantly different ($p<0.05$).

2.4.2.3 Antioxidant activities by FRAP and ORAC assays

Application of enzymes is considered to be an important factor to extract antioxidant compounds. There was no significant increase ($p>0.05$) in FRAP value between enzymatic extraction with and without microwave intensification except from an Alcalase treatment. Fig. 2.3 showed that enzymatic extraction using Viscozyme, Celluclast, and the mixture of both enzymes with and without microwave intensification were the most efficient to achieve the highest FRAP value (26.1 to 27.8 mmol $FeSO_4$ equivalents.100 g^{-1} DW). It was significantly higher ($p<0.05$), by almost 3-fold, than that of the water extract (11.3 mmol $FeSO_4$ equivalents.100 g^{-1} DW). Conversely, all proteases, carbohydrase (Ultraflo), and a mixture of Flavourzyme and Ultraflo did not enhance the extraction of antioxidant compounds. A similar trend was observed with TPC that acidic extraction provided a high FRAP value (21.1 mmol $FeSO_4$ equivalents.100 g^{-1} DW) in seaweed extracts.

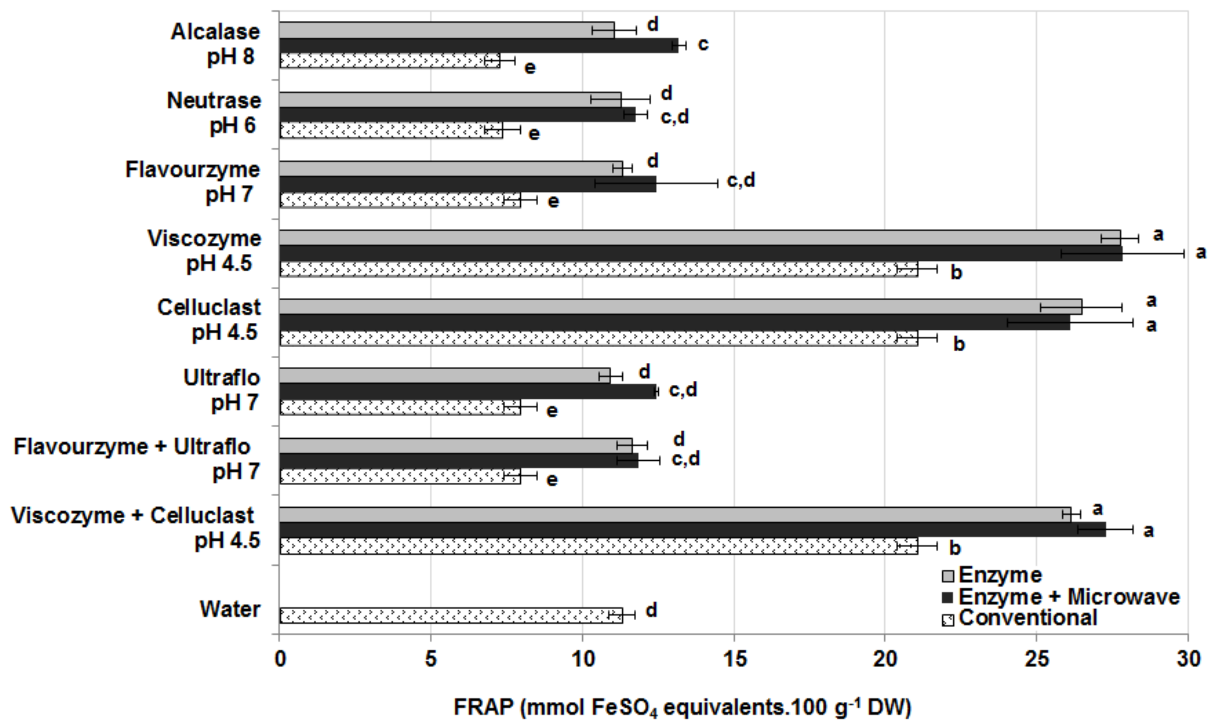

Figure 2.3 Antioxidant activities (FRAP assay) of different extraction processes: enzyme-assisted extraction (24 h), microwave-assisted enzymatic extraction (3 h), and conventional acid-base at different pH and water extractions (24 h); values with different letters are significantly different ($p<0.05$).

In this study, the ORAC assay was performed using Trolox (a water-soluble analog of vitamin E) as a standard to determine the TE. Four seaweed extracts prepared by Viscozyme and a mixture of Viscozyme and Celluclast with and without microwave intensification showed significantly higher ($p<0.05$) ORAC values (695.5 to 773.5 μmol TE.g^{-1} DW) than others (Fig. 2.4), the results being similar in the FRAP assay. However, Celluclast and acidic extraction did not show much improvement in ORAC values (512.4 to 602.8 μmol TE.g^{-1} DW). The protease treatment by Alcalase intensified with microwave showed the greatest increase in ORAC value at 775.5 μmol TE.g^{-1} DW. There was a significant increase ($p<0.05$) in ORAC values of seaweed extracts intensified with microwave treatment compared to enzymatic extraction only excluding Viscozyme or with a mixture of Viscozyme and Celluclast treatments.

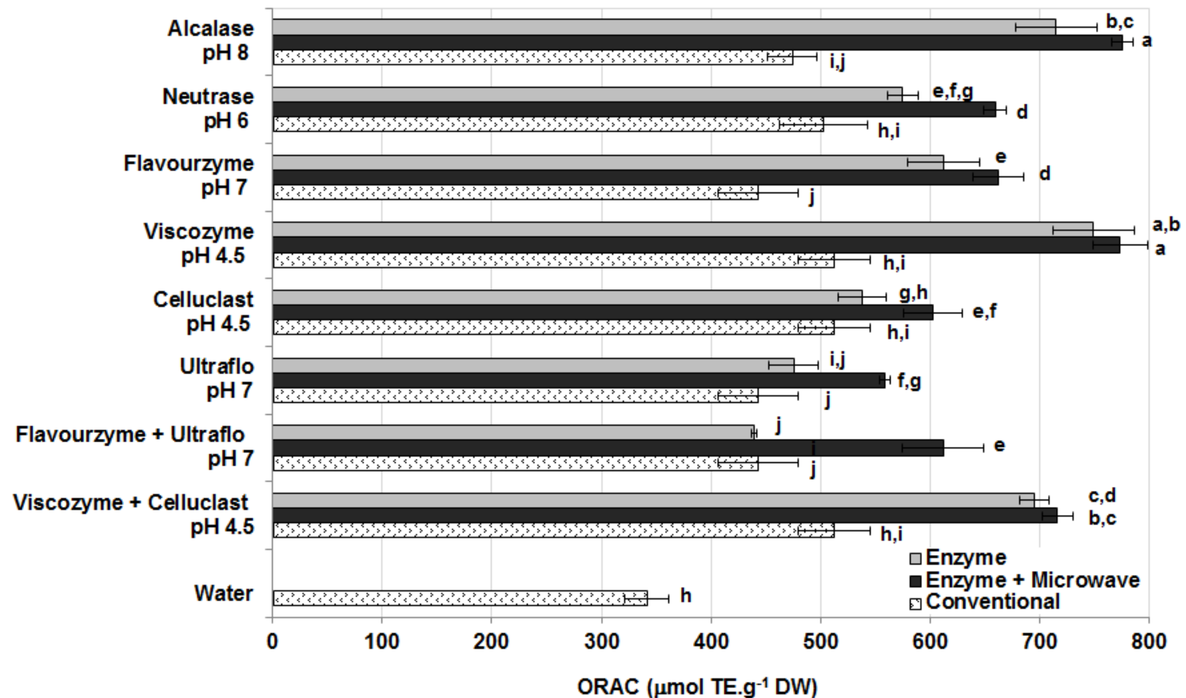

Figure 2.4 Antioxidant activities (ORAC assay) of different extraction processes: enzyme-assisted extraction (24 h), microwave-assisted enzymatic extraction (3 h), and conventional acid-base at different pH and water extractions (24 h); values with different letters are significantly different ($p<0.05$).

It is noted that the antioxidant activities represented by FRAP and ORAC did not correlate. The correlation analysis (Fig. 2.5) using the Pearson's correlation coefficients (r) indicated that the antioxidant activities as measured by the FRAP assay positively correlated with the TPC ($p<0.01$) at 0.911. However, a low correlation was found between the ORAC value and the TPC with the correlation coefficients (r) at 0.632.

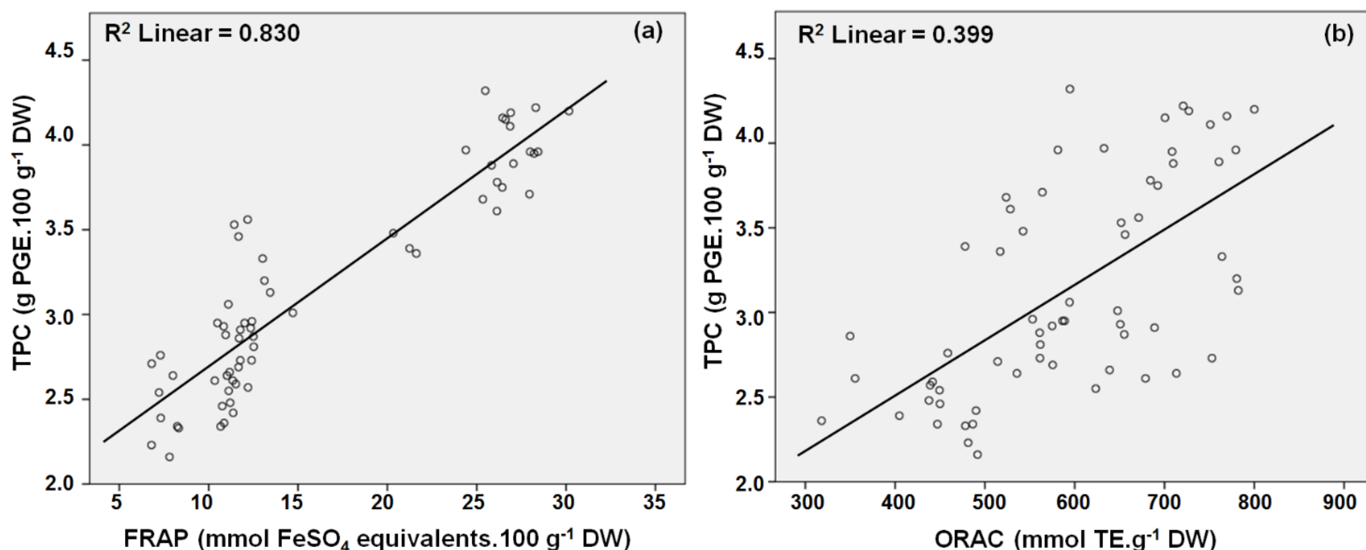

Figure 2.5 Correlations between total phlorotannin content (TPC) and antioxidant activities
(a) FRAP assay and (b) ORAC assay of different extraction processes: enzyme-assisted extraction (24 h), microwave-assisted enzymatic extraction (3 h), and conventional acid-base at different pH and water extractions (24 h); n=63

2.4.3 Optimization of Viscozyme extraction on TPC and antioxidant activities

The extraction time is an important parameter to optimize the extraction process. In this study, extracts prepared by Viscozyme-assisted extraction showed the highest TPC and antioxidant activities. This process was selected for further optimization with extraction time varying from 5 min to 24 h (Fig. 2.6). The increase in extraction time showed a slight increase in the extraction yield (51.5 to 56.9%). Although antioxidant activities measured by ORAC assay gradually increased with extraction duration (622 to 742.6 μmol TE.g^{-1} DW), antioxidant activities measured in the FRAP assay remained constant during this extraction period (27.6 to 29.2 mmol $FeSO_4$ equivalents.100 g^{-1} DW). Similarly, a high TPC (4.3 g PGE.100 g^{-1} DW) was also observed with a short extraction time at 1 h. Therefore, it was revealed that high TPC and antioxidant activities could be achieved in short extraction time.

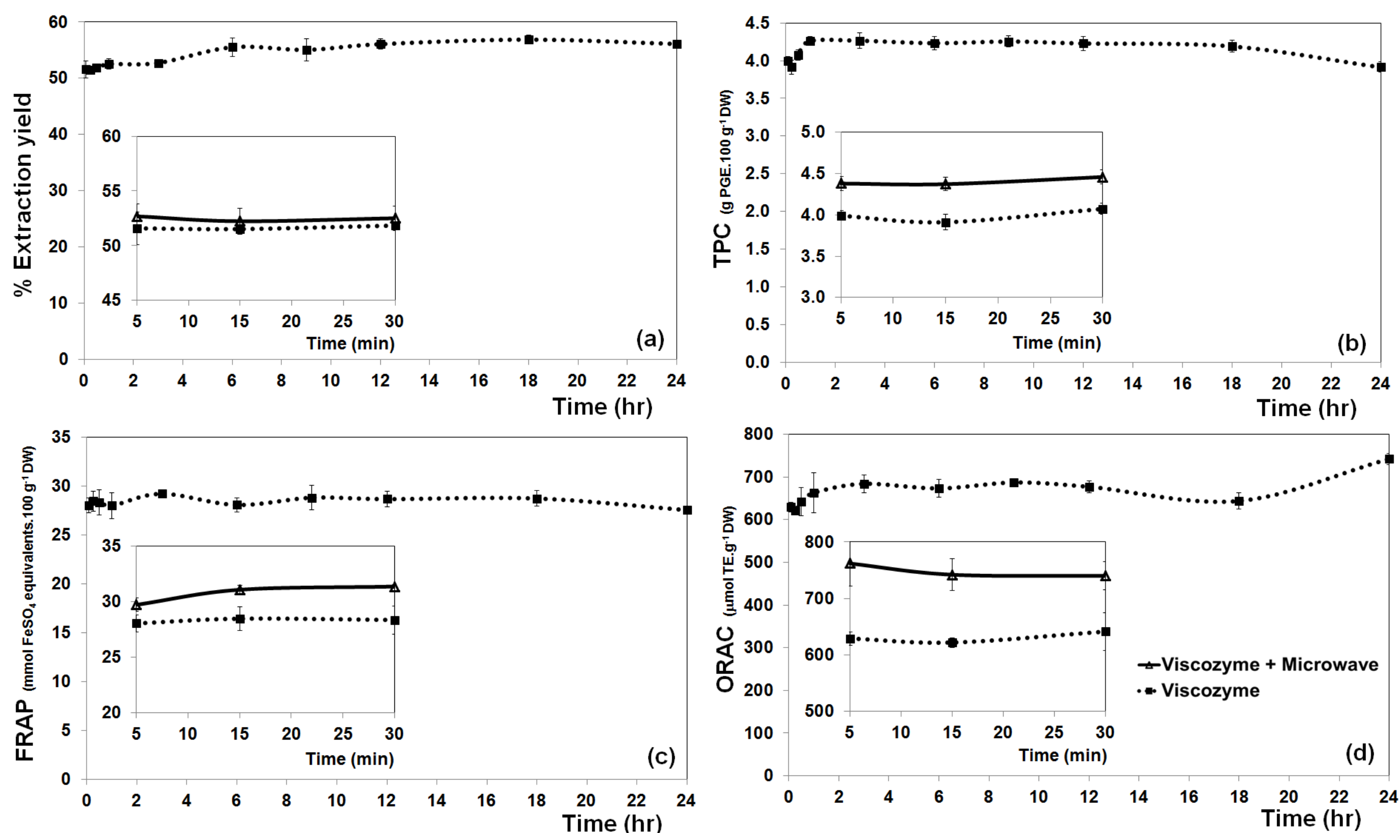

Figure 2.6 Effect of extraction duration (5 min to 24 h) tested of Viscozyme-assisted extraction on (a) %extraction yield, (b) total phlorotannin content, and antioxidant activities (c) FRAP assay and (d) ORAC assay: the same plot, zoomed in to better show the short time extraction from 5 to 30 min of Viscozyme with and without microwave intensification. The error bar represents the standard deviation of thee replicates.

The results of the enzymatic and microwave-assisted enzymatic extractions showed that microwave-assisted enzymatic extraction at 3 h was a more effective process to improve the TPC and antioxidant activities, compared to the enzymatic and conventional extraction at 24 h. Therefore, Viscozyme intensified with microwave for short extraction of 5 to 30 min was investigated in order to improve the extraction of phlorotannins and antioxidant compounds. Although Viscozyme intensified with microwave did not show much improvement ($p>0.05$) in the extraction yield (52.2 to 52.7%) compared to enzymatic extraction only, significantly higher ($p<0.05$) TPC (4.4 to 4.5 g PGE.100 g^{-1} DW) and antioxidant activities measured by both FRAP assay (29.7 to 31.5 mmol $FeSO_4$ equivalents.100 g^{-1} DW) and ORAC assay (740.1 to 762.1 μmol TE.g^{-1} DW) were obtained (Fig. 2.6, zoomed in plot).

2.5 Discussion

The phlorotannin content of *E. radiata* used in this investigation showed relative higher, but comparable levels than other seaweed sources (*Laminaria*/ *Saccharina* 0.2-5.3, *Fucus* <0.4-12.2, *Ascophyllum* 0.5-14, *Undaria* <0.4, *Sargassum* 1.1-12.7, and *Chondrus* and *Porphyra* <0.4% DW) (Holdt and Kraan, 2011).

Almost all enzyme treatments intensified with microwave showed significant improvement in TPC and antioxidant activities compared to enzymatic, conventional acid-base, and water extraction. Microwaves produce a very rough surface with many cavities resulting in increased porosity (Rodriguez-Jasso et al., 2011). This porous surface may allow better penetration of the enzymes and the resultant hydrolysis of cell wall structure will enhance the release of bioactive compounds.

It was noticed that extracts prepared by protease enzymes contained low total phlorotannin content. This could be partly because of the formation of protein-polyphenol complexes during extraction leading to aggregation and ultimate precipitation. Polyphenols may interact with proteins both reversible interactions with non-covalent forces (hydrogen and hydrophobic bonding) and van der Waals forces and irreversible interactions with covalent bonds (Ozdal et al., 2013). On the other hand, acidic extraction showed high TPC. The increase could be a result of the release of bound phenolic compounds under acid condition and the enhanced extractability of free and esterified phenolic compounds (Liyana-Pathirana and Shahidi, 2005). Acidification may also disintegrate cell walls and facilitate the solubilisation and diffusion of phenolic compounds (Campos et al., 2008; Shelembe et al, 2014). Moreover, low pH may prevent the oxidation of phenolic compounds due to the inhibition of enzymatic oxidation and the maintenance of the extract stability. Friedman and Jürgens (2000) reported that some phenolic compounds such as caffeic, chlorogenic, and gallic acids are not stable at high pH (7-11) because phenolic OH groups may create unstable quinone intermediates and other resonance forms which ultimately oxidized in the presence of air to diketo derivatives or other degradation products.

Viscozyme and Celluclast enzymes provided high antioxidant activities because these enzymes may work by breaking down the seaweed cell walls and complex interior storage materials to release compounds. Breakdown of these barriers can enhance the extraction of the desired bioactive compounds. It may also increase the antioxidant activities due to the release or reduction in high molecular weight polysaccharides and proteins (Heo et al., 2003a; Heo et al., 2005b). On the contrary, other enzymes did not show much improvement in the extraction of antioxidant compounds. This may be due to content of the seaweed species, but the high ORAC value of protease extracts may relate to the liberation of low molecular weight peptides and amino acids (Wang et al., 2010).

The reaction mechanism of ORAC assay was determined to follow the hydrogen atom transfer (HAT). This mechanism plays a dominant role in biological redox reactions (Ou et al., 2002). Therefore, the ORAC value of seaweed extracts was selected to compare the antioxidant activity with other well-known antioxidant-rich sources. The ORAC of Viscozyme and Alcalase extracts possessed strong antioxidant activities comparable to green and black tea (761.1±85.3 μmol TE.g^{-1} DW) (Prior and Cao, 1999) and greater than fresh blueberry (210-340 μmol TE.g^{-1} DW) (Speisky et al., 2012). In addition, they were higher than some fruits, vegetables, medicinal plants (100-500 μmol TE.g^{-1} DW) which are suggested as high antioxidant sources (Haytowitz and Bhagwat, 2010; Kameya et al., 2014; Kratchaova et al., 2010; Li et al., 2012). Therefore, *E. radiata* has high potential for use as functional food ingredients.

A correlation between TPC and antioxidant activities was examined during this investigation. High positive correlation between TPC and FRAP was found. The higher FRAP value of carbohydrase extracts could be attributed to the improved extraction efficiency of phlorotannins, whereas TPC and ORAC value showed low correlation. Therefore, it may imply that phlorotannins are not the only compounds with antioxidant potential in *E. radiata*. Some other active compounds such as pigments, low molecular weights of polysaccharides, proteins, or some organic compounds in seaweed extracts may also contribute to the overall antioxidant properties (Farvin and Jacobsen, 2013). In addition, the different results obtained with the ORAC and FRAP assays may be related to the kinetic action of antioxidants in each assay (Szydłowska-Czerniak et al., 2010). The FRAP assay is based on the reducing power of antioxidants, whereas the ORAC assay has been used to evaluate the antioxidant activity against the peroxyl radical-induced oxidation initiated by thermal decomposition of AAPH (2,20-azobis-(2-methylpropionamidine) dihydrochloride) (Ou et al., 2002). The positive correlation between total phenolic content and FRAP, and the low correlation between total phenolic content and ORAC have been observed by other researches (Dudonné et al., 2009; Li et al., 2012; Šamec et al., 2010; Szydłowska-Czerniak et al., 2010).

It was interesting to compare TPC between the microwave-assisted enzymatic extraction and the solvent extraction by acetone which is the most efficient extractant (Koivikko et al., 2005). The microwave-assisted enzymatic extraction using Viscozyme from 5 to 30 min gave TPC 4.4 to 4.5 g PGE.100 g^{-1} DW that is about 70% TPC of the acetone extraction (6.5 g PGE.100 g^{-1} DW). Therefore, enzyme intensified with microwave is an alternative process to extract bioactive compounds for food and pharmaceutical applications due to no organic solvent use.

2.6 Conclusions

High TPC and antioxidant activities were achieved by enzymatic extraction for a short duration; microwave-assisted enzymatic extraction was a more effective technique to increase the recovery of phlorotannins and antioxidant compounds. A short extraction duration of 5 to 30 min using Viscozyme intensified with microwave treatment is suggested for practical applications with extracts containing a high content of phlorotannins and high antioxidant activities measured by FRAP and ORAC assay. A major advantage of these finding is the time and cost-savings in improving extraction efficiency. Brown seaweed *E. radiata* extracts have a high potential to be developed for use as natural antioxidants and functional food ingredients with antioxidant activities comparable to other antioxidant-rich plants such as green and black tea.

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Appendix 2.1: First page of the published article

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Improved antioxidant activities of brown seaweed *Ecklonia radiata* extracts prepared by microwave-assisted enzymatic extraction

Suvimol Charoensiddhi · Chris Franco · Peng Su · Wei Zhang

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Abstract Seaweeds are important sources of bioactive compounds with potential use in functional foods and nutraceutical products. This study aims to investigate the extraction efficiency of phlorotannins and antioxidant compounds of a South Australian brown seaweed *Ecklonia radiata* by enzymatic and microwave-assisted enzymatic extraction in order to evaluate their uses as potential functional food ingredients. A selected group of carbohydrases (Viscozyme, Celluclast, and Ultraflo) and proteases (Alcalase, Neutrase, and Flavourzyme) has been applied to improve the extraction efficiency, alone and intensified with microwave heating, using conventional acid-base and water extractions as controls. The antioxidant activities of the extracts were evaluated using both ferric reducing ability of plasma (FRAP) and oxygen radical absorbance capacity (ORAC) assays. Significantly higher yields in total phlorotannin content (TPC) and antioxidant activities of the extracts were achieved by enzymatic and microwave-assisted enzymatic extraction. Microwave-assisted Viscozyme extraction for 5 to 30 min was the most effective process with an extraction yield achieved of 52 %. The extract had a TPC of 4.4 g phloroglucinol equivalents (PGE).100 g^{-1} dry weight (DW) and antioxidant activities of 29.7 mmol $FeSO_4$ equivalents.100 g^{-1} DW and 740.1 μmol Trolox equivalents (TE).g^{-1} DW. In contrast, the conventional acidic extraction for 24 hours resulted in a TPC of 3.4 g PGE.100 g^{-1} DW and antioxidant activities of 21.1 mmol $FeSO_4$ equivalents.100 g^{-1} DW and 512.4 μmol TE.g^{-1} DW. Extracts of brown seaweed *E. radiata* have potential for use in value-added products for nutritional purposes, using the microwave-assisted enzymatic extraction techniques.

Keywords Carbohydrase · FRAP · Macroalga · ORAC · Phlorotannins · Protease

S. Charoensiddhi · C. Franco · P. Su · W. Zhang
Centre for Marine Bioproducts Development, Flinders University, Adelaide, South Australia 5042, Australia

S. Charoensiddhi · C. Franco · P. Su · W. Zhang (✉)
Department of Medical Biotechnology, School of Medicine, Flinders University, Adelaide, South Australia 5042, Australia
e-mail: wei.zhang@flinders.edu.au

Introduction

The relationship between diet, health, and disease prevention is being widely recognized by consumers. There has been an increasing interest in research, development, and commercialization of functional foods, nutraceuticals, and dietary supplements around the world (Bernal et al. 2011; Freitas et al. 2012). Sources of functional ingredients from many different fruits, vegetables, cereals, and mushrooms have been explored in preference to marine macroalgae or seaweeds (Rasmussen and Morrissey 2007). As a new potential source of functional ingredients, a number of studies have reported that marine macroalgae produce a large variety of bioactive metabolites which are not produced by terrestrial plants (Plaza et al. 2008).

Among all macroalgae, brown seaweeds (Phaeophyceae) have relatively high diversity and endemism; of the 231 species reported in Southern Australia, 57 % are considered endemic (Womersley 1990). Despite this natural abundance, Australia remains a net importer of seaweed products, with 5000 T valued at over AUS$17 million imported during the 2008–2009 financial year (Lee 2010). Furthermore, much of what is harvested locally is underutilized and processed primarily into low-value commodities such as fertilizers and animal feeds (Lorbeer et al. 2013). Recent research on seaweed-derived functional food ingredients has shown that brown seaweeds are a rich source of nutraceuticals with a variety of bioactive compounds, mainly sulfated polysaccharides (fucoidan), phenolic compounds (phlorotannin), and

3. ENZYME-ASSISTED EXTRACTION OF CARBOHYDRATES FROM THE BROWN ALGA *ECKLONIA RADIATA*: EFFECT OF ENZYME TYPE, PH AND BUFFER ON SUGAR YIELD AND MOLECULAR WEIGHT PROFILES

Brown seaweeds are a rich source of polysaccharides, which account for up to 70% of their dry weight, with alginate, fucoidan, and laminarin typically constituting the main components. As detailed in chapter 1, diverse bioactivities, as well as useful physical properties have been reported for these polysaccharides, meaning they could have high potential for use as functional food ingredients.

Previous studies have shown that the buffer and pH conditions used during enzyme-assisted extractions influence the biological activities of oligo- and polysaccharides from seaweeds. However, specific impacts of these process parameters have not been reported, which is critical knowledge for process optimisation and design. Therefore, in this chapter we investigated the effects of selected parameters (enzyme, pH, and buffer) on the recovery of polysaccharides from brown seaweed *E. radiata*.

This article was published in “Process Biochemistry” (Vol. 51; 2016, pp. 1503-1510), and the first page of the publication is attached in Appendix 3.1.

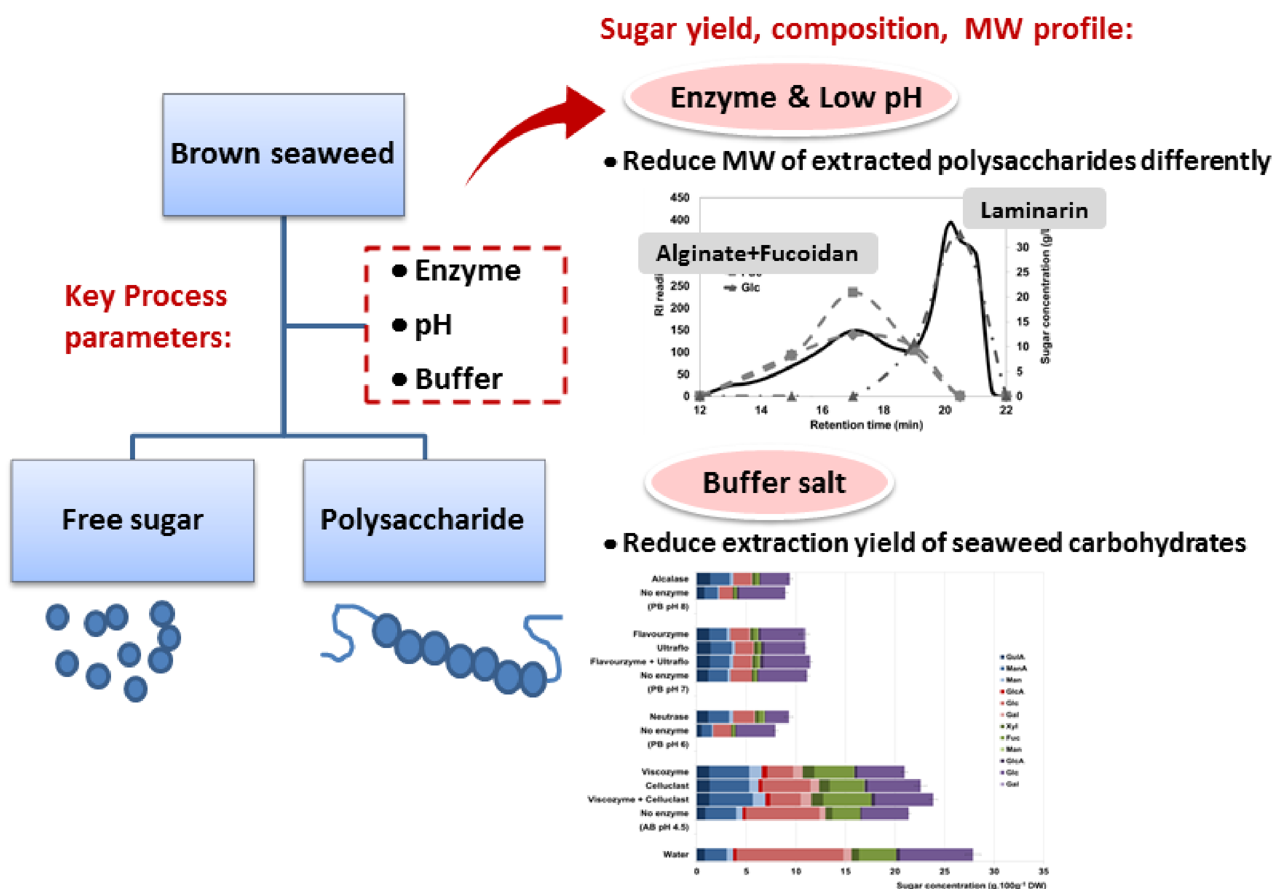
Author contributions: SC designed and performed all experiments as well as analysed data and wrote all primary contents. WZ helped decide on the research directions, scope, and plan. WZ and CF provided advice on the experimental results. AL assisted in the sugar analysis using high performance liquid chomatography (HPLC), and made suggestions for the experiment design. JL provided technical information and assistance to operate the high performance size exclusion chomatography (HP-SEC). VB provided advice on analysis using HP-SEC. All of the co-authors assisted with the revision of manuscript before and during the publication process.

Enzyme-assisted extraction of carbohydrates from the brown alga *Ecklonia radiata*: Effect of enzyme type, pH and buffer on sugar yield and molecular weight profiles

Suvimol Charoensiddhi[1,2], Andrew J. Lorbeer[1,2], Jelle Lahnstein[3], Vincent Bulone[3], Chistopher M.M. Franco[1,2], Wei Zhang[1,2]

[1] Centre for Marine Bioproducts Development, and [2] Department of Medical Biotechnology, School of Medicine, Flinders University, Adelaide, South Australia 5042, Australia
[3]ARC Centre of Excellence in Plant Cell Walls, School of Agriculture,
Food and Wine, University of Adelaide, Adelaide, South Australia 5064, Australia

Graphical abstract



3.1 Abstract

The aim of this study was to understand the carbohydrate yield, composition, and molecular weight (MW) profiles of extracts from the brown alga *Ecklonia radiata* prepared using an enzyme-assisted extraction approach. The most significant effect on the total sugar yield was observed when comparing the use of salt-containing buffers (7.9-23.9 g.100g^{-1} dry weight (DW)) to pH-adjusted water (23.6-27.9 g.100g^{-1} DW), with the buffers significantly reducing the extraction efficiency in all instances. The inclusion of selected enzymes had little or no impact on total sugar yield, when compared with the corresponding solvent or buffer-only extractions. Marginal differences on total sugar yield were found when using different carbohydrate hydrolytic enzymes and proteases, and when changing pH in pH-adjusted water extractions. Both enzymes and pH influence sugar composition, and significantly affect MW profile of the polysaccharide fractions but in different ways. The acidic extraction at pH 4.5 yields lower MW components. Enzyme-assisted extraction reduced the MW of the extracted polysaccharides by 20-50% compared to pH-adjusted water only extractions.

Key words Alginate; Carbohydrate extraction; Enzymatic hydrolysis; Fucoidan; Macroalgae

3.2 Introduction

Brown seaweeds (Phaeophyceae) have high diversity in Southern Australia, with 231 species reported, of which 57% are considered endemic [1]. Brown seaweeds are a rich source of functional food ingredients and nutraceuticals with a variety of bioactive compounds such as phenolic compounds (phlorotannins), carotenoids (e.g. fucoxanthin), polyunsaturated fatty acids (PUFAs) including omega-3 fatty acids, and bioactive proteins and peptides. The major components in brown seaweeds are polysaccharides, accounting for up to 70% of the dry weight, which also demonstrate many biological activities [2].

The principal polysaccharides in brown seaweeds are alginate and fucoidan, whilst laminarin is the main storage polysaccharide. Alginate is the major component of the brown seaweed cell wall, representing 15-50% of total dry weight. It is a linear acidic polysaccharide consisting of guluronic and mannuronic acids with β-(1,4)-linkages [3]. On the other hand, fucoidan is a water-soluble branched polysaccharide, which contains esterified sulfate groups and may cross-link alginate and cellulose. Fucoidan is mainly composed of L-fucose monomers linked though α-(1,2)-linkages, and represents 5-20% dry weight of brown seaweed [4]. Laminarin consists of glucose units linked by β-(1,3)-glycosidic bonds. This polysaccharide is essentially linear, although some β-(1,6) branches do occur along the main β-(1,3)-glucan chain [5].

Seaweed oligo- and polysaccharides have received increasing interest for functional food and pharmaceutical applications due to their unique physicochemical and biological properties [6,7]. A broad range of biological activities have been reported for alginate, fucoidan, and laminarin, including prebiotic, immunomodulatory, anti-inflammatory, and antitumor activities [8,9]. However, it is a challenge to efficiently extract oligo- and polysaccharides from brown seaweed due to the high degree of structural complexity and rigidity of the seaweed cell wall [9]. Current industrial extraction processes of carbohydrates from brown seaweed mainly involve chemical extractions. Acidic conditions are commonly used in both fucoidan and alginate extraction processes. For fucoidan, dilute acid extractions are used to disrupt hydrogen bonds leading to liberation of fucoidan [10], while acid treatments are used as a pre-treatment of alginate to remove contaminants before sodium carbonate or alkaline extraction [11]. Apart from acid and alkaline extractions, laminarin can be extracted with water at high temperature [12].

An alternative technology, enzyme-assisted aqueous extraction methods have recently attracted considerable attention due to their ability to degrade plant cell wall polysaccharides and facilitate the extraction of plant derived metabolites. This technology has shown potential to improve extraction efficiency in terms of yield, time, cost, environmental impact, and safety for food applications [13-19]. The available commercial carbohydrate hydrolytic enzymes and proteases were commonly used, though not the most efficient. The use of these enzymes is practically feasible in commercial applications and cost effective for industry, while the seaweed polysaccharide-specific hydrolytic enzymes such as fucoidanases and alginases are still difficult to access. These commercial enzymes were also chosen according to the characteristic of brown seaweed cell wall. Polysaccharides such as fucoidans are tightly associated with cellulose and proteins which limit their extractability by chemicals [20]. The hydrolysis of this cellulose and protein network by available commercial carbohydrate hydrolytic enzymes and proteases may enable the weakening of the cell wall complex and liberate target polysaccharides such as fucoidan and alginate without significant degradation.

Previous studies have shown that enzyme-assisted extractions performed in different buffer and pH conditions influence the biological activities of oligo- and polysaccharides from seaweeds [21-23]. However the impact of these process parameters such as the type of enzyme, pH, and buffer on the sugar profiles and MW distribution of seaweed extracts as the critical knowledge for process optimization and design have not been reported. We hypothesised that these process parameters have significant impact on the profiles of oligo- and polysaccharides in seaweed extracts, which exhibit different functional properties [24]. Therefore, the aims of the present study were to investigate the effects of commercial hydrolytic enzymes, pH, and buffers on the extraction yield, sugar composition, and MW profiles of carbohydrate extracts produced from the brown seaweed *Ecklonia radiata* (C. Agardh) J. Agardh. *E. radiata* was chosen as it is one of the most abundant brown seaweed species in southern Australia, with potential for commercial exploitation [25].

3.3 Materials and methods

3.3.1 Seaweed

The brown seaweed *Ecklonia radiata* was harvested from freshly deposited beach-cast seaweed in Rivoli Bay, Beachport, South Australia, in March 2013. The seaweed was rinsed in fresh water to remove any visible surface contaminants, and placed on mesh racks to dry. The whole plants were blended (Blendtec, USA), then passed though a 0.25-mm sieve, and dried in an oven at 45°C. The ground powder was stored at -20°C prior to extraction.

3.3.2 Chemicals

The monosaccharide, alginate and dextran MW standards were obtained from Sigma (St. Louis, MO, USA). All other chemicals were of analytical or HPLC grade and purchased from either Merck (Darmstadt, Germany) or Sigma.

3.3.3 Enzymes

Six commercial enzyme mixtures and individual enzymes from Novozyme (Bagsvaerd, Denmark) were used for the preparation of seaweed extracts, including thee cell wall carbohydrate hydrolytic preparations, namely Viscozyme® L, Celluclast® 1.5 L, Ultraflo® L and the thee proteases Alacalase® 2.4 L FG, Neutrase® 0.8 L and Flavourzyme® 1000 L. The conditions for optimum hydrolytic activity of each of these enzyme preparations and their characteristics are summarized in Table 3.1 [26,27].

Table 3.1 Optimal hydrolytic conditions, specificity and sources of the selected enzymes used in this study

Enzyme		Optimal conditions		Buffer used	Activities		Sources
		pH	Temperature (°C)		Main	Others	
Carbohydrate hydrolase	Viscozyme	4.5	50	0.1 N AB[b]	Endo-β-glucanase: Hydrolysis of (1,3)- or (1,4)-linkages in β-D-glucans	Xylanase Cellulase Hemicellulase	*Aspergillus aculeatus*
	Celluclast	4.5	50		Cellulase: Hydrolysis of (1,4)-β-D-glucosidic linkages in cellulose and other β-D-glucans	-	*Trichoderma reesei*
	Ultraflo	7.0	60[a]	0.2 M PB[c]	Endo-β-glucanase: Hydrolysis of (1,3)- or (1,4)-linkages in β-D-glucans	Cellulase Xylanase	*Humicola insolens*
Protease	Alcalase	8.0	50	0.2 M PB	Endoprotease: Hydrolysis of internal peptide bonds	-	*Bacillus licheniformis*
	Neutrase	6.0	50				*Bacillus amyloliquefaciens*
	Flavourzyme	7.0	50		Exopeptidase: Hydrolysis of N-terminal peptide bonds	Protease	*Aspergillus oryzae*

[a] Hydrolysis with Ultraflo was performed at 50°C in this study
[b] Acetate buffer
[c] Phosphate buffer

3.3.4 Preparation of seaweed extracts

The extraction processes used in this study are summarized in Fig. 3.1.

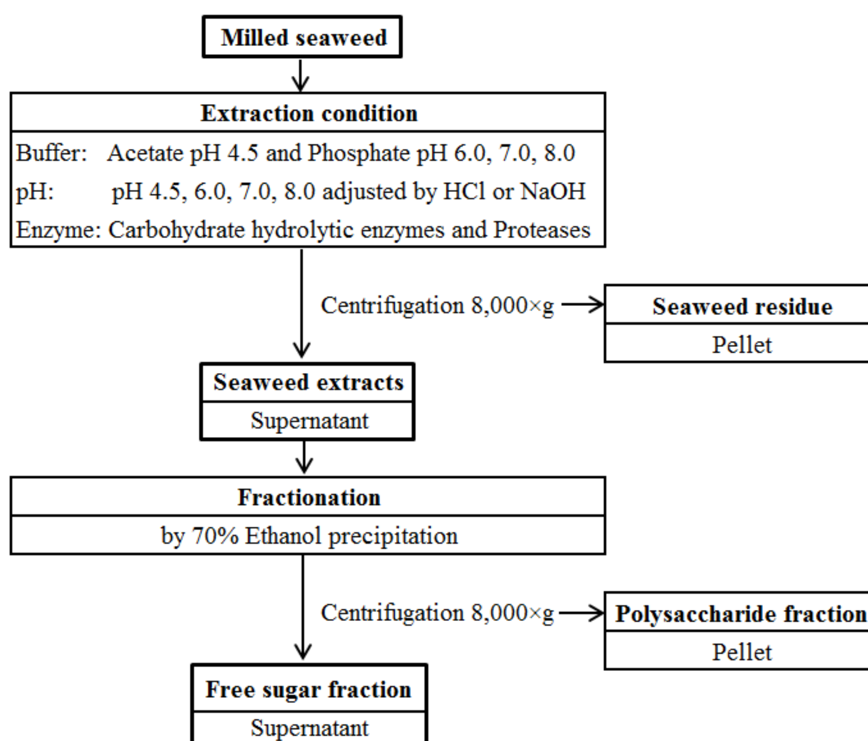


Figure 3.1 Extraction and fractionation processes of the carbohydrate components from brown seaweed *Ecklonia radiata*

3.3.4.1 Enzyme-assisted extraction

The enzyme-assisted extracts were prepared according to the method of Charoensiddhi et al. [27] with some modifications. Briefly, the dried seaweed powder was dispersed in either the relevant buffer solutions (details as in Table 3.1) or water adjusted to the optimal pH with the addition of 1 M HCl or 1 M NaOH, at alga solid to solvent ratio of 1:100 (w/v). The suspension was incubated for 10 min under continuous shaking in an orbital mixer incubator (Ratek Instruments, Australia), and the pH was then adjusted again in order to achieve a stable pH at the optimal conditions (no further adjustment was necessary to maintain the pH at 6.0). The enzyme preparation (10% v/w of alga) was then added, and the hydrolysis was performed at 50°C for 24 h (orbital incubator, continuous shaking). The enzyme was inactivated by boiling the sample at 100°C for 10 min and cooling immediately in an ice bath. The extract was centrifuged at 8000 g for 20 min at 4°C. The supernatant was then adjusted to pH 7.0 and lyophilized (Virtis freeze-dryer, USA) prior to further fractionation.

3.3.4.2 Extractions with conventional buffer and pH-adjusted water

Controls devoid of enzymes were performed under the same conditions as for the enzyme-assisted extractions.

3.3.4.3 Fractionation

The method of Lorbeer et al. [28] and Fleita et al. [29] was modified as follows (Fig. 3.1). The freeze dried extracts were resuspended in 70% (v/v) ethanol and centrifuged at 8000 g for 20 min at 4°C in order to separate the ethanol-insoluble fraction enriched in polysaccharides (pellet) from the free soluble mono/oligosaccharide compounds (supernatant). Both samples were dried in a centrifugal evaporator (Labconco, USA) and a freeze-dryer and kept at -20°C for further analysis.

3.3.5 Carbohydrate analyses

Carbohydrate analyses and determination of the MW distribution of the polysaccharides were carried out essentially as described in Lorbeer et al. [28], with the following modifications.

3.3.5.1 Monosaccharide analysis

The supernatant and pellet prepared by the ethanol precipitation step (Fig. 3.1) were subjected to acid hydrolysis by incubating the samples at room temperature for 1 hour in 72% (v/v) sulfuric acid, followed by a treatment in 1 M sulfuric acid at 100°C for 3 h. The monosaccharides released were derivatized with 1-phenyl-3-methyl-5-pyrazolone according to the method of Comino et al. [30]. The derivatives were separated and analyzed by HPLC (system: Prominence UFLC XR, Shimadzu; column: Kinetex 2.6u C18 100A, 100×3 mm, Phenomenex; detection: Prominence SPD-20A UV-VIS Detector, Shimadzu). Compounds were identified and quantified based on their retention time and peak areas, respectively, and comparison with monosaccharide standards (D-mannose, D-ribose, L-rhamnose, D-glucuronic acid, D-galacturonic acid, D-galactose, D-xylose, L-arabinose, L-fucose, D-glucose, and the internal standard 2-deoxyglucose). Sodium alginate from Sigma was subjected to the same procedure to allow for identification and quantification of the alginate-derived uronic acids. The quantitative results were then adjusted for acid degradation by treating standard sugars with the same hydrolysis protocol, and taking into consideration of any sugars from each enzyme preparation. In this study, alginate content was inferred from the guluronic and mannuronic acid quantitation. The fucose was likely to indicate the presence of fucoidan, whilst the majority of the glucose was likely derived from laminarin. The fucoidan from brown seaweed contains substantial amounts of L-fucose and sulfate ester groups. Other monosaccharides such as glucose, galactose, xylose, and mannose are found at very low concentrations [31].

3.3.5.2 Molecular weight determination

The MW profiles of the polysaccharide fractions were analyzed by size-exclusion chomatography on an HPLC (Agilent), using in-line PolySep GFC-P5000 and PolySep-GFC-P6000 columns (Phenomenex), with a mobile phase consisting of 0.1 M sodium nitrate. Elution was monitored using a refractive index detector (RID-10A, Shimadzu). The column was calibrated with standard dextrans from Sigma (peak MWs of 65, 195, 400, and 1050 kDa), and a standard curve was then established.

3.3.6 Statistical analysis

Experiments were conducted in triplicate and results are expressed as mean ± SD. One-way analysis of variance (ANOVA) was used to compare the means. Differences in monosaccharide content and relative MW were considered significant at $p<0.05$ using Duncan's test in the IBM SPSS Statistics 22 software (IBM Corporation Software Group, NY).

3.4 Results

3.4.1 Enzyme-assisted extraction of carbohydrates in buffer solution

Fig. 3.2a shows the effect of enzyme-assisted extractions of carbohydrates in buffer solution on total sugar yield and overall sugar composition in the seaweed extracts obtained, by comparison with the corresponding controls performed in the absence of enzymes and in the water. Carbohydrate hydrolases and proteases seemed to differently affect total sugar yield and composition. All carbohydrate hydrolytic preparations, except Ultraflo, produced around 2 times more total sugars (21.0-23.9 g.100g^{-1} DW) than proteases (9.3-11.0 g.100g^{-1} DW). The use of Neutrase, Celluclast, and the mixture of Viscozyme and Celluclast slightly improved the total sugar yield relative to the corresponding controls, while the other enzyme preparations did not. Total sugar yield (21.4 g.100g^{-1} DW) from the extract prepared at pH 4.5 were also significantly higher compared to extracts obtained at pH 6-8 (7.9-11.1 g.100g^{-1} DW). However, the use of different buffers may also affect the total sugar yield and composition. Indeed, considerable differences were observed in total sugar yield and composition between phosphate and acetate buffer extractions, regardless of whether enzymes were present or not. The polysaccharide and free sugar fractions obtained from the extracts prepared in acetate buffer contained significantly higher amounts of sugars (15.9-17.6 g.100g^{-1} DW and 4.9-6.3 g.100g^{-1} DW, respectively) compared to extractions performed in phosphate buffer (3.9-6.8 g.100g^{-1} DW of polysaccharide and 2.5-5.2 g.100g^{-1} DW of free sugar fractions). However, conventional water extraction (initial pH of approximately 6) led to the highest total sugar yield (27.9 g.100g^{-1} DW). As the buffer extraction at pH 6 yielded 7.9 g only of total sugar per 100g DW, it can be concluded that the use of phosphate buffer severely inhibited the extraction of carbohydrates. Therefore, further extractions were performed using pH-adjusted water in the presence or absence of enzymes.

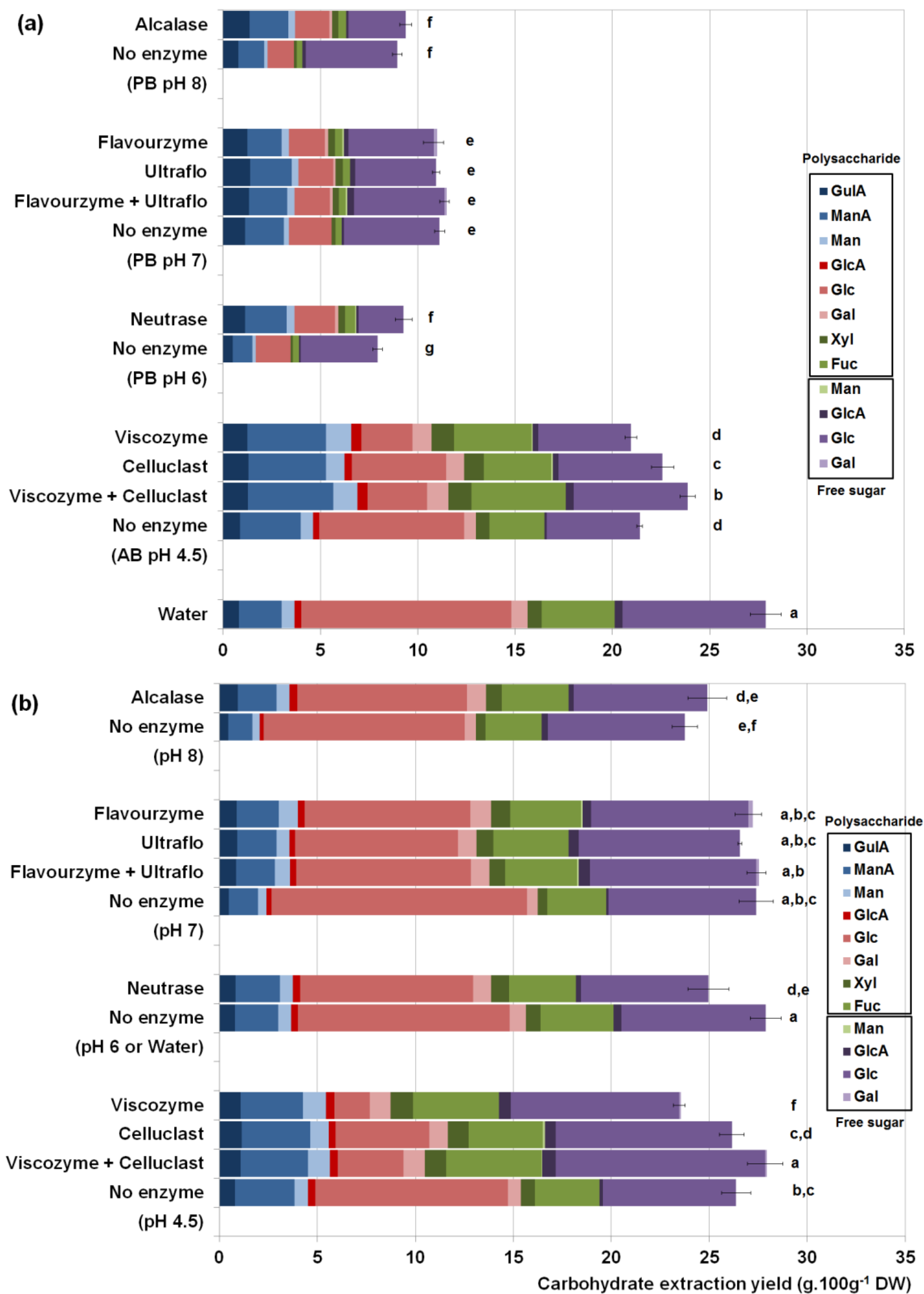


Figure 3.2 Total carbohydrate extraction yield and overall sugar composition of extracts obtained in the presence or absence of commercial enzymes, in (a) phosphate (PB) and acetate (AB) buffer solutions, and (b) pH-adjusted water. The polysaccharide and free-sugar enriched fractions are combined on the same bar for each treatment. Experiments were conducted in triplicate, the data were reported as mean ± SD. Values with different letters are significantly different ($P<0.05$), independent association between 3.2 (a) and (b). GulA, ManA, Man, GlcA, Glc, Gal, Xyl,

and Fuc represent guluronic acid, mannuronic acid, mannose, glucuronic acid, glucose, galactose, xylose, and fucose, respectively.

3.4.2 Enzyme-assisted extraction in pH-adjusted water

Fig. 3.2b shows total sugar yield and sugar composition of extracts obtained by enzyme-assisted extraction in pH-adjusted water. In each case, the total sugar yields of both polysaccharide and free sugar fractions significantly increased when the buffer was replaced with pH-adjusted water. This result confirmed that the extraction process was indeed inhibited by the buffer solutions. This effect was particularly obvious with the pH 6-8 treatments, where the removal of the phosphate buffer gave a ~2.5-fold increase in total sugar yield (from 7.9-11.1 to 23.8-27.9 $g.100g^{-1}$ DW). Therefore, the extractions performed in pH-adjusted water were used for further MW profile analysis.

Marginal differences were observed in the total sugar yield between the carbohydrate hydrolytic enzymes (23.6-27.9 $g.100g^{-1}$ DW) and protease (24.9-27.2 $g.100g^{-1}$ DW) extractions using pH-adjusted water. Enzyme-assisted extractions did not improve the extraction yield of total sugars compared with respective pH-adjusted water extractions, except for the mixture of Viscozyme and Celluclast, which led to a slight improvement from 26.4 to 27.9 $g.100g^{-1}$ DW. Also, marginal differences in total sugar yield (23.8-27.9 $g.100g^{-1}$ DW) were observed in the pH range 4.5-8. The lowest yield from pH-adjusted water extractions was observed at pH 8. Monosaccharide composition analysis indicated that glucose was the major component of all seaweed extracts for both the polysaccharide and free sugar fractions (Fig. 3.2a and b). Apart from glucose, the polysaccharide fractions also consisted of relatively high proportions of fucose, followed by guluronic acid and mannuronic acid, and small amounts of mannose, glucuronic acid, galactose, and xylose.

The sugar compositions of the polysaccharide and free sugar fractions obtained by enzyme-assisted extraction in pH-adjusted water are shown in Fig. 3.3a and b, respectively. All enzymatic extractions, except that performed with Neutrase, improved the extraction of guluronic and mannuronic acid as well as fucose. In particular, the carbohydrate hydrolytic enzymes (Viscozyme, Celluclast, and the mixture of Viscozyme and Celluclast) led to a yield of these sugars of 4.3-4.9 $g.100g^{-1}$ DW. The glucose content of the polysaccharide fractions, however, was reduced with these enzyme preparations, as glucose-containing polysaccharides were selectively digested. As such, all treatments based on carbohydrate hydrolases, with the exception of the Ultraflo alone, increased the amount free glucose and total free sugar (8.6-11.5 $g.100g^{-1}$ DW) obtained compared to other enzyme-assisted and pH-adjusted water extractions (6.5-8.8 $g.100g^{-1}$ DW). The polysaccharide fractions obtained at pH 4.5, 6, and 7 led to similar total sugar yields (19.4 to 20.1 $g.100g^{-1}$ DW), which were higher than that at pH 8 (16.5 $g.100g^{-1}$ DW) (Fig. 3.3a). The highest guluronic and mannuronic acid (3.0-3.9 $g.100g^{-1}$ DW) and fucose contents (3.3-3.7 $g.100g^{-1}$ DW) were found in the seaweed extracts obtained at pH 4.5 and 6, while the extract at pH 7 exhibited the highest glucose

content (13.0 g.100g^{-1} DW). The free sugar fractions obtained at pH 4.5-8 showed a small difference in total sugar and glucose yields (6.8-7.8 g.100g^{-1} DW) (Fig. 3.3b).

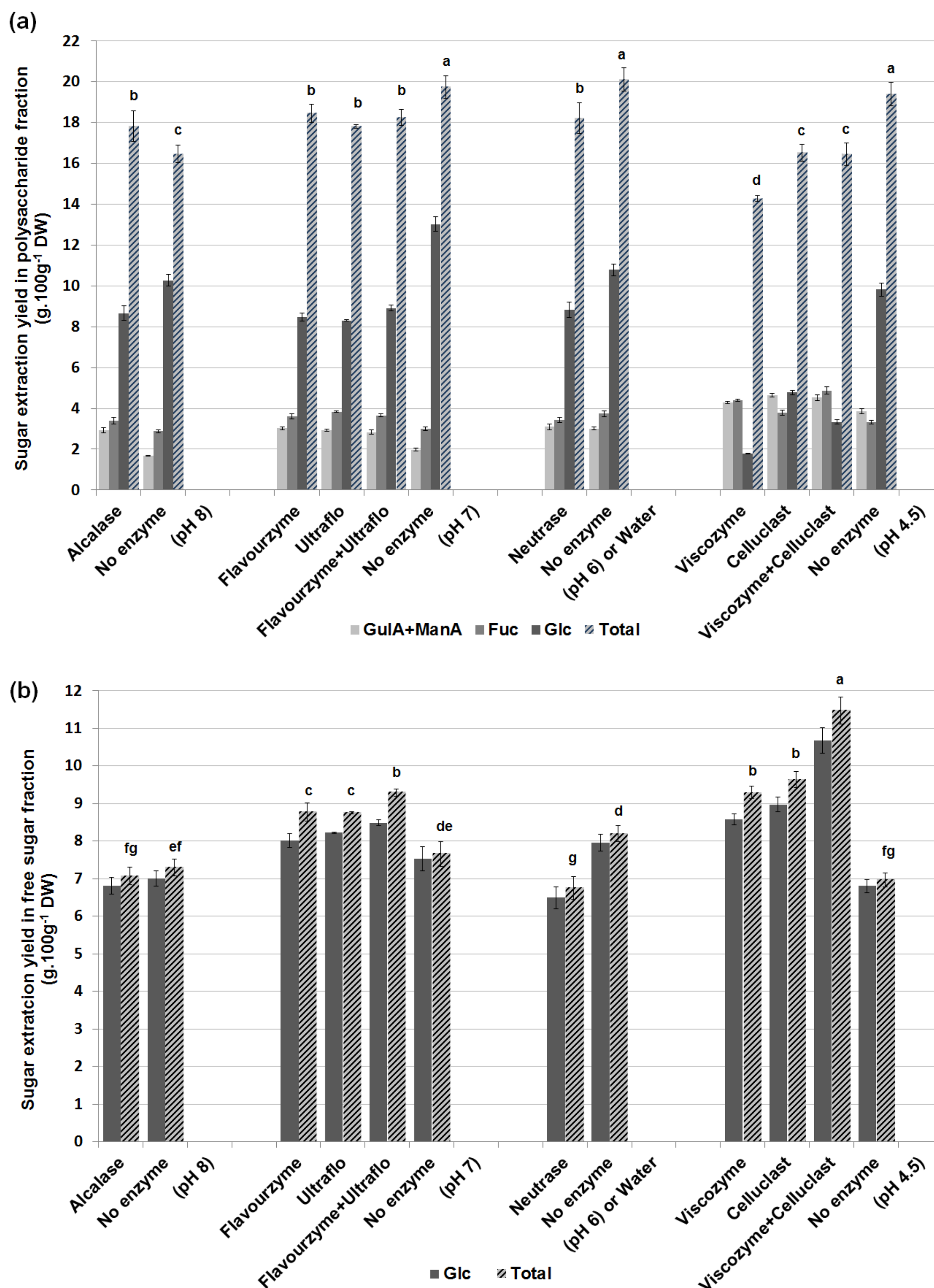


Figure 3.3 Monosaccharide components of (a) the polysaccharide fractions and (b) the free sugar fractions from the enzyme-assisted extractions and the pH-adjusted water extractions (controls) at various pHs; experiments were conducted in triplicate, the data were reported as mean ± SD. Values with different letters are significantly different (P<0.05). The abbreviations used for the different sugars are the same as in Fig. 3.2.

3.4.3 Polysaccharide composition and molecular weight profile of seaweed extracts

Polysaccharide MWs were estimated by size exclusion chomatography, relative to dextran standards. The sugar compositions of each collected fraction were also analyzed in order to identify the main polysaccharides corresponding to different peaks. Both enzymes and pH had significant effect on the MW and polysaccharide content of the seaweed extracts, but acted differently. Similar MW profiles were found in seaweed extracts obtained at pH 6, 7, and 8 (Fig. 3.4a). Thee peaks were identified on those chomatograms. Peak 1a (relative MW >1050 kDa, dextran equivalent) contained alginate and fucoidan; Peak 2a (relative MW ranging from 295 to 619 kDa) contained mainly alginate and fucoidan; and Peak 3a (relative MW <65 kDa) contained laminarin. On the other hand, the extract obtained at pH 4.5 contained two peaks with a narrower and lower MW range than the other extracts (Fig. 3.4b). This composition appeared to be a mixture of alginates and fucoidans with relative MWs of 294-493 kDa (Peak 1b), and laminarins with an average relative MW <65 kDa (Peak 2b).

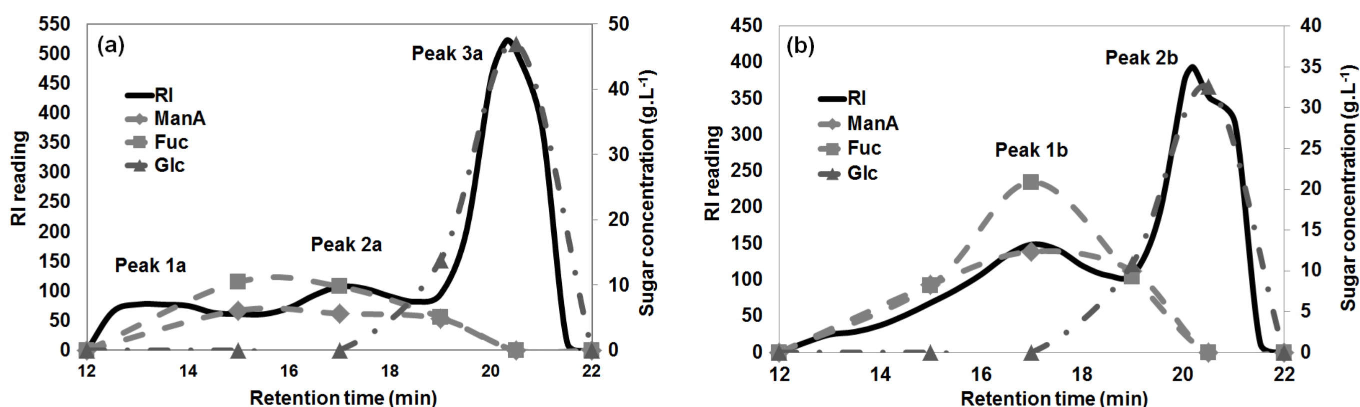


Figure 3.4 Molecular weight profiles and main sugar components (as indicated by the sugar analysis of collected fractions) of polysaccharides in the fractions prepared by extraction at (a) pH 8 (similar to the chomatogram of pH 6 and 7) and (b) pH 4.5, in the absence of enzymes. The mannuronic acid (ManA), fucose (Fuc), and glucose (Glc) content in the samples reflect the major presence of alginate, fucoidan, and laminarin, respectively.

The sugar composition of the samples obtained after size-exclusion chomatography of the polysaccharide fractions arising from the enzyme-assisted extractions and their respective pH-adjusted water extractions are summarized in Table 3.2. Seaweed extracts at pH 6-8 contained fucose as a main sugar in the high relative MW range (Peak 1), while glucose was the only monosaccharide detected in the low relative MW range (Peak 3). For the intermediate relative MW range (Peak 2), mannuronic acid, glucose, and xylose were found in most samples, in addition to the main component fucose. Similar sugar profiles were observed after extraction at pH 4.5. A main sugar in the intermediate relative MW range (Peak 1) was fucose, followed by mannuronic acid and xylose, and a small amount of galactose. Glucose was the only sugar detected in the relatively low

MW range (Peak 2). The use of enzyme preparations significantly reduced the average MWs of the extracted polysaccharides by about 20-40% for carbohydrate hydrolases and 20-50% for proteases. Sugar compositions of each of the fractions prepared using carbohydrate hydrolases or proteases were similar to that of the corresponding pH-adjusted water control.

Table 3.2 Sugar composition (molar ratio) and relative molecular weights of polysaccharides (size exclusion chomatography); values with different letters are significantly different (P<0.05).

Extraction condition		Sugar composition (Molar ratio)											Relative molecular weight (kDa)*		
		Peak 1					Peak 2					Peak 3	Peak 1	Peak 2	Peak 3
		ManA	Glc	Gal	Xyl	Fuc	ManA	Glc	Gal	Xyl	Fuc				
Alcalase	pH 8.0	0.3	-	-	-	1.0	0.6	0.5	0.3	0.8	1.0			295^{f}	
No enzyme		0.2	-	-	-	1.0	0.7	0.9	-	0.9	1.0			$385^{c,d,e}$	
Flavourzyme	pH 7.0	0.3	-	-	-	1.0	0.5	0.3	-	0.7	1.0			$329^{e,f}$	
Ultraflo		0.3	-	-	-	1.0	0.6	0.3	-	0.7	1.0			$445^{b,c}$	
Flavourzyme +Ultraflo		0.3	-	-	-	1.0	0.6	0.3	-	0.6	1.0	Only Glc detected	>1050	$339^{d,e,f}$	<65
No enzyme		0.3	-	-	-	1.0	0.7	0.7	-	0.8	1.0			619^{a}	
Neutrase	pH 6.0	0.4	-	-	-	1.0	0.6	0.4	-	0.6	1.0			$402^{c,d}$	
No enzyme		0.4	-	-	-	1.0	0.6	0.5	-	0.5	1.0			602^{a}	
Viscozyme	pH 4.5	0.6	-	0.1	0.4	1.0						-	304^{f}		-
Celluclast		0.7	-	0.1	0.3	1.0						-	395^{cd}		-
Viscozyme +Celluclast		0.6	-	0.1	0.3	1.0	Only Glc detected					-	294^{f}	<65	-
No enzyme		0.8	0.3	-	0.4	1.0						-	493^{b}		-

* Mean value from thee independent tests, reported in terms of dextran-equivalent hydrodynamic volume
* Sugar abbreviations are as defined in the legend of Fig. 3.2.

3.5 Discussion

The use of enzymes, especially carbohydrate hydrolases (Viscozyme, Celluclast, and the mixture of these enzymes) in pH-adjusted water, led to an increased glucose yield from the free sugar fraction, and improved extraction of fucoidan and alginate in the polysaccharide fraction, as illustrated in Fig. 3.3. This indicates that these enzymes could hydrolyse glucose-containing polysaccharides in the seaweed cell walls, perhaps assisting with partial disintegration of the cell wall matrix, particularly cellulose and laminarin. The apparent reduction in MW of all the extracted polysaccharides suggests that these enzymes have the ability to hydrolyze certain bonds within fucoidan and/or alginate molecules. However, the overall hydrolytic efficiency of the enzymes appeared to be relatively low. Current commercially available hydrolytic enzyme degradation could be hindered by the limited access of the enzymes to their specific substrates. The hydrolysis of cellulose networks in the cell wall may release alginate and fucoidan with their well-preserved MW and properties. The partially depolymerized polysaccharide such as fucoidan with a high MW (~390-2200 kDa) showed the potential to induce anticancer activity [32], for instance. On the other hand, the development of more specific seaweed polysaccharide hydrolytic enzyme preparations is also required in order to improve extraction efficiency, and aid in the production of lower MW oligosaccharides with a variety of biological activities [33]. Therefore, it is important to select the appropriate enzymes to best suit the desired applications of the final products. Other possibilities for low hydrolytic efficiency of carbohydrate hydrolases may be due to the physical and chemical barriers of seaweeds for the extraction process including insoluble and soluble fibre, other cell wall materials, and the gelling characteristic of alginate [34]. In addition, the enzyme activities may also be inhibited by the presence of different cellular components including polyphenols. However the purpose of this study was to use the enzymes to assist the extraction of seaweed carbohydrates, rather than degradation and hydrolyze seaweed polysaccharides. The seaweed polysaccharides-specific hydrolytic enzymes such as fucoidanases and alginase could be explored in the future for the production of low MW functional oligosaccharides.

pH significantly affected the total sugar yield and carbohydrate composition in buffer extractions. A significant increase in total sugar yield was obtained at pH 4.5 compared to pH 6-8. However, this might have been due to the use of different concentrations of buffer salts. A minor impact of pH was observed in pH-adjusted water only extraction. When using pH-adjusted water, higher contents of fucose were observed in the extracts obtained at pH 4.5 and pH 6. This result is in agreement with the previous observation that fucoidan is readily soluble in water and acid solutions [35]. The same trend was also observed at lower pH, with higher contents of guluronic and mannuronic acids. Acidic conditions generally inhibit the extraction of alginates, by converting alginate salts into insoluble alginic acids. However, the acid extraction at pH 4.5 did not affect the precipitation or hydrogel formation because the pH of the alginate containing solution is still higher than pK_a of the carboxyl groups of mannuronic acid (pK_a 3.38) and guluronic acid (pK_a 3.65) [36]. Additionally, Lorbeer et al.

[37] showed that some alginates are still extracted at a pH of 5, and the extraction of these polysaccharides decreases significantly as the pH decreases towards 2. Alginate is generally extracted using strong alkali conditions (pH ~10, 80°C) [11]. In our study, the use of pH 8 was probably not optimal to extract alginate efficiently; and high viscosity might affect the extraction efficiency [38]. The polysaccharide extracted at pH 7 exhibited high glucose content, indicating the extraction of laminarin. As shown by Kadam et al. [12], laminarin could be efficiently extracted using high temperature conditions (50-90°C) with water as a solvent. Besides the effect of extraction conditions on sugar yield and composition, MW profiles of the polysaccharide fractions were analyzed. The lower MW polysaccharides were more abundant when the extraction was conducted at pH 4.5. This result suggests that mildly acidic conditions are sufficient to partially hydrolyze fucoidan, resulting in a reduction in MW, and the application of diluted acid at ambient temperature may also result in the partial cleavage of the sulfate ester bonds in fucoidan [10,39].

As opposed to water, the use of buffers severely inhibited the extraction of polysaccharides regardless of the presence of enzymes. This may be explained by the different osmotic environment resulting from the exposure of the algal biomass in buffers or water. As marine brown algae have adapted to salty environments, placing them into pure water may cause an osmotic shock and the rapid influx of water into their cells, thereby facilitating the disruption of the structural integrity of the cell wall and facilitating extraction. Therefore, the higher solute content of the buffers (especially phosphate, which was twice as concentrated as the acetate buffer) may have protected the alga from such osmotic shock, when compared to the pH-adjusted water. This observation is in agreement with previous reports where osmotic shock was used as one of the most efficient approach to facilitate cell disruption [40,41]. Apart from the osmotic effect, the change of ionic strength affects the solubility of alginate, the main carbohydrate component of brown seaweed. The high amount of monovalent salts is one of a limiting condition for the solubilization of alginate. The decrease in alginate chain solubility and precipitation may occur by high concentrations of salts. The salting-out effects and the reduction in dissolution kinetics are also exhibited in the salt concentration less than 0.1 M [42]. Hence, it can be concluded that pH-adjusted water extraction without buffer is a key for the efficient extraction of seaweed polysaccharides.

With this approach, glucose was the major sugar in both the polysaccharide and free sugar fractions of all seaweed extracts. Higher contents of fucose, guluronic and mannuronic acids were found in the polysaccharide fractions only. Glucose was detected in the lower MW fractions after size-exclusion chomatography, while fucose and mannuronic acid occurred essentially in the higher MW fractions. The apparent MW of laminarin is typically around 5000 Da [43], suggesting that higher amounts of laminarin were extracted. Alginates and fucoidans, which typically have a higher MW than laminarin [44], were detected in the high MW fractions after size exclusion chomatography, as indicated by the presence of the alginate-derived uronic acids, and fucose, respectively. However, the detection of some glucose in the high MW fractions might have resulted from the minor presence

of glucosyl residues in fucoidans, or glucose-containing polysaccharides remaining associated with the alginate or fucoidan.

3.6 Conclusions

Enzyme type and pH had minor impact on the total sugar yield but affected differently the sugar composition of the carbohydrate extracts prepared by enzyme-assisted extraction of brown seaweed. They both played a key role in the reduction of the MW of the extracted polysaccharides, but differently. All seaweed extracts contained high MW polysaccharides that corresponded to alginates and fucoidans, and the low MW fractions were rich in laminarin. High concentrations of buffer salts were found to severely inhibit polysaccharide extraction, so buffers should be avoided to maximize the efficiency of the enzyme-assisted extraction of seaweed carbohydrates. These results are critical for the design and optimization of enzyme-assisted extraction processes of oligo- and polysaccharide from brown seaweed and the exploitation of seaweed carbohydrates in food industry.

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Appendix 3.1: First page of the published article

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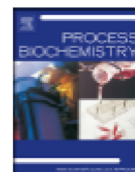

Enzyme-assisted extraction of carbohydrates from the brown alga *Ecklonia radiata*: Effect of enzyme type, pH and buffer on sugar yield and molecular weight profiles

Suvimol Charoensiddhi[a,b], Andrew J. Lorbeer[a,b], Jelle Lahnstein[c], Vincent Bulone[c], Christopher M.M. Franco[a,b], Wei Zhang[a,b,*]

[a] Centre for Marine Bioproducts Development, School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia
[b] Department of Medical Biotechnology, School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia
[c] ARC Centre of Excellence in Plant Cell Walls, School of Agriculture, Food and Wine, University of Adelaide, Adelaide, South Australia 5064, Australia

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ABSTRACT

The aim of this study was to understand the carbohydrate yield, composition, and molecular weight (MW) profiles of extracts from the brown alga *Ecklonia radiata* prepared using an enzyme-assisted extraction approach. The most significant effect on the total sugar yield was observed when comparing the use of salt-containing buffers (7.9–23.9 g × 100 g^{-1} dry weight (DW)) to pH-adjusted water (23.6–27.9 g × 100 g^{-1} DW), with the buffers significantly reducing the extraction efficiency in all instances. The inclusion of selected enzymes had little or no impact on total sugar yield, when compared with the corresponding solvent or buffer-only extractions. Marginal differences on total sugar yield were found when using different carbohydrate hydrolytic enzymes and proteases, and when changing pH in pH-adjusted water extractions. Both enzymes and pH influence sugar composition, and significantly affect MW profile of the polysaccharide fractions but in different ways. The acidic extraction at pH 4.5 yields lower MW components. Enzyme-assisted extraction reduced the MW of the extracted polysaccharides by 20–50% compared to pH-adjusted water only extractions.

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1. Introduction

Brown seaweeds (Phaeophyceae) have high diversity in Southern Australia, with 231 species reported, of which 57% are considered endemic [1]. Brown seaweeds are a rich source of functional food ingredients and nutraceuticals with a variety of bioactive compounds such as phenolic compounds (phlorotannins), carotenoids (e.g. fucoxanthin), polyunsaturated fatty acids (PUFAs) including omega-3 fatty acids, and bioactive proteins and peptides. The major components in brown seaweeds are polysaccharides, accounting for up to 70% of the dry weight, which also demonstrate many biological activities [2].

The principal polysaccharides in brown seaweeds are alginate and fucoidan, whilst laminarin is the main storage polysaccharide. Alginate is the major component of the brown seaweed cell wall, representing 15–50% of total dry weight. It is a linear acidic polysaccharide consisting of guluronic and mannuronic acids with β-(1,4)-linkages [3]. On the other hand, fucoidan is a water-soluble branched polysaccharide, which contains esterified sulfate groups and may cross-link alginate and cellulose. Fucoidan is mainly composed of L-fucose monomers linked through α-(1,2)-linkages, and represents 5–20% dry weight of brown seaweed [4]. Laminarin consists of glucose units linked by β-(1,3)-glycosidic bonds. This polysaccharide is essentially linear, although some β-(1,6) branches do occur along the main β-(1,3)-glucan chain [5].

Seaweed oligo- and polysaccharides have received increasing interest for functional food and pharmaceutical applications due to their unique physicochemical and biological properties [6,7]. A broad range of biological activities have been reported for alginate, fucoidan, and laminarin, including prebiotic, immunomodulatory, anti-inflammatory, and antitumor activities [8,9]. However, it is a challenge to efficiently extract oligo- and polysaccharides from brown seaweed due to the high degree of structural complexity and rigidity of the seaweed cell wall [9]. Current industrial extraction processes of carbohydrates from brown seaweed mainly involve chemical extractions. Acidic conditions are commonly used in both fucoidan and alginate extraction processes. For fucoidan, dilute acid

* Corresponding author at: Centre for Marine Bioproducts Development, School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia.
E-mail address: wei.zhang@flinders.edu.au (W. Zhang).

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4. IMPACT OF EXTRACTION PROCESSES ON PREBIOTIC POTENTIAL OF THE BROWN SEAWEED *ECKLONIA RADIATA* BY *IN VITRO* HUMAN GUT BACTERIA FERMENTATION

Interest in prebiotics as functional foods has increased due to their recognised health benefits. The high content of polysaccharides in brown seaweeds; their complex structures, which make them resistant to degradation by human digestive enzymes; and their susceptibility to fermentation by gut microbes make brown seaweeds a promising potential source of prebiotics.

In this study, we tested the prebiotic potential of various *E. radiata* extracts, which had been recovered using the extraction processes described in this chapter and the preceding chapter. An *in vitro* human gut model was used, which involved the anaerobic fermentation of the extracts with faecal inocula that mimic the microflora of the human large intestine.

This study was orally presented at the 1st Australia New Zealand Marine Biotechnology Society Symposium in Adelaide on April 2016, and published in the "Journal of Functional Foods" (Vol. 24; 2016, pp. 221-230), and the first page of the publication is attached in Appendix 4.1.

Author contributions: SC designed and performed all experiments as well as analysed data and wrote all primary contents. WZ and MC provided advice on research directions and the scope of the manuscript. WZ, MC, and CF provided advice on the experimental plan and results. MV provided training for the molecular experiments (DNA extraction and Q-PCR) and the use of an anaerobic chamber. All of the co-authors assisted with the revision of manuscript before and during the journal peer-review process.

Impact of extraction processes on prebiotic potential of the brown seaweed *Ecklonia radiata* by *in vitro* human gut bacteria fermentation

Suvimol Charoensiddhi [a,b], Michael A. Conlon [c], Michelle S. Vuaran [c], Chistopher M.M. Franco [a,b], Wei Zhang [a,b,]

[a] Centre for Marine Bioproducts Development, School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia
[b] Department of Medical Biotechnology, School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia
[c] CSIRO Food and Nutrition, Kintore Avenue, Adelaide, SA 5000, Australia

4.1 Abstract

The prebiotic potential of the extracts of a South Australian brown seaweed *Ecklonia radiata* obtained using different processes was investigated. Six extracts of this seaweed were prepared by enzymatic, acidic, and water extraction processes. The extracts were added to an *in vitro* anaerobic fermentation system containing human faecal inocula to assess their ability to generate short chain fatty acids (SCFA) and to promote the growth of selected bacterial genera (as assessed by quantitative PCR). Following 24 h fermentation, all seaweed extracts significantly increased ($P<0.05$) total SCFA production (50.7-72.7 μmol/mL) and the total number of bacteria ($\log_{10}$ 10.2-10.4 cells/mL) when compared to controls (blank and cellulose). The extracts prepared using Celluclast-assisted extraction showed the greatest potential for improving gut health as these induced significantly higher production of butyrate (9.2 μmol/mL) and growth of bacteria regarded as beneficial, including *Bifidobacterium* ($\log_{10}$ 6.6 cells/mL) and *Lactobacillus* ($\log_{10}$ 5.3 cells/mL).

Key words Carbohydrate; Enzyme-assisted extraction; Gut microbe; Macroalgae; Prebiotic activity; Short chain fatty acid

4.2 Introduction

The relationship between diet and health is being widely recognised by consumers, and this lead to demand for food ingredients with functionality and health benefits. There is growing evidence that indigestible dietary polysaccharides can have a significant benefit to health though their ability to stimulate the growth of beneficial microbes in the large bowel (Conlon & Bird, 2015), and hence may fulfil the definition of a prebiotic. Examples of prebiotic polysaccharides include inulin, fructooligosaccharides, galactooligosaccharides and lactulose (Kolida & Gibson et al., 2008; Al-Sheraji et al., 2013). Other dietary components, including polyphenols and proteins, can also reach the large bowel and potentially exert a health benefit via activities of the gut microbiota (Windey et al., 2012; Cardona et al., 2013).

Brown seaweeds (Phaeophyceae) have a relatively high diversity among all the macroalgae. Of the 231 species reported in Southern Australia 57% are considered endemic (Womersley, 1990). Recent research on seaweed-derived functional food ingredients has shown that brown seaweeds have high nutraceutical potential, containing a variety of bioactive compounds such as phloroglucinol-based polyphenolic compounds (phlorotannins), carotenoids (fucoxanthin), polyunsaturated fatty acids (PUFAs), and bioactive peptides (Holdt & Kraan, 2011). In addition, brown seaweeds are a rich source of polysaccharides possessing various biological functions (Synytsya et al., 2015). The complex structure of brown seaweed polysaccharides, mainly alginate, fucoidan, and laminarin, makes them resistant to degradation by human digestive enzymes, and as a consequence can be regarded as dietary fibres. Most of these can be fermented by gut microbiota which may provide a health benefit to humans though a prebiotic effect (O'Sullivan et al., 2010; Zaporozhets et al., 2014). Recent studies report that polysaccharides and oligosaccharides derived from seaweeds are potential modulators of intestinal metabolism including fermentation, inhibitors of pathogen adhesion and evasion, and may even treat inflammatory bowel disease (Deville et al., 2007; Ramnani et al., 2012; Kuda et al., 2015; Lean et al., 2015). In addition, the wide variety of phlorotannins (polyphenols) present in brown seaweed (Li et al., 2011) may not be readily absorbed in the small intestine, and would reach the large intestine where they can be converted by microbial activity into beneficial bioactive phenolic metabolites (Cardona et al., 2013).

South Australia has one of the world's highest diversity of brown seaweed, and *Ecklonia radiata* (C. Agardh) J. Agardh is one of the most abundant species (Lorbeer et al., 2013). The aim of this present study was to examine whether *E. radiata* can be utilised as a source of prebiotic components, and how different extraction processes can impact on their prebiotic potential. Given the high degree of structural complexity and rigidity of seaweed cell walls (Deniaud-Bouët et al., 2014) that present a major obstacle to the efficient extraction of polysaccharides and other bioactive compounds, enzyme-assisted extraction (EAE) was used. EAE has attracted considerable interest in the release of high molecular weight intracellular compounds such as polysaccharides, polyphenols, and

proteins (Kadam et al., 2013; Wijesinghe & Jeon, 2012), however only a few studies have examined the prebiotic potential of enzyme-assisted extracts from seaweeds (Michel et al., 1996; Wu et al., 2007; Wu et al., 2012; Rodrigues et al., 2015). There is no study on the impact of different enzyme-assisted extraction processes on the prebiotic potential of seaweed extracts using a human gut fermentation model, hence this study is valuable for the development of an efficient process for seaweed derived marketable functional foods. Fractions containing isolated components of the seaweed were tested for their prebiotic potential by including them in an *in vitro* anaerobic fermentation containing human faecal inocula that mimics the microflora of the human large bowel. The production of key beneficial gut fermentation products, namely short chain fatty acids (SCFA), and the growth of selected microorganisms are used as indicators of potential benefit.

4.3 Materials and methods

4.3.1 Seaweed

Brown seaweed (*Ecklonia radiata*- identification confirmed by the State Herbarium of South Australia) was collected from freshly deposited beach-cast seaweed in Rivoli Bay, Beachport, South Australia in March 2013. It was rinsed in fresh water to remove any visible surface contaminants, and placed on mesh racks to dry. The whole plants were collected at one time to provide a consistent sample for the whole studies. It was dry blended (Blendtec, Orem, UT, USA), then passed though a 0.25 mm sieve, and dried in an oven at 45°C. The ground powder was stored at -20°C prior to extraction and fermentation.

4.3.2 Enzymes

Two commercial enzymes used for the preparation of seaweed extracts, carbohydrases (Celluclast® 1.5 L) and proteases (Alacalase® 2.4 L FG,) were kindly provided by Novozymes (Bagsvaerd, Denmark).

4.3.3 Chemicals and substrates

All chemicals used are of analytical or gas chomatography grade from Merck and Sigma. The positive control substrates for comparison purposes in batch fermentation were glucose, resistant starch Hi-maize® 958 (Starch Australasia, Lane Cove, NSW, Australia), and inulin. α-Cellulose and blank were used as negative controls.

Eight seaweed samples used in the experiments were prepared by different extraction processes and summarised in Table 4.1. The criteria to select these samples was based on the key nutrients and potential fermentable components in the seaweed extracts, mainly carbohydrates and proteins. The indigestible dietary polysaccharides are the most important sources for prebiotics, and their characteristics such as low and high molecular weight may also affect the fermentability by gut microorganisms. From our preliminary study (data not shown), the Celluclast-treated and water

extracts contained high unbound and bound sugar contents, which could represent low and high molecular weight polysaccharides, respectively. The conventional water extraction was chosen for further fractionation with ethanol to obtain two extract fractions: unbound small sugars and bound high molecular weight sugars. In this study, the seaweed residue obtained from water extraction and seaweed raw material were included as representatives of non-digestible and polysaccharides with complex structure, respectively. In order to understand the effect of the enzyme-assisted extraction process, the conventional acidic extraction at an optimum pH of Celluclast was used as a control. The last potential ingredient is protein, and a high content of protein and amino acids was found in the Alcalase-treated extract, so this sample was also selected for inclusion in this experiment.

Table 4.1 Characteristics of seaweed samples prepared by different processes used in the batch fermentation

Seaweed sample/ Substrate	Process	Characteristics
Celluclast	Enzyme-assisted extraction by Celluclast or Alcalase	Low molecular weight polysaccharides
Alcalase		Low molecular weight proteins
Acid	Conventional extraction by acid or water	High molecular weight polysaccharides and proteins
Water		
Free sugar fraction	Water extraction and fractionation	Unbound small sugars
Polysaccharide fraction		Bound high molecular weight polysaccharides
Seaweed residue	Residues from water extraction	Non-digestible polysaccharides
Seaweed powder	Seaweed raw material	Complex structure polysaccharides

4.3.4 Analyses of the composition of dried seaweed extracts

Protein, total starch, total dietary fibre, and total non-digestible non-starch polysaccharide (NNSP) of all seaweed extracts were analysed using established AOAC methods 968.06 (1998), 996.11 (2005), 985.29 (2003) and 994.13 (1999), respectively. Total phlorotannin was determined by Folin Ciocalteu's phenol reagent. The absorbance was measured at 725 nm. A standard curve using phloroglucinol (Sigma-Aldrich, St. Louis, MO, USA) was used for calibration, and the results were expressed as g phloroglucinol equivalent (Wang et al., 2012). Total sugar content was analysed by HPLC after sulfuric acid hydrolysis and 1-phenyl-3-methyl-5-pyrazolone derivatisation (Comino et al., 2013). Compounds were quantified with monosaccharide standards. All results were expressed as g/100 g dry weight (DW).

4.3.5 Preparation of seaweed samples

4.3.5.1 Enzyme-assisted extraction

The enzyme-assisted extracts were prepared according to the method of Charoensiddhi et al. (2015) with some modifications. Briefly, the dried seaweed was dispersed in the pH-adjusted water using 1 M HCl or NaOH at the optimum pH of each enzyme (pH 4.5 for Celluclast and pH 8.0 for Alcalase) with the ratio of 1:100. The suspension was incubated in an orbital mixer incubator (Ratek Instruments, Boronia, VIC, Australia) for 10 min, and the pH was then adjusted again in order to achieve a stable optimum pH. After that, the enzyme was added. The enzymatic hydrolysis was performed under optimal conditions of the particular enzyme at 50°C for 24 h in an orbital mixer incubator. The enzyme was inactivated by boiling the sample at 100°C for 10 min and cooling immediately in an ice bath. The extract was centrifuged at 8000 g for 20 min at 4°C. The supernatant was then adjusted to pH 7.0, freeze-dried by a freeze-dryer (Virtis, Stone Ridge, NY, USA), and stored at -20°C until ready for fermentation.

4.3.5.2 Conventional water and acid extraction

Conventional extractions were prepared by the same method of enzymatic extraction: dried seaweed powder was extracted with water (pH 6) and acid solution at pH 4.5 adjusted with 1 M HCl for 24 h at 50°C in an orbital mixer incubator. Only the residue from the water extraction was freeze dried and stored at -20°C for further fermentation.

4.3.5.3 Fractionation

The fractionation method was modified from Lorbeer et al. (2015) and Fleita et al. (2015). The freeze dried supernatants from water extraction were mixed well with 70% (v/v) ethanol for 1 h, and then centrifuged at 8000 g for 20 min at 4°C in order to separate (1) supernatant (free sugar fraction) and (2) residue (polysaccharide fraction). The ethanol remaining in the residue and supernatant was evaporated in a Centrivap vacuum concentrator (Labconco, Kansas City, MO, USA). The free sugar and polysaccharide fractions were then freeze dried and kept at -20°C until used for fermentation.

4.3.6 Faecal sample preparation

Fresh faecal samples were provided by thee individual human volunteers. The volunteers were healthy, not on any dietary restrictions, and had not taken antibiotics at least 3 month prior to donating. Faecal samples were transferred to an anaerobic chamber where equivalent amounts of faeces from each donor were mixed together and diluted to 10% (w/v) with sterile anaerobic phosphate buffered saline (PBS) (0.01 M, pH 7.2). The slurry was homogenised and constantly stirred during inoculation into each fermentation test.

4.3.7 Batch fermentations

Anaerobic batch fermentations modified method from Zhou et al. (2013) were used to assess the effect of seaweed samples on fermentation characteristics and composition of gut microbiota. Substrates were fermented *in vitro* in a batch system under anaerobic conditions for 24 h. Fresh faecal samples were used as inoculum. Anaerobic conditions were maintained thoughout the set-up of fermentations using an anaerobic chamber (Bactron IV Anaerobic Chamber Sheldon Manufacturing Inc., Cornelius, OR, USA) to maximise the bacterial viability of the inoculum. Seaweed substrates at a concentration of 1.5% (w/v) in fermentation media were used in each test, and no substrate was added for the blank. Positive and negative control fermentations supplemented with glucose, resistant starch, inulin, and cellulose at the same concentration were also included. Test substrates and controls were fermented in quadruplicate. The fermentation medium contained the following (per L of distilled water): 2.5 g trypticase, 125 μL micromineral solution, 250 mL buffer solution, 250 mL macromineral solution, and 1.25 mL resazurine solution 0.1% (w/v). The micromineral solution contained 132 g $CaCl_2.2H_2O$, 100 g $MnCl_2.4H_2O$, 10 g $CoCl_2.6H_2O$, and 80 g $FeCl_3.6H_2O$ per L distilled water. The buffer solution contained 4 g NH_4HCO_3 and 35 g $NaHCO_3$ per L of distilled water. The macromineral solution was prepared with 5.7 g Na_2HPO_4, 6.2 g KH_2PO_4, and 0.6 g $MgSO_4.7H_2O$ per L distilled water. Thirty thee and a half millilitres of reducing solution (6.25 g cysteine hydrochloride, 6.25 g $Na_2S.9H_2O$ and 40 mL 1 M NaOH per L of distilled water) were added to 1 L of fermentation medium and sterilised at 121°C for 15 min. The pH of the fermentation medium was adjusted to 7.2. Substrates were hydrated at 37°C for 1 h in fermentation medium within an anaerobic chamber. Each fermentation test was inoculated with 10% (w/v) of faecal inoculum. All fermentation tests were incubated in an orbital mixer incubator at 37°C and mixed gently at 80 rpm over a period of 24 h, and the end products were obtained for SCFA analysis and bacterial number determination.

4.3.8 Short chain fatty acid analysis

The short chain fatty acids (SCFA) were analysed according to the method of McOrist et al. (2008) with slight modifications. Heptanoic acid (3 mL) as internal standard was added to each 1 mL of fermentation samples. Samples were mixed thoroughly and centrifuged at 2000 g, 4°C for 10 min. Then 10 μL of 1 M phosphoric acid was added to 300 μL of the supernatant. Fermentation samples were kept on ice to prevent SCFA volatilisation thoughout the processing. The supernatant was filtered, and 0.2 μL was injected into a Gas Chomatograph (model 7890A; Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector and a capillary column (Zebron ZB-FFAP, 30 m × 0.53 mm × 1.0 μm, Phenomenex, Lane Cove, NSW, Australia). Helium was used as the carrier gas; the initial oven temperature was 90°C held for 1 min and was increased at 20°C/min to 190°C held for 2.5 min; the injector and detector temperature was 210°C; Gas flow and septum purge at 7.7 and 3.0 mL/min, respectively. A standard SCFA mixture containing acetic, propionic,

butyric, *iso*-butyric, valeric, *iso*-valeric, and caproic acids was used for calculation, and fatty acid concentrations were calculated in μmol/mL by comparing their peak areas with standards.

4.3.9. Microbial population enumeration by quantitative real-time PCR (Q-PCR)

After 24 h of fermentation, 2 mL aliquots of the fermentation samples were centrifuged at 2655 g, 4°C for 5 min. DNA was extracted using bead beating followed by the PowerMag® Microbiome RNA/DNA Isolation Kit (27500-4-EP; MO BIO Laboratories, Inc., Carlsbad, CA, USA) optimized for epMotion® platforms with slight modifications as follows. Briefly, 0.8 g of glass beads and 650 μL of pre-warmed PowerMag® Microbiome Lysis Solution were added to faeces. Bead beating was performed at 3450 rpm for 3 min (MiniBeadBeater-16, Biospec Products, Bartlesville, OK, USA), followed by centrifugation at 2655 g for 5 min. The supernatant was recovered, and 30 μL of Proteinase K >600mAU/mL (Qiagen, Hilden, Germany) was added. The sample was heat treated at 70°C for 10 min. Immediately on completion of the heating step, 150 μL of PowerMag® Inhibitor Removal Solution was added. Samples were then incubated at -20°C for 5 min, and centrifuged at 20,817 g for 5 min. The supernatant was placed into a MO BIO 2 mL Deep Well Plate, and 5μL RNAse (10 mg/mL) was added to each sample. The remaining extraction procedure was done using the manufacturers protocol (epMotion-protocol-27500-V2.dws) optimized for epMotion® 5075 (Eppendorf AG, Hamburg, Germany) and the final elution of DNA was performed with ClearMag® RNase-Free Water. DNA concentrations and purity were determined spectrophotometrically (NanoDrop 1000 spectrophotometer, Thermo Fisher Scientific, Wilmington, DE, USA). Samples were stored at -80°C. PCR amplification and detection were done by Q-PCR (Bio-Rad Laboratories, Hercules, CA, USA) with a series of genus-specific primer pairs according to the modified method from references in Table 4.2. Briefly, each reaction mixture contained 5 μL of SsoFast™ EvaGreen® Supermix fluorescent nucleic acid dye (Bio-Rad Laboratories, Hercules, CA, USA), 0.2 μL of bovine serum albumin (BSA) (Promega, Madison, WI, USA), 0.06-0.12 μL of each specific primer at a concentration of 300-600 nM, and sterile water to make the total volume 7 μL. For quantification, a total 3 μL of 3 ng/μL of extracted DNA from each sample was used. The fluorescent products were detected at the last step of each cycle. The Cq values and Melting curve analysis after amplification were used to calculate the bacterial population and distinguish target from non-target PCR products. Standard curves were constructed from eight 10-fold dilutions for *Bacteroidetes*, *Bifidobacterium*, *Clostridium coccoides*, *Clostridium leptum*, *Entercoccus*, *Escherichia coli*, *Faecalibacterium prausnitzii*, *Lactobacillus*, *Ruminococcus bromii*, and Total bacteria. These target bacterial genera were selected in this study for their relevance to gut health.

Table 4.2 Primers and amplification conditions for quantitative real-time PCR assays in this study

Target bacteria	Primers	Sequence (5′–3′)	nM	Annealing		Reference
				Temp (°C)	Time (s)	
Bifidobacteria	Bif-F Bif-R	TCGCGTC(C/T)GGTGTGAAAG CCACATCCAGC(A/G)TCCAC	600	56	20	Chistophersen et al., 2013
Clostridium coccoides group	Ccoc-F Ccoc-R	AAATGACGGTACCTGACTAA CTTTGAGTTTCATTCTTGCGAA	500	60	20	
Clostridium leptum group	Clept-F Clept-R	CTTTGAGTTTCATTCTTGCGAA GCACAAGCAGTGGAGT	500	56	20	
Escherichia coli	E.coli F E.coli R	CATGCCGCGTGTATGAAGAA CGGGTAACGTCAATGAGCAAA	300	60	20	
Faecalibacterium prausnitzii	FPR-1F FPR-2R	AGATGGCCTCGCGTCCGA CCGAAGACCTTCTTCCTCC	500	60	20	
Lactobacillus group	Lacto-F Lacto-R	AGCAGTAGGGAATCTTCCA CACCGCTACACATGGAG	600	56	20	
Entercoccus spp.	Entero F Entero R	CCCTTATTGTTAGTTGCCATCATT ACTCGTTGTACTTCCCATTGT	600	59	15	Rinttila et al., 2004
Ruminococcus bromii	He-10 F He-10 R	GGTCTTGACATCCAACTAACGAAGT TTTTGTCAACGGCAGTCCTAT	500	60	30	Mondot et al., 2011
Bacteroidetes	Bact934F Bact1060R	GGARCATGTGGTTTAATTCGATGAT AGCTGACGACAACCATGCAG	600	60	30	Guo et al., 2008
Total bacteria	Eub 338F Eub 518R	ACTCCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	500	53	15	

4.3.10 Statistical analysis

Results were expressed in quadruplicate as mean ± SEM for SCFA analysis and bacterial enumeration. One-way analysis of variance (ANOVA) was used to compare the means. Differences in SCFA and bacterial enumeration were considered significant at $P<0.05$ by Duncan's test in the IBM SPSS Statistics 22 (IBM Corporation Software Group, Somers, NY, USA).

4.4 Results

4.4.1 Composition of seaweed extracts

The levels of key fermentable components in each seaweed extract are shown in Table 4.3. Total fibre and/or sugars were the major components (of components tested) in all seaweed extracts. The polysaccharide fraction contained the highest total fibre, NNSP, and sugar content while the free sugar fraction contained the lowest levels of these components. The seaweed extract prepared by the Alcalase-assisted extraction consisted of the highest protein content. A small amount of phlorotannin and starch was also found in all seaweed extract.

Table 4.3 Key nutrients and potential fermentable components (g/100 g DW) of seaweed extracts; values are means of duplicate analyses.

Sample	Total fibre	Total NNSP	Total starch	Total sugar	Protein	Total phlorotannin
Celluclast	18.2	4.1	1.2	24.9	1.9	3.9
Alcalase	16.2	6.0	0.9	21.4	5.2	3.5
Acid	23.9	6.7	1.1	26.1	2.0	4.3
Water	20.7	7.1	1.2	23.5	2.3	4.0
Free sugar fraction	2.3	0.6	1.0	15.6	1.8	3.6
Polysaccharide fraction	48.7	16.1	1.3	43.0	3.8	4.5

4.4.2 SCFA production

The concentrations of SCFA produced following 24 h fermentation of all samples are shown in Table 4.4. Fermentations with all the seaweed samples produced significantly higher levels of total SCFA, acetic acid and propionic acid compared to negative controls (blank and cellulose). Of the seaweed samples tested, the highest levels of acetic, propionic, and butyric acids resulted from fermentation with the polysaccharide fraction, free sugar fraction, and Celluclast-assisted extract, respectively. Total SCFA produced from each of the seaweed extracts, except polysaccharide fraction, was approximately 50-70% higher than that of resistant starch and 30-40% lower than that of inulin, the latter generating the highest levels of total SCFA, acetic acid, and butyric acid of all the substrates. All seaweed samples produced low levels of *iso*-butyric, valeric, *iso*-valeric, and caproic acids. The initial pH of this batch fermentation was 7.2 and this decreased in conjunction with SCFA production during fermentation.

Table 4.4 SCFA concentration (μmol/mL ± SEM) and pH value in batch fermentations at 24 h of seaweed samples and controls; values in quadruplicate with different letters are significantly different (P<0.05).

Substrates (seaweed samples and controls)	pH	Total SCFA	Acetic acid	Propionic acid	Butyric acid	*iso*-Butyric acid	Valeric acid	*iso*-Valeric acid	Caproic acid
Celluclast	5.75^{de} ± 0.03	63.42^{c} ± 1.76	22.81^{cd} ± 0.91	29.61^{c} ± 2.60	9.22^{b} ± 1.38	0.31^{d} ± 0.07	0.78^{e-g} ± 0.26	0.51^{fg} ± 0.08	0.19^{b} ± 0.08
Alcalase	5.80^{e} ± 0.03	66.01^{c} ± 1.82	23.67^{c} ± 0.61	36.79^{b} ± 0.57	3.85^{d-f} ± 0.64	0.34^{d} ± 0.02	0.58^{f-h} ± 0.18	0.70^{e} ± 0.02	0.09^{b} ± 0.02
Acid	5.68^{d} ± 0.02	62.86^{c} ± 0.20	20.59^{d-f} ± 0.21	36.25^{b} ± 0.80	4.27^{de} ± 0.48	0.31^{d} ± 0.05	0.81^{ef} ± 0.11	0.55^{f} ± 0.06	0.08^{b} ± 0.02
Water	5.74^{de} ± 0.05	57.83^{d} ± 2.91	21.05^{de} ± 0.87	31.74^{c} ± 3.81	4.12^{de} ± 0.48	0.17^{e} ± 0.01	0.29^{hi} ± 0.08	0.40^{g} ± 0.04	0.06^{b} ± 0.01
Free sugar fraction	5.21^{c} ± 0.01	72.72^{b} ± 1.65	18.32^{f} ± 0.58	49.89^{a} ± 1.12	3.84^{d-f} ± 0.07	0.08^{ef} ± 0.03	0.40^{g-i} ± 0.03	0.13^{h} ± 0.01	0.06^{b} ± 0.00
Polysaccharide fraction	6.07^{g} ± 0.02	50.70^{e} ± 1.10	27.05^{b} ± 0.58	18.2^{d} ± 0.38	2.87^{d-f} ± 0.20	0.58^{c} ± 0.00	0.98^{de} ± 0.26	0.86^{d} ± 0.01	0.14^{b} ± 0.03
Seaweed residue	6.37^{i} ± 0.01	39.46^{f} ± 1.31	22.68^{cd} ± 0.77	10.13^{ef} ± 0.45	2.63^{e-g} ± 0.08	0.75^{b} ± 0.01	1.76^{b} ± 0.02	1.22^{c} ± 0.01	0.28^{b} ± 0.00
Seaweed powder	5.94^{f} ± 0.02	48.94^{e} ± 1.50	20.25^{d-f} ± 0.80	18.63^{d} ± 0.51	4.83^{d} ± 0.25	0.54^{c} ± 0.01	3.00^{a} ± 0.03	0.93^{d} ± 0.01	0.75^{a} ± 0.03
Blank	7.02^{j} ± 0.03	17.87^{h} ± 0.20	8.53^{g} ± 0.14	3.03^{g} ± 0.08	1.95^{fg} ± 0.03	0.91^{a} ± 0.01	1.39^{bc} ± 0.01	1.90^{a} ± 0.03	0.17^{b} ± 0.04
Cellulose	7.05^{j} ± 0.01	18.59^{h} ± 0.14	9.09^{g} ± 0.07	3.24^{g} ± 0.04	2.02^{fg} ± 0.03	0.91^{a} ± 0.01	1.31^{cd} ± 0.06	1.89^{a} ± 0.01	0.12^{b} ± 0.02
Glucose	3.86^{a} ± 0.02	34.32^{g} ± 1.71	20.03^{ef} ± 1.24	13.17^{e} ± 0.22	0.79^{g} ± 0.20	0.04^{f} ± 0.02	0.13^{i} ± 0.04	0.11^{h} ± 0.04	0.06^{b} ± 0.00
Resistant starch	6.21^{h} ± 0.05	39.89^{f} ± 2.91	21.97^{c-e} ± 1.97	6.42^{fg} ± 0.24	6.78^{c} ± 0.89	0.74^{b} ± 0.04	1.73^{b} ± 0.10	1.35^{b} ± 0.09	0.90^{a} ± 0.35
Inulin	4.74^{b} ± 0.03	109.71^{a} ± 1.16	47.57^{a} ± 0.84	39.15^{b} ± 1.47	22.11^{a} ± 1.21	0.05^{f} ± 0.00	0.66^{e-h} ± 0.03	0.09^{h} ± 0.01	0.08^{b} ± 0.01

4.4.3 Bacterial enumeration

The bacterial populations resulting from fermentations are shown in Table 4.5. Total bacteria numbers significantly increased after 24 h fermentation of most of the seaweed samples when compared to negative controls. Seaweed extracts prepared by Celluclast-assisted extraction, but not other extracts tested, significantly increased numbers (by up to 10-fold) of potentially beneficial groups of bacteria, namely *Bifidobacterium*, *Bacteroidetes*, *Lactobacillus*, and *C. coccoides*. There were no significant effects on the bacterial populations of *F. prausnitzii*, *C. leptum* and *R. bromii*, but numbers of *E. coli* and *Enterococcus* in almost all seaweed extracts increased compared to negative controls. In contrast, the polysaccharide fraction, seaweed residue, and seaweed powder did not result in a significant increase in total bacteria, and decreased numbers in most target bacteria were also observed. The positive control inulin increased almost all target bacteria compared to the other treatment groups and controls.

Table 4.5 Bacterial populations (log_{10} cells/mL ± SEM) in batch fermentations at 24 h of seaweed tested samples and controls; values in quadruplicate with different letters are significantly different ($P<0.05$); *ND is not detected.

Substrates (seaweed samples and controls)	***Total Bacteria***	***Bifidobacteria***	***Bacteroidetes***	***Lactobacillus***	***F. prausnitzii***	***C. coccoides***	***C. leptum***	***R. bromii***	***E. coli***	***Enterococcus***
Celluclast	10.20^{a} ± 0.11	6.55^{ab} ± 0.08	6.02^{b} ± 0.09	5.28^{cd} ± 0.19	6.40^{b} ± 0.10	7.04^{ab} ± 0.11	6.35^{cd} ± 0.16	5.57^{ef} ± 0.15	7.12^{ab} ± 0.26	6.60^{a} ± 0.16
Alcalase	10.39^{a} ± 0.05	5.10^{de} ± 0.23	4.85^{c} ± 0.17	4.58^{ef} ± 0.24	6.53^{b} ± 0.09	6.65^{b} ± 0.20	6.43^{cd} ± 0.07	5.71^{d-f} ± 0.20	7.41^{ab} ± 0.17	6.03^{ab} ± 0.26
Acid	10.28^{a} ± 0.06	5.76^{cd} ± 0.12	5.27^{c} ± 0.09	5.02^{de} ± 0.10	6.48^{b} ± 0.04	7.07^{ab} ± 0.04	6.25^{cd} ± 0.26	5.75^{c-f} ± 0.14	7.52^{a} ± 0.07	6.63^{a} ± 0.11
Water	10.33^{a} ± 0.08	5.01^{e} ± 0.32	4.87^{c} ± 0.18	4.45^{f} ± 0.31	6.28^{bc} ± 0.16	6.61^{b} ± 0.30	6.04^{d} ± 0.20	5.61^{ef} ± 0.16	7.65^{a} ± 0.27	6.38^{ab} ± 0.33
Free sugar fraction	10.24^{a} ± 0.06	5.69^{d} ± 0.11	5.21^{c} ± 0.10	5.02^{de} ± 0.07	6.53^{b} ± 0.09	6.96^{ab} ± 0.09	6.68^{b-d} ± 0.07	5.87^{b-e} ± 0.16	7.14^{ab} ± 0.08	6.36^{ab} ± 0.11
Polysaccharide fraction	9.90^{ab} ± 0.13	3.21^{f} ± 0.61	4.08^{d} ± 0.12	ND*	6.15^{bc} ± 0.17	ND	ND	4.87^{g} ± 0.29	7.01^{a-c} ± 0.17	5.80^{b} ± 0.33
Seaweed residue	9.55^{b} ± 0.04	3.79^{f} ± 0.23	4.04^{d} ± 0.13	3.45^{g} ± 0.14	5.92^{c} ± 0.13	4.42^{d} ± 0.37	5.05^{e} ± 0.56	5.35^{f} ± 0.11	5.51^{e} ± 0.13	3.99^{d} ± 0.15
Seaweed powder	8.30^{c} ± 0.64	ND	1.51^{e} ± 0.70	ND	4.58^{d} ± 0.44	ND	ND	3.89^{h} ± 0.16	ND	ND
Blank	9.52^{b} ± 0.05	5.60^{de} ± 0.08	5.05^{c} ± 0.08	4.91^{d-f} ± 0.06	6.38^{b} ± 0.08	6.87^{b} ± 0.09	6.66^{b-d} ± 0.09	6.15^{b-d} ± 0.09	6.45^{cd} ± 0.05	4.84^{c} ± 0.04
Cellulose	9.51^{b} ± 0.04	5.51^{de} ± 0.15	5.09^{c} ± 0.06	4.73^{ef} ± 0.13	6.21^{bc} ± 0.13	5.74^{c} ± 0.75	6.23^{cd} ± 0.28	6.20^{bc} ± 0.06	6.09^{de} ± 0.41	5.02^{c} ± 0.31
Glucose	9.94^{ab} ± 0.05	6.54^{ab} ± 0.09	5.88^{b} ± 0.06	6.65^{a} ± 0.11	6.25^{bc} ± 0.12	6.48^{bc} ± 0.17	6.47^{cd} ± 0.14	5.82^{b-f} ± 0.12	6.43^{cd} ± 0.27	6.63^{a} ± 0.10
Resistant starch	9.94^{ab} ± 0.10	6.34^{bc} ± 0.12	5.91^{b} ± 0.12	5.68^{bc} ± 0.03	7.08^{a} ± 0.06	7.32^{ab} ± 0.07	7.62^{a} ± 0.13	7.06^{a} ± 0.08	6.45^{cd} ± 0.13	5.74^{b} ± 0.04
Inulin	10.35^{a} ± 0.08	7.06^{a} ± 0.10	6.72^{a} ± 0.09	5.93^{b} ± 0.12	7.09^{a} ± 0.06	7.81^{a} ± 0.06	7.28^{ab} ± 0.06	6.25^{b} ± 0.06	6.79^{bc} ± 0.07	6.26^{ab} ± 0.11

4.5 Discussion

In this study, different extract samples of a brown seaweed *Ecklonia radiata*, prepared by different extraction processes, were examined as potential sources of prebiotics. A range of fermentable components, including polysaccharides, which are likely to undergo fermentation by microorganisms in the large intestine, were found to be present in these samples. Their addition to an *in vitro* anaerobic fermentation system containing human faecal inocula demonstrated that the components within the seaweed are able to produce SCFA, microbial fermentation products which are known to have multiple gut health benefits *in vivo*. Changes in the profiles of targeted microbial populations were also observed following fermentation of some of the seaweed extract samples, including an increase in *Bifidobacterium* and *Lactobacillus*, which are commonly regarded as markers of prebiosis. These findings demonstrate for the first time that *Ecklonia radiata* has the potential to be used as a source of dietary supplements with gut health benefits in humans.

The SCFA are the primary products of polysaccharide (and to some extent protein) fermentation by microbiota in the large bowel. These fermentation products are known to have a wide range of effects that are vital to the proper functioning and integrity of colorectal tissues, including stimulation of fluid uptake, cell differentiation, mucus production and apoptosis of damaged cells (Topping & Clifton, 2001; Conlon & Bird, 2015). The most abundant and important SCFA are acetic acid, propionic acid, and butyric acid. These forms can have different physiological impacts within the gut. Butyric acid is of particular interest because it is the primary energy source of colonocytes, and is especially important for maintaining tissue integrity though apoptosis of cells with high levels of DNA damage (Canani et al., 2011). Recent studies in animals and humans suggest increasing intake of dietary fibres such as resistant starch may help reduce the risk of colorectal cancer though production of butyric acid and other SCFA (Slavin, 2013). Acetic acid has also recently been shown to inhibit the growth of enteropathogenic bacteria (Fukuda et al., 2011). The role of propionic acid in the gut is less clearly defined. We demonstrated that SCFA was produced in response to the *in vitro* fermentation of all seaweed samples tested.

Our analysis of the microbiota using targeted QPCR, which focused on a limited number of key microbes of relevance to human health, revealed significant differences. Relative to the negative control fermentations, the Cellulclast-derived extracts resulted in significant increases in *Bifidobacterium* and *Bacteroidetes*, whereas a significant increase in *Lactobacillus* was also observed relative to the cellulose fermentation. *Bifidobacterium* and *Lactobacillus* are genera often used as markers of prebiosis (Kleerebezem & Vaughan 2009; Bird et al., 2010). Consequently, our findings suggest components in extracts of the brown seaweed we have tested have the capacity to be used as prebiotics in humans. We also determined numbers of *F. prausnitzii*, as these bacteria are key butyric acid producers with anti-inflammatory properties, and could be used as indicators of

a prebiotic effect (Bird et al., 2010). However, we did not observe significant increases in *F. prausnitzii* with the Cellulclast derived extract. This did not fit well with the SCFA data, as these extracts induced the greatest production of butyric acid. It is quite likely that populations of other butyrate-producing bacteria such as *Roseburia* and *Eubacterium* (Flint et al., 2015) were also increased in number, but these were not analysed here. However, we did also target the *C. coccoides* and *C. leptum* groups of bacteria, which are known to contain numerous butyrate producers (Louis & Flint, 2009). We observed an increase in the *C. coccoides* group in response to almost all seaweed extract samples relative to the cellulose control but not relative to the blank fermentation. The *C. leptum* group was unaffected by the extract samples. The growth of *Bacteroidetes* was also stimulated by the Cellulclast extracts. Bacteria of this phylum play important roles in degradation of polysaccharides, and so it is not surprising that some of our extracts, abundant in polysaccharides, enhance their growth. One of the most well-documented of these bacteria in humans is *Bacteroides thetaiotaomicron*, which has hundreds of enzymes capable of degrading polysaccharides (Xu et al., 2003). It is also worth noting that many Japanese, who consume significant amounts of seaweed daily, possess a gut microbe of this genus (*B. plebeius*) with enzymes (porphyranases and agarases) suited to degrade certain seaweed polysaccharides (Hehemann et al., 2010). Increases in the numbers of *E. coli* and *Enterococcus* were observed with almost all seaweed samples compared to the blank controls. Although these bacteria are commonly known for their pathogenic potential, they can also have some benefits, as the probiotic market includes *E. coli* Nissle 1917 and some *Enterococcus* species (Iannitti & Palmieri, 2010; Gerritsen et al., 2011). The seaweed samples we used contain compounds, such as phenolic compounds, which are able to slow down or partially inhibit bacterial growth (Eom et al., 2012; Jun et al., 2015) and this may have influenced outcomes in our study. There is a significant inter-individual variation in human gut microbiota populations (Minot et al., 2011) and the capacity to produce SCFA within stool (McOrist et al., 2008). Consequently, stool microbes from any one individual may not act upon substrates, such as from seaweed, in a manner which occurs in stool from other individuals. The *in vitro* fermentations used in this study contained stool inocula pooled from thee individuals to help broaden the suite of microbes available to act upon seaweed derived substrates.

Although seaweed samples were prepared though various processes, analysis of these samples demonstrated that they all contained variable amounts of potentially fermentable components, including fibre, starch, sugar, protein and polyphenols. Four of seaweed extracts contained similar (but still variable) levels of dietary fibre, NNSP, and starch, perhaps not surprisingly, produced similar levels of total SCFA. However, there was no obvious relationship between carbon source content in seaweed extracts, SCFA production, and total bacterial numbers. The polysaccharide fraction that contained approximately twice the amount of these components on a dry weight basis produced significantly lower total SCFA and bacterial number following fermentation. This is in contrast with the free sugar fraction with the lowest levels of dietary fibre that gave the highest total SCFA

production and increases in total bacteria number. This indicates that dietary fibre content alone is not a primary predictor of SCFA production following *in vitro* fermentation of the seaweed samples in this experiment. Other measured components do not appear to be clear predictors either. Interactions between components are likely to have a significant impact upon fermentation and these may be at play here. Other factors need to be taken into consideration when considering *in vitro* fermentation outcomes.

We have carried out fermentation over a 24 h period but this may not be optimal for all substrates. The rate and extent of fermentation of the substrates by the microbes can depend on the complexity and accessibility of components added. Dietary fibres such as those derived from plant cell walls are more likely to be resistant to breakdown due to their complexity whereas simpler carbohydrates would be readily metabolised by microbes. In our study, it is possible that 24 h was not a sufficient time to enable a more complete breakdown of some complex polysaccharides from seaweed which may explain why the polysaccharide fraction with the highest levels of dietary fibre, as well as seaweed residue and powder, generated the lowest total SCFA and bacterial number of the seaweed extracts tested. In addition, the chemical structure of the dietary fibres and other fermentable substrates within the different samples may differ and contribute to variations in the observed effects.

Of the seaweed extracts tested the most promising prebiotic potential was observed with those processed with carbohydrase (Celluclast)-assisted extraction. Many naturally occurring seaweed fibres are high molecular weight polymers which pass though the gut with little bacterial utilisation (MacArtain et al, 2007), but these treatments probably assist by breaking down the seaweed cell walls and complex interior storage materials to release oligomers that can be fermented by the microbial populations. A number of studies on plant polysaccharides have indicated that low molecular weight or hydrolysed oligosaccharides increase fermentability by gut microbial communities (Van Laere et al., 2000; Hughes et al., 2007; Hughes et al., 2008). However, current commercial enzymes which contained major activities of β-glucanase, cellulase, xylanase, and hemicellulase can only work by breaking down some complex materials such as laminarin and cellulose. Polysaccharides like alginate and fucoidan, a main structural component of the seaweed cell wall, still remain unhydrolysed. Therefore, the development of low molecular weight seaweed extracts and oligosaccharide fractions using more specific enzymes was important in order to improve the efficiency for fermentability by gut microbes.

Although components of the seaweed were shown here to stimulate SCFA production and growth of potentially beneficial gut bacteria in an *in vitro* system, it is possible that such effects will not manifest themselves *in vivo* following consumption of these components. It is quite probable that some components would not reach the large bowel after they are eaten as they would undergo digestion in the upper gut and be absorbed. However, many complex carbohydrates are poorly digested and can reach the colon where they undergo fermentation. The digestibility of isolated

components of the seaweed extracts should be determined in future studies for a more accurate assessment of their prebiotic potential.

4.6 Conclusion

The results presented in this study have shown that different extraction processes have a significant impact on the prebiotic potential of seaweed extracts. Enzyme-assisted extraction is an effective technique to improve the components in brown seaweed *Ecklonia radiata* that when added to an *in vitro* fermentation system containing human stool can undergo fermentation and stimulate production of SCFA. This, together with a capacity to promote the growth of beneficial microbes such as *Bifidobacterium* and *Lactobacillus*, suggests the components of this seaweed could potentially be consumed by humans as prebiotics.

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Appendix 4.1: First page of the published article

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Impact of extraction processes on prebiotic potential of the brown seaweed *Ecklonia radiata* by *in vitro* human gut bacteria fermentation

Suvimol Charoensiddhi [a,b], *Michael A. Conlon* [c], *Michelle S. Vuaran* [c], *Christopher M.M. Franco* [a,b], *Wei Zhang* [a,b,*]

[a] *Centre for Marine Bioproducts Development, School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia*
[b] *Department of Medical Biotechnology, School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia*
[c] *CSIRO Food and Nutrition, Kintore Avenue, Adelaide, South Australia 5000, Australia*

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ABSTRACT

The prebiotic potential of the extracts of a South Australian brown seaweed *Ecklonia radiata* obtained using different processes was investigated. Six extracts of this seaweed were prepared by enzymatic, acidic, and water extraction processes. The extracts were added to an *in vitro* anaerobic fermentation system containing human faecal inocula to assess their ability to generate short chain fatty acids (SCFAs) and to promote the growth of selected bacterial genera (as assessed by quantitative PCR). Following 24 h fermentation, all seaweed extracts significantly increased ($P < 0.05$) total SCFA production (50.7–72.7 μmol/mL) and the total number of bacteria ($\log_{10}$ 10.2–10.4 cells/mL) when compared to controls (blank and cellulose). The extracts prepared using Celluclast-assisted extraction showed the greatest potential for improving gut health as these induced significantly higher production of butyrate (9.2 μmol/mL), and the growth of bacteria regarded as beneficial, including *Bifidobacterium* ($\log_{10}$ 6.6 cells/mL) and *Lactobacillus* ($\log_{10}$ 5.3 cells/mL).

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1. Introduction

The relationship between diet and health is being widely recognised by consumers, and this leads to the demand for food ingredients with functionality and health benefits. There is growing evidence that indigestible dietary polysaccharides can have a significant benefit to health through their ability to stimulate the growth of beneficial microbes in the large bowel (Conlon & Bird, 2015), and hence may fulfil the definition of a prebiotic. Examples of prebiotic polysaccharides include inulin, fructooligosaccharides, galactooligosaccharides and lactulose (Al-Sheraji et al., 2013; Kolida & Gibson, 2008).Other dietary components, including polyphenols and proteins, can also reach the large bowel and potentially exert a health benefit via activities of the gut microbiota (Cardona, Andrés-Lacueva, Tulipani, Tinahones, & Queipo-Ortuño, 2013; Windey, De Preter, & Verbeke, 2012).

* *Corresponding author.* Centre for Marine Bioproducts Development, School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia. Tel.: +61 8 72218557; fax: +61 8 7221 8555.
E-mail address: wei.zhang@flinders.edu.au (W. Zhang).
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5. POLYSACCHARIDE AND PHLOROTANNIN-ENRICHED EXTRACTS OF THE BROWN SEAWEED *ECKLONIA RADIATA* INFLUENCE HUMAN GUT MICROBIOTA AND FERMENTATION *IN VITRO*

The previous chapter demonstrated that enzyme-assisted extraction of *E. radiata* yielded extracts that could undergo anaerobic fermentation in the presence of human gut microbes, stimulating the production of short chain fatty acids, and selectively promoting the growth of beneficial gut microbes. This finding encouraged further investigations, documented here, with the aim of gaining an understanding into how specific fractions, including phlorotannin and polysaccharides, can influence the prebiotic potential.

The findings from this study were orally presented at the 22nd International Seaweed Symposium (ISS) in Copenhagen, Denmark on June 2016, and published in the conference special issue of the "Journal of Applied Phycology" (DOI 10.1007/s10811-017-1146-y). The first page of the publication is attached in Appendix 5.1.

Author contributions: SC designed and performed all experiments as well as analysed the data and wrote all primary contents. WZ and MC provided advice on research directions and scope. WZ, MC, and CF provided advice on the experimental plan and synthesis of results. MV assisted with training in the techniques used for the molecular experiments (DNA extract and Q-PCR) and the use of the anaerobic chamber. All of the co-authors assisted with the revision of the manuscript before and during journal submission.

Polysaccharide and phlorotannin-enriched extracts of the brown seaweed *Ecklonia radiata* influence human gut microbiota and fermentation *in vitro*

Suvimol Charoensiddhi [a,b], Michael A. Conlon [c], Michelle S. Vuaran [c], Chistopher M.M. Franco [a,b], Wei Zhang [a,b]

[a] Centre for Marine Bioproducts Development, School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia
[b] Department of Medical Biotechnology, School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia
[c] CSIRO Health and Biosecurity, Kintore Avenue, Adelaide, SA 5000, Australia

5.1 Abstract

This study aimed to understand the prebiotic potential and contribution of four extract fractions from the brown seaweed *Ecklonia radiata*. Four seaweed fractions were tested for their digestibility and prebiotic effects using an *in vitro* anaerobic fermentation system containing human faecal inocula. After 24 h fermentation, thee seaweed fractions, except the phlorotannin-enriched fraction (PF), significantly increased ($P<0.05$) total short chain fatty acid (SCFA) production (68.9-97.3 $\mu mol.mL^{-1}$) compared to the negative controls comprising either of a blank (36.3 $\mu mol.mL^{-1}$) or cellulose (39.7 $\mu mol.mL^{-1}$). The low molecular weight (MW) polysaccharide-enriched fraction (LPF) stimulated the growth of beneficial bacteria including *Bifidobacterium*, *Lactobacillus*, and *Clostridium coccoides*. The high MW polysaccharide-enriched fraction (HPF) showed the greatest potential for improving gut health as this fraction was not digestible by enzymes present in the small intestine, and induced significantly higher butyric acid production (8.2 $\mu mol.mL^{-1}$) than the positive control, inulin (2.3 $\mu mol.mL^{-1}$). These findings further demonstrate that *E. radiata*-derived polysaccharides have the potential to be used as dietary supplements with gut health benefits, worthy of further *in vivo* studies.

Key words Digestibility; Fibre; Macroalgae; Polyphenols; Prebiotic; Short chain fatty acids

5.2 Introduction

Recently, indigestible dietary oligo- and polysaccharides have attracted attention as functional food ingredients that provide health benefits beyond basic nutrition (de Jesus Raposo et al., 2016). These polysaccharides often stimulate the growth and activity of beneficial gut microbes, potentially qualifying as prebiotics. They may act as substrates for fermentation in the large bowel, leading to the production of short chain fatty acids (SCFA) with multiple functions that help maintain health (Conlon and Bird, 2015). Commonly used prebiotics include inulin, fructooligosaccharides, galactooligosaccharides, and lactulose (Al-Sheraji et al., 2013). A growing consumer awareness of the benefits of prebiotics is leading to commercial interest in the isolation and development of polysaccharides and other compounds from novel sources such as marine seaweeds for use as prebiotics.

There is a high diversity of brown seaweeds (Phaeophyceae) in Southern Australia; of the 231 species reported, 57% are considered endemic (Womersley, 1990). They are a rich source of functional food ingredients and bioactive compounds, with polysaccharides being a major component, accounting for up to 70% of the dry weight (Holdt and Kraan, 2011). As a result, brown seaweeds have a high total dietary fibre content of 33-75%, with soluble dietary fibre accounting for 26-60% of the dry weight (Lahaye, 1991). Brown seaweed-derived carbohydrates have been shown to be fermented by gut microbiota and so could act as prebiotics and provide a health benefit to humans (O'Sullivan et al., 2010; Zaporozhets et al., 2014). Recent studies indicate that polysaccharides and oligosaccharides derived from seaweeds can modulate intestinal metabolism including fermentation, inhibit pathogen adhesion and evasion, and potentially treat inflammatory bowel disease (Deville et al., 2007; Ramnani et al., 2012; Kuda et al., 2015; Lean et al., 2015). Ingested polyphenols with complex structures can also reach the large intestine where they can be converted into beneficial bioactive metabolites by microbes (Cardona et al., 2013), and this has been shown to occur for brown seaweed phlorotannins (Corona et al., 2016). Hence, brown seaweed phlorotannins may also be valuable as a food ingredient capable of providing a health benefit.

Several studies have examined the prebiotic activity of brown seaweeds using *in vitro* fermentation systems which mimic the human gut (Michel et al., 1996; Deville et al., 2007; Ramnani et al., 2012; Li et al., 2016; Rodrigues et al., 2016) but none examined the digestibility and prebiotic potential of different fractions extracted from brown seaweed. Our previous study demonstrated for the first time that crude extracts of the brown seaweed *Ecklonia radiata* have the potential to be used as a source of dietary supplements with gut health benefits in humans (Charoensiddhi et al., 2016a). The aim of the present study was to further understand the prebiotic potential of some key fractions of *E. radiata* extract by refining the extraction process. Of particular novelty is the use of microwave-assisted enzymatic extraction to disassemble the high degree of structural complexity and rigidity of seaweed cell walls reported by Deniaud-Bouët et al. (2014) and liberate target polysaccharides and

polyphenols without significant degradation. We will evaluate the digestibility and prebiotic potential of four major extract fractions, namely a crude extract fraction (CF), a phlorotannin-enriched fraction (PF), a low MW polysaccharide-enriched fraction (LPF), and a high MW polysaccharide-enriched fraction (HPF). The four seaweed fractions were tested for their prebiotic potential by including them in an *in vitro* anaerobic fermentation containing human faecal inocula. The production of SCFA and the growth of selected gut microorganisms are used as indicators of prebiotic potential.

5.3 Materials and methods

5.3.1 Seaweed

Brown seaweeds (*Ecklonia radiata*- identification confirmed by the State Herbarium of South Australia) were collected from freshly deposited beach-cast seaweed in Rivoli Bay, Beachport, South Australia in March 2013. They were rinsed in fresh water to remove any visible surface contaminants, and placed on mesh racks to dry. All the seaweed materials were collected at the one time to provide consistent samples for all these studies. They were blended (Blendtec, Orem, UT, USA) and dried in an oven at 45°C. The ground powder was stored at -20°C prior to extraction and fermentation.

5.3.2 Chemicals and substrates

All chemicals used are of analytical or chomatography grade from Merck and Sigma. The positive control substrate for comparison purposes in batch fermentation is inulin (Sigma). Blank and α-cellulose (Sigma) were used as negative controls. A commercial carbohydrate hydrolytic enzyme (Viscozyme® L) used for the preparation of seaweed extracts was kindly provided by Novozymes (Bagsvaerd, Denmark).

5.3.3 Preparation of seaweed fractions

From our preliminary study, the crude extract prepared by a microwave-intensified Viscozyme treatment process (Fig. 5.1a) showed the potential for improving gut health as this extract induced high levels of SCFA production and growth of beneficial bacteria (data removed from chapter 4 due to IP issues, but appear later within this chapter). In this study, a process was developed to sequentially obtain thee extract fractions (Fig. 5.1b) in order to understand which specific fractions in the seaweed are responsible for the prebiotic activity.

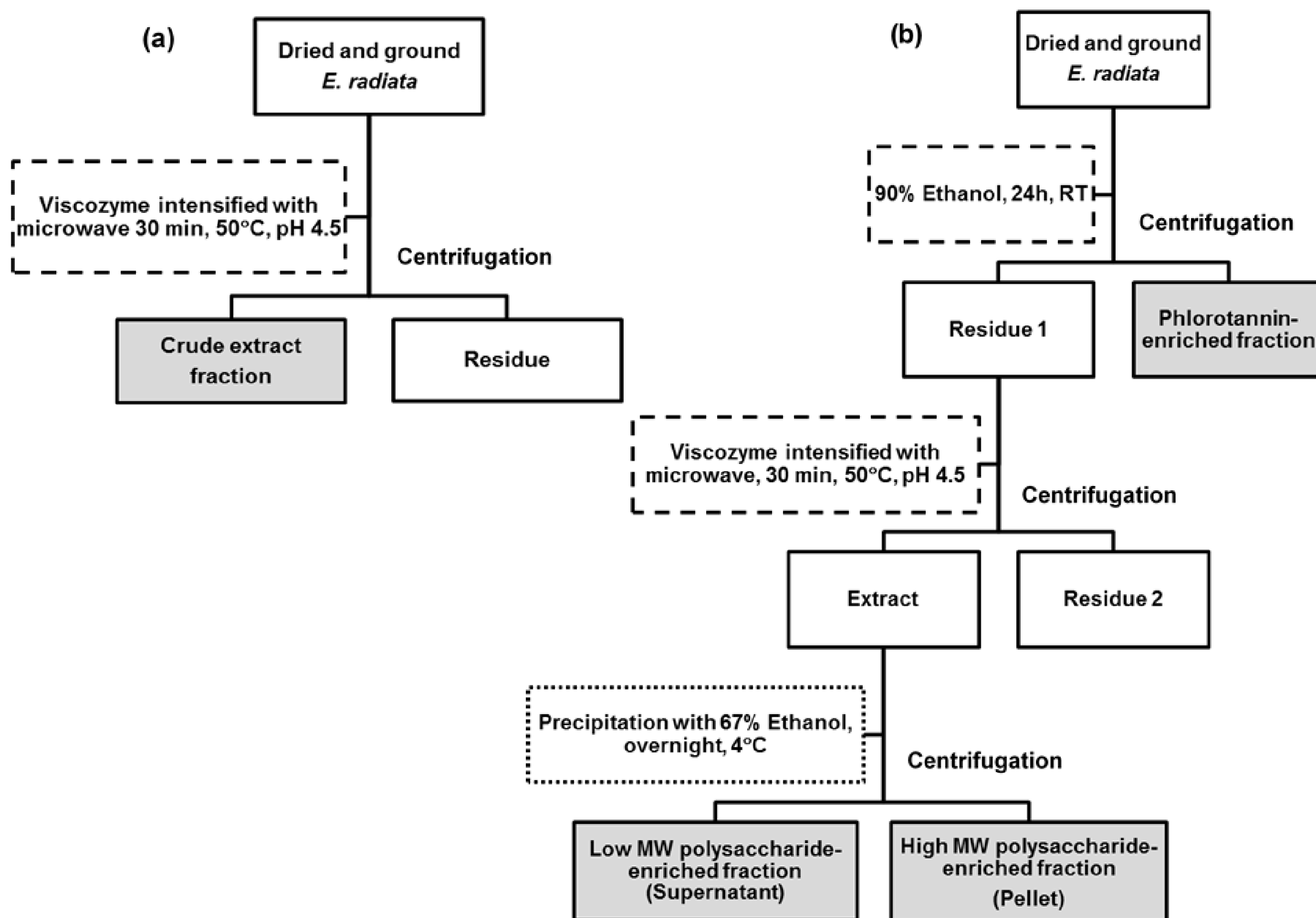

Figure 5.1 Overview of the process flow chart to prepare (a) the crude extract fraction (CF) and (b) the phlorotannin-enriched fraction (PF), the low MW polysaccharide-enriched fraction (LPF), and the high MW polysaccharide-enriched fraction (HPF) by sequential extraction and fractionation of brown seaweed *E. radiata*

5.3.3.1 Crude extract fraction (CF)

The CF was selected for inclusion in this experiment in order to demonstrate the impact of a mixture of components in the seaweed extract on prebiotic potential. This extract was prepared according to the method of Charoensiddhi et al. (2015) and Charoensiddhi et al. (2016b) with some modifications. Briefly, the dried and ground seaweed was dispersed in the pH-adjusted water in the ratio 1:10 (w/v). The pH was adjusted using 1M HCl to achieve the optimum pH of Viscozyme at 4.5. The enzyme solution was added at 10% (v/w), and the enzymatic hydrolysis was performed under optimal conditions at 50°C for 30 min in a StartSYNTH Microwave Synthesis Labstation (Milestone Inc., Shelton, CT, USA). The temperature was controlled using an infrared sensor and automatic power adjustment. The enzyme was inactivated by boiling the sample at 100°C for 10 min and cooling immediately in an ice bath. The extract was centrifuged at 8000×g for 20 min at 4°C. The supernatant

was collected and adjusted to pH 7.0, freeze dried, and stored at -20°C to be denoted as CF for further analysis and fermentation.

5.3.3.2 Phlorotannin-enriched fraction (PF)

The complex phlorotannins in brown seaweed have the potential to be fermented by gut microbes. They can be extracted efficiently with 90% ethanol with a small amount of polysaccharide contamination (Table 5.1). The other advantage is that ethanol is the preferred organic solvent for the extraction of food-grade components. In this study, dried and ground seaweed was first extracted with 90% (v/v) ethanol at an alga solid to solvent ratio of 1:10 (w/v). The suspension was incubated at room temperature for 24 h under continuous shaking in an orbital mixer incubator (Ratek Instruments, Boronia, VIC, Australia), and then centrifuged at 8000×g for 20 min at 4°C in order to separate (1) the supernatant and (2) the residue 1. The ethanol in the supernatant was evaporated in a rotary evaporator (Rotavapor-R, Flawil, Switzerland), and the residual aqueous extract was freeze dried and stored at -20°C denoted as PF for further use. The residue 1 was dried in an oven at 50°C overnight for further microwave-assisted enzymatic extraction of seaweed polysaccharides.

5.3.3.3 Low and high MW polysaccharide-enriched fractions (LPF and HPF)

The indigestible dietary polysaccharides are the most important sources for prebiotics, and their characteristics such as low and high MW may also affect the fermentability by gut microorganisms. LPF and HPF from seaweeds were prepared by the same method of preparing crude extract, but using the dried residue 1 after PF extraction instead of dried seaweed. After the enzymatic hydrolysis and centrifugation at 8000×g for 20 min at 4°C to separate the supernatant and the residue 2, the supernatant was adjusted to pH 7.0. Ethanol was then added to the supernatant to a concentration of 67% (v/v) to precipitate HPF. HPF was precipitated at 4°C overnight (modified method from Lorbeer et al. (2015)), and then collected from LPF (supernatant) by centrifugation at 8000×g for 20 min at 4°C. The ethanol in the supernatant (LPF) was evaporated in a rotary evaporator. Both LPF and HPF were freeze dried and stored at -20°C until further use.

5.3.4 Analyses of major compositions of dried seaweed fractions

The composition of dried seaweed fractions (expressed as g.100 g^{-1} dry fractions) were investigated according to the methods described in Charoensiddhi et al. (2016a,b). Total protein, starch, dietary fibre, and non-digestible non-starch polysaccharide (NNSP) of all four seaweed fractions were determined using established AOAC methods. Total phlorotannin was analysed by Folin Ciocalteu's phenol reagent, and the results were expressed as g phloroglucinol equivalent. Total sugar content was determined by HPLC after sulfuric acid hydrolysis and 1-phenyl-3-methyl-5-pyrazolone derivatisation according to the method of Comino et al. (2013). Briefly, the derivatives were separated and analyzed by HPLC (system: Prominence UFLC XR, Shimadzu; column: Kinetex 2.6u C18 100A, 100×3 mm, Phenomenex; detection: Prominence SPD-20A UV-VIS Detector, Shimadzu)

column, operated at a flow rate of 0.8 mL.min^{-1} and 30°C. The gradient eluents used were (A) 10% acetonitrile, 40 mM ammonium acetate, and (B) 70% acetonitrile with absorbance detection at 250 nm. Compounds were identified and quantified based on their retention time and peak areas, respectively, and comparison with monosaccharide standards (D-mannose, D-ribose, L-rhamnose, D-glucuronic acid, D-galacturonic acid, D-galactose, D-xylose, L-arabinose, L-fucose, D-glucose, and the internal standard 2-deoxyglucose). Sodium alginate from Sigma was subjected to the same procedure to allow for identification and quantification of the alginate-derived uronic acids.

5.3.5 *In vitro* batch fermentations

Anaerobic batch fermentations were used to assess the effect of seaweed fractions on fermentation characteristics and composition of gut microbiota. The preparation of faecal sample and fermentation medium, SCFA analysis, and bacterial enumeration were conducted according to the method described in Charoensiddhi et al. (2016a) with some modifications.

In brief, seaweed fractions were fermented *in vitro* in a batch system under anaerobic conditions for 6, 12, and 24 h. Fresh faecal samples from thee healthy individuals 10% (w/v) in sterile anaerobic solution of glycerol 20% (v/v) and cysteine hydrochloride 0.05% (w/v) were used as inoculum. Anaerobic conditions were maintained thoughout the fermentations using an anaerobic chamber. Seaweed fraction substrates at a concentration of 1.5% (w/v) in fermentation media were used in each test. For comparative purposes, no substrate was added for the blank, but positive and negative controls supplemented with inulin and cellulose, respectively, were included at the same concentration. Test substrates and controls were fermented in triplicate. Each fermentation test was inoculated with 10% (w/v) of faecal inoculum, and all fermentation tests were incubated in an orbital shaker incubator at 37°C, 80 rpm.

Fermentations were sampled at 6, 12, and 24 h for SCFA analysis using gas chomatography. Fatty acid concentrations were calculated in μmol.mL^{-1} by comparing their peak areas with standards (acetic, propionic, butyric, iso-butyric, valeric, iso-valeric, and caproic acids). The bacterial numbers were determined by quantitative real-time PCR (Q-PCR) after 24 h fermentation with a series of microbe-specific primer pairs according to the modified methods from Guo et al. (2008) for *Firmicutes*, Bartosch et al. (2004) for *Bacteroides-Prevotella*, and Charoensiddhi et al. (2016a) for the rest of the target bacteria (*Bacteroidetes*, *Bifidobacterium*, *Clostridium coccoides*, *Entercoccus*, *Escherichia coli*, *Faecalibacterium prausnitzii*, and *Lactobacillus*). These target bacterial genera were selected in the study due to their relevance to gut health.

5.3.6 *In vitro* digestibility of seaweed components

The digestibility of seaweed fractions were determined using an *in vitro* human digestion model developed by CSIRO Health and Biosecurity. Briefly, approximately 4.5 g of each seaweed fraction was treated successively with protease, pancreatic, amyloglucosidase enzymes to mimic upper gastrointestinal digestion. After 16 h at 37°C, the undigested material was precipitated via the addition of acetonitrile to 66% (v/v) and collected by centrifugation at 2000×g for 10 min. Precipitates were washed with acetone and dried to give the final products. Under these conditions, soluble and insoluble dietary fibre and larger peptides should be precipitated. Sugars, small oligosaccharides, and fructans would be removed with the supernatant. Digestibility was calculated by subtracting the dried weight of sample residue after precipitation from 4.5 g of dried seaweed fraction, and was expressed as a percentage.

5.3.7 Statistical analysis

Results were expressed in triplicate as mean ± SEM for SCFA analysis and bacterial enumeration. One-way analysis of variance (ANOVA) was used to compare the means. Differences in SCFA and bacterial enumeration were considered significant at $P<0.05$ by Duncan's test in the IBM SPSS Statistics 22 (IBM Corporation Software Group, Somers, NY, USA).

5.4 Results

5.4.1 Composition of four brown seaweed fractions

The levels of the main fermentable components in each seaweed extract fraction are shown in Table 5.1. Fibre and sugar were the major components (of components tested) in CF. Relative to CF, HPF contained approximately 4-fold higher total dietary fibre and NNSP content and 2-fold higher sugar content, while the LPF contained lower levels of fibre. PF contained the highest total phlorotannin content, but lowest fibre and sugar contents. Protein was not detected in any seaweed fraction, and a small amount of starch was found in LPF and HPF. Each of the four seaweed extract fractions had different sugar composition profiles. Glucose was the major component of all seaweed fractions. Apart from glucose, CF also consisted of fucose and small amounts of mannuronic acid, mannose, galactose, and xylose, while relatively high proportions of fucose and mannuronic acid were found in HPF, followed by guluronic acid, mannose, glucuronic acid, galactose, and xylose.

Table 5.1 Main nutrients and potential fermentable components (g.100 g^{-1} dry fraction) of four seaweed fractions, the crude extract fraction (CF), the phlorotannin-enriched fraction (PF), the low MW polysaccharide-enriched fraction (LPF), and the high MW polysaccharide-enriched fraction (HPF). Values are means of analytical duplicate analyses (n=2).

Seaweed composition	Seaweed fraction			
	CF	PF	LPF	HPF
Protein	0	0	0	0
Fibre	14.4	3.4	0.5	62.4
NNSP	5.6	0	0	22.8
Starch	0	0	0.4	0.3
Total sugar	20.6	3.4	22.7	42.1
Guluronic acid	0	0	0	1.9
Mannuronic acid	0.2	0	0	7.2
Mannose	0.5	0	0	2.1
Glucuronic acid	0	0	0	1.1
Glucose	17.2	3.4	22.7	17.1
Galactose	0.5	0	0	1.7
Xylose	0.3	0	0	1.5
Fucose	1.8	0	0	9.4
Phlorotannin	4.6	13.4	2.5	1.7

5.4.2 SCFA production

Total SCFA including acetic, propionic, and butyric acids were monitored during 6, 12, and 24 h fermentation (Fig. 5.2). Acetic and propionic acids were the predominant SCFA produced in all fermentations. All seaweed fractions and controls produced low levels of *iso*-butyric, valeric, *iso*-valeric, and caproic acids (0.02-3.43 μmol.mL^{-1}), so these results were not shown. Concentrations of acetic acid, propionic acid and butyric acid fermentations increased over the 24 h fermentations of all four seaweed fractions, with the highest concentrations observed at 24 h in each case. No significant increase in butyric acid concentration was observed in fermentations with PF, the positive control (inulin), and negative controls (cellulose and blank). After 24 h fermentation, total SCFA production of CF supplemented fermentation (97.3 μmol.mL^{-1}) was significantly higher than that of other seaweed fractions (19.6-89.0 μmol.mL^{-1}). Fermentations with all seaweed fractions, except PF, significantly increased total SCFA production (68.9-97.3 μmol.mL^{-1}) by almost 2 to 3-fold compared to negative controls (cellulose 39.7 μmol.mL^{-1} and blank 36.3 μmol.mL^{-1}). Relative to positive control (inulin), total SCFA produced from CF and LPF supplemented fermentations was approximately 10-20% higher, while fermentation with HPF was 20% lower than that of inulin. Of the seaweed fractions tested, the highest levels of acetic (40.8 μmol.mL^{-1}), propionic (54.6 μmol.mL^{-1}), and butyric (17.3 μmol.mL^{-1}) acids resulted from fermentations with the HPF, CF, and LPF, respectively. Importantly, higher levels of propionic and butyric acids were observed in CF, LPF, and HPF supplemented

fermentations compared to both positive and negative controls. The acetic acid to propionic acid ratio was different among tested fractions and controls, valued at 0.47 ± 0.04, 1.32 ± 0.09, 0.62 ± 0.06, 2.15 ± 0.06, 4.08 ± 0.18, 5.73 ± 0.13, and 5.64 ± 0.05 for CF, PF, LPF, HPF, inulin, cellulose, and blank, respectively. The initial pH of this batch fermentation was 7.2. The pH of all seaweed fractions decreased, in agreement with the increase of SCFA production during fermentation (data not shown).

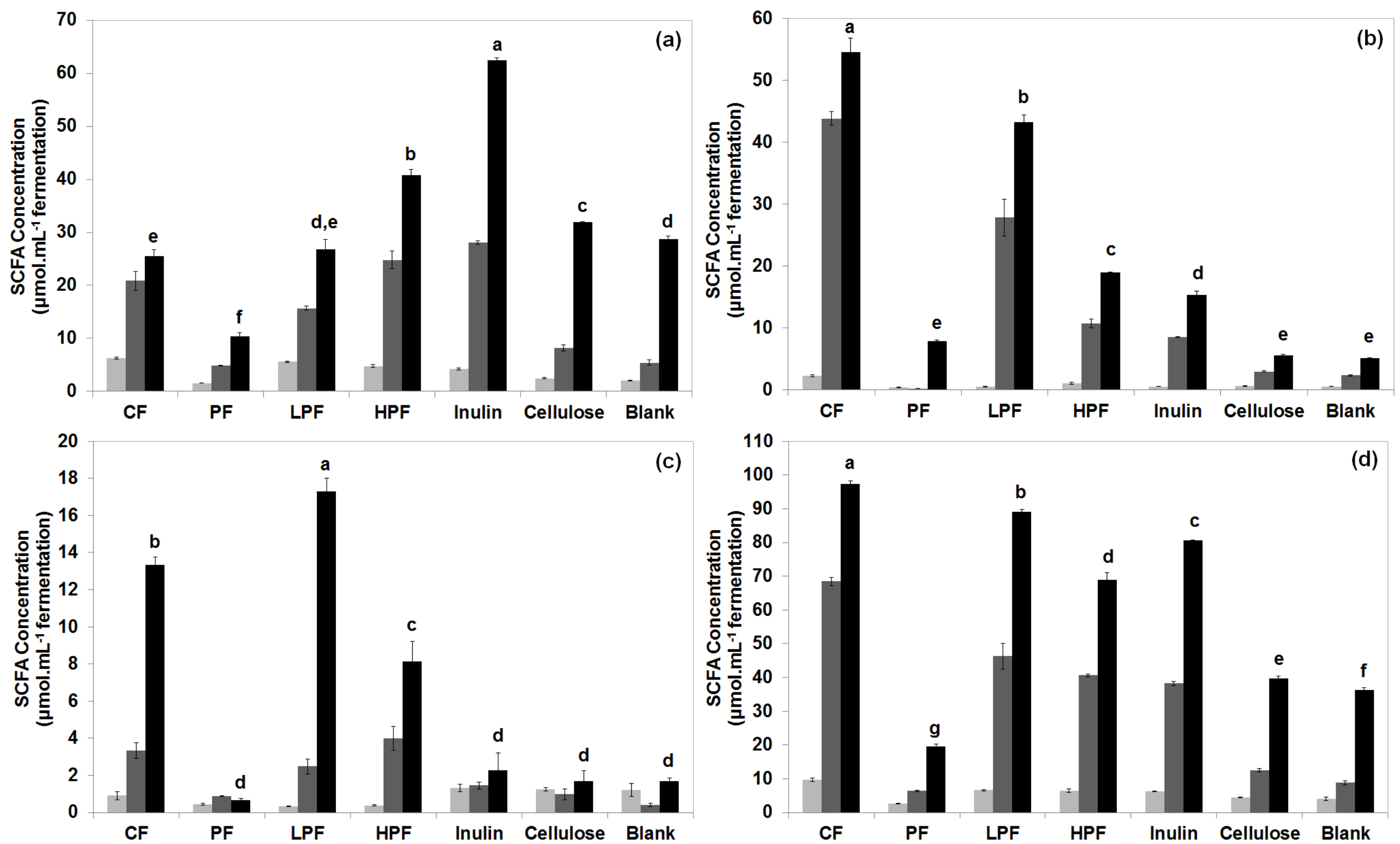

Figure 5.2 Concentration (µmol.mL^{-1} ± SEM) of (a) acetic acid, (b) propionic acid, (c) butyric acid, and (d) total SCFA in *in vitro* faecal fermentations supplemented with four seaweed fractions, the crude extract fraction (CF), the phlorotannin-enriched fraction (PF), the low MW polysaccharide-enriched fraction (LPF), and the high MW polysaccharide-enriched fraction (HPF), and controls at 6 h, 12 h, 24 h fermentation; values are means of experimental triplicate analyses (n=3), and bars with different letters are significantly different from each other (P<0.05).

5.4.3 Bacterial enumeration

The bacterial populations resulting from fermentations with four seaweed fractions and controls are shown in Table 5.2. Relative to negative controls at 24 h fermentation, the numbers of beneficial bacteria such as *Bifidobacterium*, *Lactobacillus*, and *C. coccoides* in the LPF supplemented fermentation significantly increased approximately 10-fold, while increased numbers of *C. coccoides* in CF supplemented fermentation were also observed. Although the positive control inulin significantly increased the numbers of most target bacteria compared to negative controls and other seaweed fractions, higher numbers of *Lactobacillus*, *F. prausnitzii*, *C. coccoides*, *Firmicutes*, and *E. coli* were observed for the LPF supplemented fermentation compared to inulin fermentation. In contrast, the numbers of *Enterococcus* in fermentations with PF and CF significantly decreased, approximately 10-fold, but the numbers of *E. coli* in most seaweed fractions and positive control increased in comparison to negative controls. A significant increase in the bacterial populations of *F. prausnitzii* was observed for PF and LPF supplemented fermentations compared to both negative controls and only cellulose, respectively. Most target bacteria tested could not be detected in HPF supplemented fermentation determined by Q-PCR except for *Bacteroidetes*, *Firmicutes*, and *E. coli*. The *Firmicutes* to *Bacteroidetes* ratio was calculated for each fermentation substrates. These ratios were 1.14 ± 0.001, 1.20 ± 0.008, 1.18 ± 0.006, 1.08 ± 0.008, 1.13 ± 0.004, 1.22 ± 0.004, and 1.25 ± 0.005 for CF, PF, LPF, HPF, inulin, cellulose, and blank, respectively.

Table 5.2 Bacterial populations (log_{10} cells.mL^{-1} ± SEM) in batch fermentations at 24 h of four seaweed fractions, the crude extract fraction (CF), the phlorotannin-enriched fraction (PF), the low MW polysaccharide-enriched fraction (LPF), and the high MW polysaccharide-enriched fraction (HPF), and controls; values are means of experimental triplicate analyses (n=3) from which DNA was extracted in duplicate. PCR amplification was carried out in triplicate from each of these 6 DNA extracts. Means in a column with superscripts indicate significant differences (P<0.05). *N/A is technical difficulty.

Substrates (seaweed fractions and controls)	*Bifidobacterium*	*Lactobacillus*	*F. prausnitzii*	*C. coccoides*	*Bacteroidetes*	*Bacteroides-Prevotella*	*Firmicutes*	*Enterococcus*	*E. coli*
CF	6.15^{d} ± 0.07	4.67^{e} ± 0.09	6.34^{bc} ± 0.05	8.29^{b} ± 0.03	7.36^{a} ± 0.03	7.68^{c} ± 0.11	8.40^{ab} ± 0.04	4.73^{b} ± 0.15	7.16^{c} ± 0.04
PF	6.38^{c} ± 0.07	4.92^{d} ± 0.08	6.57^{a} ± 0.05	7.97^{c} ± 0.05	6.52^{c} ± 0.04	7.01^{d} ± 0.02	7.85^{c} ± 0.08	4.15^{c} ± 0.13	8.09^{a} ± 0.02
LPF	7.11^{b} ± 0.12	6.56^{a} ± 0.05	6.42^{ab} ± 0.08	8.56^{a} ± 0.06	7.21^{b} ± 0.05	7.89^{b} ± 0.06	8.53^{a} ± 0.04	5.99^{a} ± 0.04	7.31^{b} ± 0.05
HPF	N/A	N/A	N/A	N/A	7.28^{ab} ± 0.04	N/A	7.86^{c} ± 0.03	N/A	6.96^{d} ± 0.04
Inulin	7.99^{a} ± 0.05	6.07^{b} ± 0.05	6.17^{cd} ± 0.04	7.57^{e} ± 0.06	7.34^{a} ± 0.02	8.09^{a} ± 0.04	8.30^{b} ± 0.05	5.79^{a} ± 0.03	7.10^{c} ± 0.03
Cellulose	6.34^{cd} ± 0.06	5.11^{c} ± 0.06	6.07^{d} ± 0.06	7.40^{f} ± 0.05	6.40^{d} ± 0.05	7.04^{d} ± 0.04	7.82^{c} ± 0.06	5.81^{a} ± 0.05	6.81^{e} ± 0.03
Blank	6.38^{c} ± 0.05	5.14^{c} ± 0.02	6.33^{bc} ± 0.05	7.77^{d} ± 0.05	6.30^{d} ± 0.04	6.98^{d} ± 0.04	7.89^{c} ± 0.03	5.91^{a} ± 0.10	6.94^{d} ± 0.03

5.4.4 The digestibility test of seaweed fractions

The digestibility of four seaweed fractions in the simulated human digestive system was assessed and the results are shown in Fig. 5.3. When HPF was subjected to hydrolytic enzymes imitating the upper gastrointestinal, no change was observed in dried weight of the residue. Therefore, HPF was not digestible by enzymes present in the small intestine whereas the CF, PF, and LPF were digested at 71.5%, 87.3%, and 86.1%, respectively.

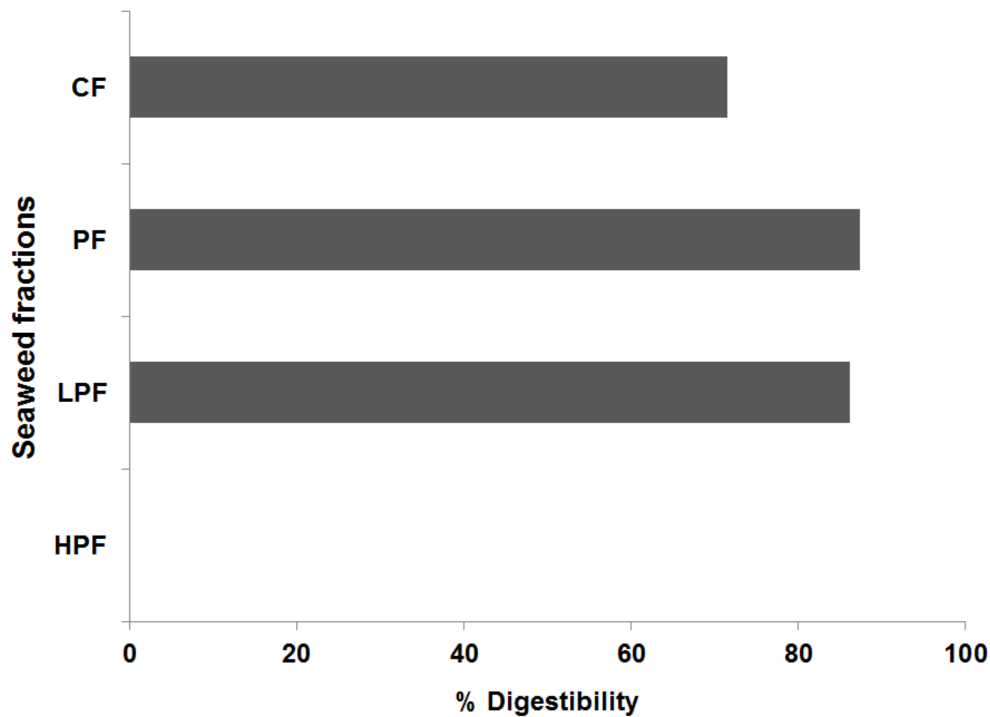

Figure 5.3 The digestibility of four seaweed fractions, the crude extract fraction (CF), the phlorotannin-enriched fraction (PF), the low MW polysaccharide-enriched fraction (LPF), and the high MW polysaccharide-enriched fraction (HPF), tested by *in vitro* simulation of the enzymes involved in upper gastrointestinal digestion; values are means of analytical duplicate analyses (n=2).

5.5 Discussion

Our previous study (Charoensiddhi et al., 2016a) using an *in vitro* anaerobic fermentation system containing human faecal inocula demonstrated that crude extracts of the brown seaweed *E. radiata* could be fermented to produce beneficial SCFA and also promoted the growth of some targeted beneficial bacteria, and could be considered to be prebiotics. However, the fractions of the complex extract mixture responsible for prebiotic activity were not clearly defined. In this study, key potential fermentable components, particularly low and high MW polysaccharides and polyphenols, were fractionated from *E. radiata* for further investigation of their prebiotic potential *in vitro*. The digestibility of these seaweed fractions was also tested to understand the likelihood of components reaching the large bowel and the resident microbiota.

Each of the seaweed fractions tested in this study increased SCFA levels when fermented over 24 h *in vitro*. However, the rates and extent of production varied, and is likely to reflect the different compositions and complex structures of components added (Rodrigues et al., 2016). The increase in SCFA levels was considerably more pronounced in the fermentations supplemented with CF, LPF, and HPF. As expected, the most abundant SCFA produced during each of the *in vitro* fermentations were acetic acid, propionic acid and butyric acid, with low concentrations of the branched chain fatty acids. These SCFA can have different physiological impacts within the gut (Topping and Clifton, 2001). Butyric acid is the primary energy source of cells lining the colon, and helps maintain colonic tissue integrity though stimulation of apoptosis in cells with high levels of DNA damage (Canani et al., 2011). Acetic acid can inhibit the growth of enteropathogenic bacteria (Fukuda et al., 2011), and propionic acid produced in the gut may influence hepatic cholesterol synthesis (Raman et al., 2016). Interestingly, the acetic acid to propionic acid ratio of fermentations with CF, LPF, and HPF were significantly lower than those for the positive control inulin and negative controls after 24 h fermentation. The decrease in this SCFA ratio has been proposed as a possible indicator of the inhibition of cholesterol and fatty acids biosynthesis in the liver, leading to a decrease in lipid levels in blood (Delzenne and Kok, 2001; Salazar et al., 2008). In contrast to the fractions enriched in polysaccharides, fermentation of the PF fraction which was enriched with phlorotannins resulted in very low levels of SCFA in comparison to other seaweed fractions, levels also below those of the negative and positive controls. Phlorotannins in brown seaweed appear to have some antibacterial activities (Dierick et al., 2010) which could explain the low SCFA production, although our microbiology analyses suggest that the inhibition of growth occurs for selected populations. Some phlorotannins were present in other fractions tested, including those enriched in polysaccharides, but this did not prevent the polysaccharides from inducing significant production of SCFA. The influence of dietary fibre and NNSP content might have a greater contribution to SCFA production than from the phlorotannin content. However, elimination of phlorotannins from the polysaccharide mixes in the future could potentially enhance their prebiotic effects.

Several studies have demonstrated the degradation of polysaccharides from brown seaweeds in the human gastrointestinal tract. Salyers *et al.* (1978) reported that species of *Bacteroides* can induce production of enzymes which degrade laminarin and alginate. *Bacteroides distasonis*, *Bacteroides thetaiotaomicron*, and *Bacteroides* group "0061" are able to break down laminarin to glucose (G1) and higher oligomers (G2-G6), while *Bacteroides ovatus* is involved in alginate degradation. Studies have also reported the use of enzymes produced by bacteria from marine environments to hydrolyse fucoidan into oligomers (Kusaykin et al., 2016). However, the results from some studies also indicate the potential of fucoidan degradation by gut bacteria. A study in pigs (Lynch et al., 2010) demonstrated that a fucoidan-supplemented diet increased *Lactobacillus* populations and SCFA production, and Shang *et al.* (2016) demonstrated that fucoidan increased the abundance of *Lactobacillus* and *Ruminococcaceae*, and decreased the number of *Peptococcus*, in mice. We have used Q-PCR to investigate the effects of the seaweed fractions on selected bacteria present in human stool. Relative to the negative controls, all seaweed fractions increased the number of *Bacteroidetes* and/or *Firmicutes*, which together comprise about 90% of the large intestinal microbiota and represent the majority of the gut bacterial phyla in humans (Qin et al., 2010). Of the seaweed fractions tested, LPF induced the greatest increase in *Bifidobacterium* and *Lactobacillus*, which are the most commonly recognised bacterial markers of prebiosis (Kleerebezem and Vaughan, 2009; Bird et al., 2010), and significantly enhanced the growth of the butyric acid producing *C. coccoides* group (Louis and Flint, 2009) compared to controls, as well as the key butyrate producing bacteria *F. prausnitzii* relative to the cellulose control. In addition, the *Bacteroides-Prevotella* population, which plays important roles in the hydrolysis and fermentation of dietary fibre (Balamurugan et al., 2010), increased over 24 h of fermentation with CF, LPF, and the positive control, but not HPF. This group of microbes and numerous others that we targeted were not detectable in the HPF fermentation, suggestive of components in the fraction interfering with many of the Q-PCR assays. Nevertheless, *Bacteroidetes* and *Firmicutes* were detectable in samples taken from the HPF fermentations. Increases in *Bacteroidetes* but not *Firmicutes* relative to the controls indicate that bacteria of the former phylum may be responsible for the increased SCFA induced by HPF. The relative proportions of *Firmicutes* and *Bacteroidetes* appears to have implications for human health (Gerritsen et al., 2011) with a low *Firmicutes* to *Bacteroidetes* ratio associated with a reduced risk of obesity or excessive body weight (Ley et al., 2005; Ley et al., 2006). In our study, the lowest *Firmicutes* to *Bacteroidetes* ratio was observed in response to HPF fermentation. We also examined changes in *Enterococcus and E.coli*, which are often linked to poor gut health outcomes. A decrease in the numbers of *Enterococcus* was observed with PF and CF supplemented fermentations while an increase in the numbers of *E. coli* was observed with most seaweed fractions compared to negative controls. The PF and CF fractions contained a higher phlorotannin content in comparison to other seaweed fractions, and these components may be able to slow down or partially inhibit pathogenic bacterial growth (Eom et al., 2012). This potential to influence the growth of gut pathogens could at least partly explain the observation that brown seaweed-derived phlorotannins

could also prevent inflammatory diseases of the mammalian intestine (Bahar et al., 2016). Although *E. coli* are commonly known for their pathogenic potential, they can also have some benefits as a consequence of non-pathogenic strains outcompeting the pathogenic forms, as is thought to occur for the probiotic *E. coli* Nissle 1917 (Iannitti and Palmieri, 2010; Gerritsen et al., 2011). Hence the increase in *E.coli* in response to the seaweed fractions in our fermentations could conceivably be beneficial, although impacts on pathogenic strains would need to be tested specifically in future studies.

The composition of the four seaweed fractions tested demonstrated that they all contained variable proportions of potentially fermentable components, mainly fibres, sugars, and polyphenols. Although components of the seaweeds were shown to stimulate SCFA production and growth of potentially beneficial gut bacteria in an *in vitro* system, it is possible that such effects will not manifest themselves *in vivo* following consumption of these components. The digestibility of components will determine how much material reaches the large bowel and is available for fermentation. In this study, HPF that contained a high dietary fibre and NNSP content, was not digestible by the enzymes responsible for breakdown of polysaccharides in the small intestine. This result was in agreement with polysaccharide compositions of this fraction inferred from sugar analysis. HPF contained a high proportions of fucose, guluronic and mannuronic acid, and glucose which might imply the presence of fucoidan, alginate, and laminarin. The mixture and cross-linkages between these sulphated, branched polysaccharides, and other cell wall components such as proteins and polyphenols contributes to the more complex structure of seaweed cell wall polysaccharides compared to terrestrial plants (Jeon et al., 2012). The resistance of these polysaccharides from brown seaweeds to digestion by enzymes in the upper gastrointestinal tract have been reported in many publications (Deville et al., 2004; Zaporozhets et al., 2014; de Jesus Raposo et al., 2016). This contrasts with the other fractions tested which were highly digestible. Although CF and LPF were better than HPF at stimulating SCFA production and some microbial growth using our *in vitro* fermentation system, HPF may prove to be more effective in promoting SCFA production *in vivo* as its minimal digestion will enable far more to reach the colon. HPF is also likely to have other advantages. In particular the high levels of fibre is expected to increase the stool bulk (and consequently dilute toxins) due to their capacity to hold water (Praznik et al., 2015).

5.6 Conclusion

The results presented in this study have shown that different seaweed fractions, and hence components derived from the brown seaweed *E. radiata*, also differ in their prebiotic potential as assessed using an *in vitro* fermentation system containing human stool. The HPF constituents show promise as prebiotics as they were resistant to digestion by enzymes responsible for polysaccharide digestion in the small intestine and were readily fermentable, stimulating the production of beneficial SCFA, including butyric acid. However, the impacts on gut microbe populations below the phylum

level were not as clear as for other fractions and may be a result of the narrow range of targets examined. The LPF constituents also show potential, as they were the most potent at stimulating growth of the traditional markers of prebiosis *Lactobacillus* and *Bifidobacterium*, and stimulated the production of SCFA in our *in vitro* system. However, the high digestibility of LPF indicates that far less of these components would reach the large bowel *in vivo*, reducing its effectiveness as a prebiotic relative to HPF components. Further investigations, which are carried out *in vivo*, are required to substantiate the prebiotic potential of HPF and LPF fractions derived from *E. radiata*. The phlorotannin fraction of the seaweed was also shown to influence the gut microbiota populations as evidenced by inhibition of the growth of potentially pathogenic bacteria and inhibition of fermentation *in vitro*, and this may have beneficial uses *in vivo*.

5.7 Acknowledgements

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Appendix 5.1: First page of the published article

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22ND INTERNATIONAL SEAWEED SYMPOSIUM, COPENHAGEN

Polysaccharide and phlorotannin-enriched extracts of the brown seaweed *Ecklonia radiata* influence human gut microbiota and fermentation in vitro

Suvimol Charoensiddhi[1,2] · Michael A. Conlon[3] · Michelle S. Vuaran[3] · Christopher M. M. Franco[1,2] · Wei Zhang[1,2]

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Abstract This study aimed to understand the prebiotic potential and contribution of four extract fractions from the brown seaweed *Ecklonia radiata*. Four seaweed fractions were tested for their digestibility and prebiotic effects using an in vitro anaerobic fermentation system containing human faecal inocula. After 24 h fermentation, three seaweed fractions, except the phlorotannin-enriched fraction (PF), significantly increased ($P < 0.05$) total short chain fatty acid (SCFA) production (68.9–97.3 μmol mL^{-1}) compared to the negative controls comprising either of a blank (36.3 μmol mL^{-1}) or cellulose (39.7 μmol mL^{-1}). The low molecular weight (MW) polysaccharide-enriched fraction (LPF) stimulated the growth of beneficial bacteria including *Bifidobacterium*, *Lactobacillus*, and *Clostridium coccoides*. The high MW polysaccharide-enriched fraction (HPF) showed the greatest potential for improving gut health as this fraction was not digestible by enzymes present in the small intestine, and induced significantly higher butyric acid production (8.2 μmol mL^{-1}) than the positive control, inulin (2.3 μmol mL^{-1}). These findings further demonstrate that *E. radiata*-derived polysaccharides have the potential to be used as dietary supplements with gut health benefits, worthy of further in vivo studies.

Keywords Digestibility · Fibre · Macroalgae · Polyphenols · Prebiotic · Short chain fatty acids

✉ Wei Zhang
wei.zhang@flinders.edu.au

Suvimol Charoensiddhi
suvimol.charoensiddhi@flinders.edu.au

Michael A. Conlon
Michael.Conlon@csiro.au

Michelle S. Vuaran
Michelle.Vuaran@csiro.au

Christopher M. M. Franco
chris.franco@flinders.edu.au

[1] Centre for Marine Bioproducts Development, School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia

[2] Department of Medical Biotechnology, School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia

[3] CSIRO Health and Biosecurity, Kintore Avenue, Adelaide, SA 5000, Australia

Introduction

Recently, indigestible dietary oligo- and polysaccharides have attracted attention as functional food ingredients that provide health benefits beyond basic nutrition (de Jesus Raposo et al. 2016). These polysaccharides often stimulate the growth and activity of beneficial gut microbes, potentially qualifying as prebiotics. They may act as substrates for fermentation in the large bowel, leading to the production of short chain fatty acids (SCFA) with multiple functions that help maintain health (Conlon and Bird, 2015). Commonly used prebiotics include inulin, fructooligosaccharides, galacto-oligosaccharides, and lactulose (Al-Sheraji et al. 2013). A growing consumer awareness of the benefits of prebiotics is leading to commercial interest in the isolation and development of polysaccharides and other compounds from novel sources such as marine seaweeds for use as prebiotics.

There is a high diversity of brown seaweeds (Phaeophyceae) in Southern Australia; of the 231 species reported, 57% are considered endemic (Womersley 1990). They are a rich source of functional food ingredients and bioactive compounds, with polysaccharides being a major component, accounting for up to 70% of the dry weight (Holdt and Kraan 2011). As a result,

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6. GUT HEALTH BENEFITS OF BROWN SEAWEED *ECKLONIA RADIATA* AND ITS POLYSACCHARIDES DEMONSTRATED *IN VIVO* IN A RAT MODEL

The positive results obtained during *in vitro* experiments, as described in chapters 4 and 5 of the thesis, warranted further investigations *in vivo*. A rat model was therefore used to determine whether the consumption of the brown seaweed *E. radiata* and its high molecular weight polysaccharide-enriched extracts would lead to beneficial changes to gut health. Moreover, alginate and fucoidan were selectively separated from the polysaccharide fraction and characterised, in order to confirm their presence and investigate their contributions toward the observed prebiotic activities.

This article will be submitted to the "Journal of Functional Foods" for publication.

Author contributions: SC designed and performed all experiments (with the assistance of (1) MC who suggested an experimental design and helped with the animal ethics application, (2) the technical teams from CSIRO Health and Biosecurity, who assisted with animal handling, monitoring, and sacrificing, as well as helping with the analyses, (3) PM, who provided instrument expertise and technical information for polysaccharide characterisation, and (4) PS, who assisted with seaweed collection and seaweed extraction in an upscale process). SC also analysed data and wrote all primary contents. WZ and MC provided advice on the research directions. WZ, MC, PM, and CF provided advice on the experimental plan, as well as the results and scope of the manuscript. All of the co-authors assisted with the revision of the manuscript prior to the journal peer-review process.

Gut health benefits of brown seaweed *Ecklonia radiata* and its polysaccharides demonstrated *in vivo* in a rat model

Suvimol Charoensiddhi [a,b], Michael A. Conlon [c], Pawadee Methacanon [d], Chistopher M.M. Franco [a,b], Peng Su [a,b], Wei Zhang [a,b]

[a] Centre for Marine Bioproducts Development, School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia
[b] Department of Medical Biotechnology, School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia
[c] CSIRO Health and Biosecurity, Kintore Avenue, Adelaide, SA 5000, Australia
[d] MTEC, National Science and Technology Development Agency (NSTDA), 114 Thailand Science Park, Pahonyothin Rd., Khlong Nueng, Klong Luang, Pathum Thani 12120, Thailand

6.1 Abstract

We examined the gut health potential of the brown seaweed *Ecklonia radiata* and its polysaccharides. Rats consumed an AIN-93G-based diet without addition (control) or containing 5% (w/w) of ground dried whole seaweed (WS) or dried powdered polysaccharide fraction (PF) of the seaweed for 1 wk. The PF consisted largely of fucoidan and alginate. PF treatment increased cecal digesta weight relative to the control (1.36±0.17 vs 0.60±0.06 g/100g body weight). Beneficial cecal total short chain fatty acids increased in response to WS (213.25±14.40 µmol) and PF (208.59±23.32 µmol) compared with the control (159.96±13.10 µmol). Toxic protein fermentation product levels were decreased by WS and PF. Cecal numbers of bacteria relevant to gut health were determined using quantitative real-time PCR. Relative to the control, numbers of butyrate-producing *Faecalibacterium prausnitzii* were increased by PF supplementation, whereas WS decreased numbers of potentially pathogenic *Enterococcus*. In conclusion, *E. radiata*-derived polysaccharides have promise as prebiotic supplements.

Key words Alginate; Fucoidan; Gut microbes; Macroalgae; Prebiotic activity; Short chain fatty acid

6.2 Introduction

Prebiotics are substrates that improve the host gut health by selectively stimulating the growth and/or metabolic activity of beneficial gut microbes (Roberfroid et al., 2010). Numerous varieties of indigestible oligo- and polysaccharides have been demonstrated as potential sources of dietary fibre and prebiotics (Praznik et al., 2015). They can act as substrates for fermentation in the large bowel, leading to the production of short chain fatty acids (SCFA) with multiple functions that help maintain gut health (Conlon and Bird, 2015). Apart from polysaccharides, ingested polyphenols with complex structures can also reach the large intestine where they can be converted into beneficial bioactive metabolites by gut microbes (Cardona et al., 2013). Thus, there is a growing interest in isolating polysaccharides and polyphenols from novel sources such as marine seaweeds for use as prebiotics and gastrointestinal modification (O'Sullivan et al., 2010; Corona et al., 2016; de Jesus Raposo et al., 2016).

A brown seaweed (*Ecklonia radiata*) commonly found along the coast of Southern Australia contains many nutrients which may benefit gut health, especially polysaccharides, which account for around 70% of its dry weight, and polyphenols, namely phlorotannins, that represent up to 6.5% of the dry weight (Charoensiddhi et al., 2015). Our previous studies (Charoensiddhi et al., 2016) demonstrated the prebiotic potential of extracts of this seaweed *in vitro*. When added to an *in vitro* anaerobic fermentation system containing human faecal inocula, the extracted components underwent fermentation, increased the production of SCFA, and promoted the growth of specific beneficial gut microbes. To understand the prebiotic potential of these seaweed-derived components further, we isolated a fraction from this seaweed which was enriched in non-digestible complex polysaccharides. This fraction subsequently stimulated the production of beneficial fermentation products by gut microbes, particularly butyric acid, when added to the *in vitro* anaerobic fermentation system (Charoensiddhi et al., 2017). The prebiotic potential of extracts and fermentable components from other seaweeds such as *Osmundea pinnatifida*, *Laminaria digitata*, and *Enteromorpha prolifera* were also demonstrated *in vitro* using fermentation systems which mimic the human gut (Michel et al., 1996; Ramnani et al., 2012; Li et al., 2016; Kong et al., 2016; Rodrigues et al., 2016).

The complex interactions between dietary components, gastrointestinal physiological processes, and gut microbiota are difficult to model *in vitro*. Consequently it is important to follow up promising *in vitro* results with *in vivo* testing. Therefore, the aim of the present study in rats was to understand whether the consumption of the brown seaweed *E. radiata* and a derived polysaccharide fraction consisting largely of fucoidan and alginate will lead to beneficial changes in gut health. The production of SCFA and other fermentation products, and the impacts on the growth of selected microbes with potentially beneficial or pathogenic effects were used as indicators of prebiotic potential. Effects on digesta bulk were also examined, as increased stool bulk in humans is an important means of reducing colorectal disease, at least partly due to dilution of toxins.

6.3 Materials and methods

6.3.1 Preparation of the whole seaweed (WS)

Brown seaweed (*Ecklonia radiata*- identified by the State Herbarium of South Australia) was collected from freshly deposited beach-cast seaweed in Rivoli Bay, Beachport, South Australia in March 2016. It was rinsed in fresh water to remove visible surface contaminants, and dried on mesh racks. All seaweed materials were collected at one time to provide consistent samples for all studies. They were dry milled (Foss Cyclotec^{TM} 1093, Hilleroed, Denmark), then passed though a 0.25 mm sieve, and dried in an oven at 45°C to obtain a moisture content of approximately 10%. The WS was stored at -20°C prior to extraction and supplementation in the rat diet.

6.3.2 Chemicals and substrates

All chemicals used are of analytical or chomatography grade from Merck and Sigma. A commercial carbohydrate hydrolytic enzyme (Viscozyme® L) used for the preparation of seaweed polysaccharide fraction (PF) was kindly provided by Novozymes (Bagsvaerd, Denmark).

6.3.3 Preparation of the PF

The PF was prepared according to the method of Charoensiddhi et al. (2017) with some modifications. Briefly, the WS was firstly extracted with 90% (v/v) ethanol at a seaweed solid to solvent ratio of 1:10 (w/v) to remove phlorotannins from the seaweed biomass. The suspension was incubated at room temperature for 3 h under continuous stirring at 80 rpm, and then centrifuged at 7350×g for 10 min at 4°C. The residue was dried and further dispersed in water with a ratio 1:10 (w/v). The pH was adjusted using 1N HCl to achieve the optimum pH of Viscozyme at 4.5. The enzyme solution was added at 10% (v/w), and the enzymatic hydrolysis was performed under an optimal condition at 50°C for 3 h in a jacketed electric kettles VEL20 (Vulcan, Baltimore, MD, USA). The enzyme was inactivated by boiling the solution at 100°C for 10 min and cooling with a cooling coil. The extract was centrifuged at 7350×g for 10 min at 4°C. The supernatant was collected and adjusted to pH 7.0 using 1N NaOH. Ethanol was then added to the supernatant to a concentration of 67% (v/v) to precipitate the PF. The PF was left to precipitate at 4°C overnight, then centrifuged at 7350×g for 10 min at 4°C, freeze dried, and stored at -20°C until further use in the supplementation of the rat diet.

6.3.4 Analyses of the composition of WS and PF

The compositions of WS and PF were investigated according to the methods described in Charoensiddhi et al. (2016). Total protein, starch, dietary fibre, and non-digestible non-starch polysaccharide (NNSP) were determined using established AOAC methods. Total phlorotannin was analysed by Folin Ciocalteu's phenol reagent, and the results were expressed as g phloroglucinol equivalent. All results were expressed as g/100 g dry samples.

6.3.5 Animal experiments and diets

All experimental protocols related to animal experiments were approved by the Animal Ethics Committee of CSIRO Food and Nutritional Sciences and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Twenty four male Sprague-Dawley rats of ~200 g weight were obtained from the Animal Resource Centre, Murdoch University, Perth, Australia. They were housed in wire-bottomed cages (4 rats per cage) in a room with controlled temperature at 23°C and lighting (a 12-h-light/-dark cycle) thoughout the study. Rats were acclimatised for 1 wk following arrival, with free access to a standard commercial rat diet (Lab chow) and water. After acclimatisation, they were weighed, ear tagged, and assigned randomly to 3 groups (n=8/ group). All rats were given free access to water and powdered AIN-93G based diets (Reeves et al., 1993) with or without supplementation with 5% (w/w) of WS *E. radiata* or PF of the seaweed by reducing levels of cornstarch in the diet (Table 6.1) for 1 wk. Dietary intake was monitored every day, and the body weight for each animal was weighed on day 3 and 7 during the week of the experimental dietary treatment.

Table 6.1 Compositions of the experimental diets used in the animal studies

Ingredient (g/kg diet)	Control diet	WS diet	PF diet
Casein	200	200	200
Cornstarch	530	480	480
Sucrose	100	100	100
WS	-	50	-
PF	-	-	50
Sunflower oil	70	70	70
Wheat bran	50	50	50
L-Cystine	3	3	3
Choline bitartrate	2.5	2.5	2.5
Vitamins (AIN 93)	10	10	10
Minerals (AIN 93)	35	35	35
Tert-butyl hydroquinol	0.014	0.014	0.014

6.3.6 Sampling procedures and analysis methods

At the completion of the 1 wk dietary intervention period, fresh faeces were collected for moisture content analysis. Rats were then anesthetized with 4% isofluorane/oxygen and killed to allow the collection and weighing of gut tissues, digesta, and other organs (liver, kidney, spleen, thymus, small intestine, colon, and cecum). The lengths of small intestine and colon were also measured. Cecal digesta was frozen and stored at -80°C for subsequent analyses of SCFA, phenols and *p*-cresols, and bacterial populations.

6.3.6.1 SCFA, phenol, and p-cresol analysis

SCFA were analysed using GC according to the modified method from Vreman et al. (1978), and phenol and p-cresol were analysed using HPLC according to the modified methods from De Smet et al. (1998), Murray and Adams (1988), and King et al. (2009). Briefly, frozen cecal digesta was mixed thoroughly with internal standard solution (5.04 µmol/g cecum of heptanoic acid and 150 µg/g cecum of *o*-cresol). Samples were centrifuged at 2000×g, 4°C for 10 min, and 300 µL of supernatant was transferred to a pre-cooled tube. Then 100 µL of 10% (w/v) sulfosalicylic acid and 4 mL of ether were added and centrifuged at 2000×g, 4°C for 2 min. The ether top layer was transferred to a clean tube containing 200 µL of 0.05M sodium hydroxide in methanol and mixed. The solvents were evaporated under a stream of nitrogen at room temperature, and 100 µL of water was added and mixed to dissolve the dried residue. 30 µL of 1M phosphoric acid was added to the tube and immediately transferred to a cold GC vial and capped. Samples were loaded onto the GC (model 6890N; Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionisation detector and capillary column (Zebron ZB-FFAP, 30 m × 0.53 mm × 1.0 µm, Phenomenex, Lane Cove, NSW, Australia). Helium was used as the carrier gas; the initial oven temperature was 90°C held for 1 min and increased at 20°C/min to 190°C held for 2.5 min; the injector and detector temperature was 210°C; the gas flow and septum purge rates were at 7.7 and 3.0 mL/min, respectively. A standard SCFA mixture containing acetic, propionic, butyric, isobutyric, valeric, iso-valeric, and caproic acids was used for the calculation, and the fatty acid concentrations were calculated in µmol/g cecum by comparing their peak areas with the standards.

After the GC analysis, the vials were transferred to the HPLC (LC-10 system; Shimadzu, Kyoto, Japan) equipped with RF-10AXL fluorescence detector and C18 column (Microsorb-MV 100-5, 250 mm × 4.6 mm, Agilent Technologies, Santa Clara, CA, USA) for analysis of phenol and *p*-cresol. Mobile phase consisted of 30% (v/v) acetonitrile, pH 3.2 at a flow rate of 1 mL/min. Phenol and *p*-cresol were calculated in µg/g cecum by comparing their peak areas with the standards.

6.3.6.2 Microbial population enumeration

DNA was extracted from cecal digesta using bead beating followed by the PowerMag® Microbiome RNA/DNA Isolation Kit (27500-4-EP; MO BIO Laboratories, Inc., Carlsbad, CA, USA) optimised for epMotion (Charoensiddhi et al., 2016, 2017). The extracted DNA was further purified by removal of protein and polysaccharide contaminants to obtain a high quality of nucleic acids following to the method of Greco et al. (2014). The bacterial numbers were determined by quantitative real-time PCR (Q-PCR) with a series of microbe-specific primer pairs according to the methods described in Charoensiddhi et al. (2016, 2017).

6.3.7 Characterisation of the PF

6.3.7.1 Selective isolation of alginate and fucoidan

Alginate and fucoidans were selectively isolated from the PF in order to determine their levels (%w/w) and characteristics. The separation of alginate was carried out using the method described by McHugh (2003) and Sellimi et al. (2015). Briefly, the PF was dissolved in deionised water and adjusted to pH 8 with 2M NaOH. 2M $CaCl_2$ solution was slowly added with stirring until no further precipitation was observed, followed by centrifugation at 9000×g, 25°C for 15 min. After that $CaCl_2$ was added to the supernatant, and the suspension was centrifuged again to ensure that all of the calcium alginate had been recovered. The precipitated calcium alginate was collected for further purification, by suspending it in deionised water and reducing the pH to below 3 with the addition of 6M HCl. The alginic acid precipitate was resuspended in deionised water, and 2M NaOH was added to achieve a pH of 8. Sodium alginate derived from this process was then dialysed (MW cut off 1 kDa, Spectra/Por®7, Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) and freeze dried. Meanwhile, ethanol was added to the supernatant to 67% (v/v), and left overnight at 4°C for complete precipitation. The precipitate was separated by centrifugation at 9000×g, 25°C for 15 min, and then dissolved in deionised water. Contaminants were removed using dialysis membrane (1 kDa) and the retentate was freeze dried to obtain fucoidan fraction.

6.3.7.2 Molecular weight (MW) analysis of alginate and fucoidan

High performance gel permeation chomatography (HPGPC, Waters 600E, Milford, MA, USA) were carried out at 30°C using an Ultrahydrogel linear column (MW resolving range of 1,000-20,000,000) with a guard column and a refractive index detector. Samples were eluted at a flow rate of 0.6 mL/min with 0.05M sodium bicarbonate buffer (pH 11) as a mobile phase. The relative MWs of the samples were estimated using a calibration curve established with standard dextrans from Sigma (MWs of 4,400-401,000 Da).

6.3.7.3 FT-IR spectroscopy

The infrared spectra of alginate and fucoidan were recorded using Spectrum One FT-IR Spectrometer (Perkin Elmer, Shelton, CT, USA) equipped with a MIR TGS detector. The samples were ground together with potassium bromide (KBr), and the mixture was transferred to the compression die and pressed into a pellet under high pressure. A region from 400 to 4000 cm^{-1} was used for scanning at 4 cm^{-1} resolution over 16 scans.

6.3.7.4 NMR analysis

Alginate and fucoidan (75 mg) were dissolved in 1 mL of D_2O (99.8% D). ^1H NMR and ^{13}C NMR were recorded at 20°C on an AVANCE^{III} 500 MHz Digital NMR Spectrometer (Bruker Biospin AV-500, Rheinstetten, Germany). ^1H NMR spectra were recorded at a frequency of 500 MHz. The spectral parameters used were as follows: 1,024 numbers of scan (NS), relaxation delay of 2 sec, spin rate

of 25 Hz, and spectral size 16 K with 32 K time domain size. ^{13}C NMR spectra were recorded at a frequency of 125 MHz. The spectral parameters used were as follows: 80,000 numbers of scan (NS), relaxation delay of 1 sec, spin rate of 25 Hz and spectral size 32 K with 32 K time domain size.

6.3.7.5 Analysis of sulphate content

Sulphur was quantified by inductively coupled plasma optical emission spectroscopy (ICP-OES) using the model Activa Horiba Jobin Yvon (Kyoto, Japan). Prior to analysis, 150 mg of samples containing a mixture of 2 mL sulphuric acid %72)w/w) and 6 mL nitric acid 65%)w/w) were digested using a microwave system at 200°C for 20 min (ramp-up time is 10 min). Calibration standards were prepared with sulphur concentrations of 0, 2.5, 5.0, 7.5, and 10 mg/L. The sulphate content was deduced from the amount of sulphur determined by ICP using the following equation: %Sulphate content = 3.22 × S%. Where 3.22 is the conversion factor for the sulphur to sulphate content (as the sodium salt), and S% is the percentage of sulphur in the samples (Rioux et al., 2007).

6.3.8 Statistical analysis

Data on the body, organ, and digesta weight/ length and %fecal moisture were presented as the mean ± SEM for each treatment group (n=8). Results of SCFA, phenol, and *p*-cresol analyses were expressed as mean ± SEM, n=8 from analytical duplicate analyses. For bacterial enumeration, results were mean ± SEM, n=8 from which DNA was extracted in duplicate. PCR amplification was carried out in triplicate from each of these DNA extracts. The effect of treatments was determined by ANOVA and differences between treatments were analysed post hoc by Tukey’s test and considered significant at $P<0.05$ using IBM SPSS Statistics 22 (IBM Corporation Software Group, Somers, NY, USA).

6.4 Results

6.4.1 Composition of the WS and PF

The compositions of key fermentable components in the WS and PF are shown in Table 6.2. Dietary fibre is the major component (of the components tested) in the WS and PF. The PF contains higher dietary fibre and NNSP contents, while the WS contains higher levels of protein and phlorotannin. Starch was not detected in both WS and PF.

Table 6.2 Main nutrients and potential fermentable components (g/100 g dried seaweed or fraction) of the WS and PF, values are means of duplicate analyses (n=2).

Composition (g/100g)	**WS**	**PF**
Dietary fibre	59.5	69.5
NNSP	11.2	17.6
Starch	0	0
Protein	7.1	5.6
Phlorotannin	4.5	1.4

6.4.2 Effect of different diets on the body and organ weight

All results are summarised in Table 6.3. There were no significant differences in the final body weight between the control (AIN-93G based diet) and two treatment groups (the diets supplemented with 5% WS or PF), but the weight gain (%) observed from the start to the end of experiment for rats fed with WS diet (23%) was increased significantly ($P<0.05$) when compared to other groups (~17%). Food intake measured on a daily basis was not different between any of the groups (data not shown). Although dietary treatments had no significant effects on the weight and the ratio of body weight to liver, kidney, spleen, thymus, and small intestine, there were significant impacts on cecum and colon. Cecal tissue and digesta weights (including the ratio to body weight) of rats fed with PF diet were significantly ($P<0.05$) higher (1.4-fold for tissue; 2.3-fold for digesta) than the control and higher (1.3-fold for tissue; 1.5-fold for digesta) than the WS fed group. Although the weight of colon digesta was not significantly affected by the dietary treatments, the weight of colonic tissue in rats fed with PF diet was significantly ($P<0.05$) higher (1.2-fold) than the control group. The colon length in two treatment groups fed with WS and PF diets was significantly ($P<0.05$) longer (1.1-fold) than the control. The PF diet significantly ($P<0.05$) increased fecal moisture of rats by 6% and 11% relative to WS and control diet groups, respectively.

Table 6.3 Effect of dietary supplementation with WS and PF on the final body weight, gut tissue and digesta, organ weight/length, and fecal moisture; values are mean ± SEM, n=8. Means in a row with different superscripts indicate significant differences (P<0.05).

	Control	WS diet	PF diet
Final body weight (g)	308.38 ± 5.82	321.75 ± 6.06	307.25 ± 4.51
Body weight gain (%, 7 days)	17.09^{b} ± 1.67	23.17^{a} ± 1.16	17.65^{b} ± 1.67
Cecum weight (g)			
Tissue	0.70^{b} ± 0.03	0.76^{b} ± 0.03	0.99^{a} ± 0.03
Digesta	1.86^{b} ± 0.19	2.87^{b} ± 0.16	4.15^{a} ± 0.50
% Cecum weight (g/100g body weight)			
Tissue	0.23^{b} ± 0.01	0.24^{b} ± 0.01	0.32^{a} ± 0.01
Digesta	0.60^{b} ± 0.06	0.89^{b} ± 0.05	1.36^{a} ± 0.17
Colon weight (g)			
Tissue	1.35^{b} ± 0.08	$1.59^{a,b}$ ± 0.05	1.68^{a} ± 0.08
Digesta	0.67 ± 0.16	1.23 ± 0.28	1.15 ± 0.23
% Colon weight (g/100g body weight)			
Tissue	0.44^{b} ± 0.02	$0.49^{a,b}$ ± 0.01	0.55^{a} ± 0.02
Digesta	0.22 ± 0.05	0.38 ± 0.09	0.37 ± 0.07
Organ weight (g)			
Liver	14.16 ± 0.40	15.43 ± 0.35	14.30 ± 0.69
Kidney	1.13 ± 0.02	1.21 ± 0.04	1.16 ± 0.03
Spleen	0.69 ± 0.03	0.65 ± 0.01	0.69 ± 0.06
Thymus	0.52 ± 0.04	0.56 ± 0.04	0.55 ± 0.02
Small intestine	6.95 ± 0.22	7.27 ± 0.27	7.19 ± 0.22
% Organ weight (g/100g body weight)			
Liver	4.59 ± 0.08	4.79 ± 0.06	4.64 ± 0.17
Kidney	0.37 ± 0.01	0.38 ± 0.01	0.38 ± 0.01
Spleen	0.23 ± 0.01	0.20 ± 0.01	0.22 ± 0.02
Thymus	0.17 ± 0.01	0.17 ± 0.01	0.18 ± 0.01
Small intestine	2.25 ± 0.05	2.26 ± 0.07	2.34 ± 0.05
Organ length (cm)			
Small intestine	128.13 ± 3.36	124.00 ± 2.59	129.75 ± 3.36
Colon	20.31^{b} ± 0.19	22.38^{a} ± 0.43	22.38^{a} ± 0.40
% Fecal moisture	59.78^{c} ± 0.42	62.91^{b} ± 0.36	66.78^{a} ± 0.35

6.4.3 SCFA, phenol, and *p*-cresol production

The cecum SCFA, phenol, and *p*-cresol production in rat cecum are presented in Table 6.4. Total rat cecum SCFA (μmoL) produced from both WS and PF diets was significantly ($P<0.05$) higher, approximately 20%, than that of the control group. Relative to the control group, the levels of acetic and propionic acids in the total rat cecum of the rats fed with WS and PF diets were significantly higher at approximately 25 to 37%, while the highest level of butyric acid (increase of 20%) was observed in the rat cecum of the PF diet group. Lower levels of *iso*-butyric, *iso*-valeric, valeric, and caproic acids were recorded for two treatment diet groups when compared to the control, and the pH of rat cecum of each diet tested decreased in conjunction with SCFA production (data not shown). However, the concentrations of almost all individual and total SCFA (μmol/g cecum) in rats fed with WS and PF diets were lower when compared to the control due to taking the weight of the cecum into account. In contrast, the level and concentration of cecum phenol in rats fed with WS and PF diets were significantly ($P<0.05$) lower than the control rats, approximately decreased up to 80%, with WS having a more profound effect. Similar results were observed in the significant decrease in the level and concentration of cecum *p*-cresols of rats fed with WS and PF diets when compared to the control group. The WS diet resulted in approximately 95% decreases in level and concentration of cecum *p*-cresols, however the PF diet only resulted in a 63% decrease in concentration but the level (which takes the weight of the cecum into account) was not statistically significant.

Table 6.4 Effect of WS and PF diets on level and concentration of SCFA, phenol, and *p*-cresol in cecal digesta of rats; values are mean ± SEM, n=8 (except SCFA of the PF group n=7) (each sample analysed in duplicate). Means in a row with different superscripts indicate significant differences (P<0.05).

Gut microbial metabolites	Control	WS diet	PF diet
Cecum SCFA (µmol)			
Acetic acid	$92.44^{b} \pm 6.98$	$138.12^{a} \pm 9.07$	$122.71^{a} \pm 12.63$
Propionic acid	$30.93^{b} \pm 2.59$	$49.21^{a} \pm 3.60$	$46.93^{a} \pm 5.29$
Butyric acid	$26.95^{a,b} \pm 3.55$	$21.40^{b} \pm 2.56$	$33.73^{a} \pm 6.29$
Valeric acid	$2.99^{a} \pm 0.36$	$2.29^{a} \pm 0.31$	$1.40^{b} \pm 0.43$
Caproic acid	$0.63^{a} \pm 0.25$	0	$0.07^{b} \pm 0.07$
iso-Butyric acid	$3.12^{a} \pm 0.51$	$1.20^{b} \pm 0.22$	$1.56^{b} \pm 0.21$
iso-Valeric acid	$2.89^{a} \pm 0.50$	$1.03^{b} \pm 0.20$	$2.19^{a} \pm 0.36$
Total SCFA	$159.96^{b} \pm 13.10$	$213.25^{a} \pm 14.40$	$208.59^{a} \pm 23.32$
SCFA concentration (µmol/g cecum)			
Acetic acid	$51.23^{a} \pm 3.39$	$48.18^{a} \pm 2.11$	$27.57^{b} \pm 2.65$
Propionic acid	$16.87^{a} \pm 0.54$	$17.12^{a} \pm 0.84$	$10.31^{b} \pm 0.79$
Butyric acid	$14.32^{a} \pm 1.18$	$7.35^{b} \pm 0.68$	$7.60^{b} \pm 1.35$
Valeric acid	$1.59^{a} \pm 0.08$	$0.79^{b} \pm 0.08$	$0.34^{c} \pm 0.11$
Caproic acid	$0.35^{a} \pm 0.15$	0	$0.01^{b} \pm 0.01$
iso-Butyric acid	$1.61^{a} \pm 0.13$	$0.41^{b} \pm 0.07$	$0.34^{b} \pm 0.03$
iso-Valeric acid	$1.48^{a} \pm 0.14$	$0.35^{b} \pm 0.06$	$0.47^{b} \pm 0.05$
Total SCFA	$87.45^{a} \pm 3.86$	$74.20^{b} \pm 2.74$	$46.65^{c} \pm 4.53$
Cecum Phenol (µg)	$5.77^{a} \pm 1.61$	$1.00^{b} \pm 0.07$	$2.07^{b} \pm 0.15$
Phenol concentration (µg/g cecum)	$2.91^{a} \pm 0.70$	$0.36^{b} \pm 0.03$	$0.49^{b} \pm 0.02$
Cecum *p*-Cresol (µg)	$25.18^{a} \pm 6.18$	$1.32^{b} \pm 0.12$	$19.34^{a} \pm 5.14$
p-Cresol concentration (µg/g cecum)	$11.96^{a} \pm 2.20$	$0.47^{b} \pm 0.05$	$4.45^{b} \pm 0.97$

6.4.4 Bacterial enumeration

The selected bacterial populations in rat cecal digesta resulting from different dietary supplementation are shown in Table 6.5. Relative to the control diet group, the numbers of key butyric acid producer *F. prausnitzii* in the cecum of rats fed with PF diet significantly increased, while a decrease in numbers of *Enterococcus* in the cecum of rats fed with WS diet was observed. Additionally, significantly higher abundance, approximately 10-fold, of *E. coli* in the rat cecum fed with PF diet was observed in comparison to that of the control and WS diets. The *Firmicutes* to *Bacteroidetes* ratio was calculated for each diet group, and the lowest of these ratios was found in the rat cecum fed with WS diet. However, a decrease in the number of *Bifidobacterium* and

Lactobacillus was detected in the rats in two treatment groups when compared to the control. The lowest abundance of *C. coccoides*, *Firmicutes*, and total bacteria were noticed in rats fed with WS diet.

Table 6.5 Effect of WS and PF diets on selected bacterial population in cecal digesta of rats from which DNA was extracted in duplicate; values are mean ± SEM, n=8. PCR amplification was carried out in triplicate from each of these DNA samples. Means in a row with different superscripts indicate significant differences ($P<0.05$).

Bacteria	Log_{10} bacteria/ total cecum (g)		
	Control	**WS diet**	**PF diet**
Bifidobacterium	$6.24^{a} \pm 0.20$	$4.83^{b} \pm 0.31$	$5.41^{b} \pm 0.13$
Lactobacillus	$7.98^{a} \pm 0.11$	$7.37^{b} \pm 0.11$	$7.62^{a,b} \pm 0.12$
Faecalibacterium prausnitzii	$4.87^{b} \pm 0.11$	$4.99^{ab} \pm 0.14$	$5.32^{a} \pm 0.11$
Clostridium coccoides	$8.21^{a} \pm 0.07$	$7.80^{b} \pm 0.08$	$8.05^{ab} \pm 0.13$
Bacteroidetes	$7.40^{a} \pm 0.07$	$7.27^{a} \pm 0.08$	$7.48^{a} \pm 0.10$
Bacteroides-Prevotella	$7.43^{a} \pm 0.08$	$7.32^{a} \pm 0.10$	$7.45^{a} \pm 0.12$
Firmicutes	$8.40^{a} \pm 0.09$	$7.93^{b} \pm 0.08$	$8.50^{a} \pm 0.07$
Enterococcus	$6.04^{a} \pm 0.09$	$5.59^{b} \pm 0.08$	$5.96^{a} \pm 0.10$
Escherichia coli	$5.24^{b} \pm 0.19$	$5.10^{b} \pm 0.16$	$6.38^{a} \pm 0.14$
Total Bacteria	$8.35^{a} \pm 0.08$	$7.93^{b} \pm 0.08$	$8.44^{a} \pm 0.06$
Ratio of *Firmicutes:Bacteroidetes*	$1.13^{a} \pm 0.006$	$1.09^{b} \pm 0.005$	$1.14^{a} \pm 0.100$

6.4.5 Characterisation of the PF

After selective extractions, it was found that the alginate and fucoidan accounted for 23.8%±1.7 and 46.1%±1.7 (dry weight) of the PF, respectively. Approximately 30% of the remaining components could be accounted for by ash (~20%) and small amounts of other components (~10%) such as protein, phlorotannin, fat, etc. (data not shown). The MWs of alginate and fucoidan in the fraction were then estimated by HPGPC, relative to the dextran standards. Both alginate and fucoidan showed a single peak in the chomatograms (data not shown), indicating their homogeneity. The peak MW of alginate and fucoidan was estimated to be 237.03 and 339.78 kDa, respectively. The sulphur content of fucoidan fraction (7.65%±0.16) was analysed by ICP-OES indicated that the fucoidan fraction contained 24.62%±0.52 sodium sulphate.

The identification of alginate and fucoidan was further confirmed by spectroscopic methods (FT-IR and NMR), and their corresponding spectra are shown in Fig. 6.1 and 6.2.

Alginate The FT-IR spectrum of alginate contained intense absorption bands at 1,618 and 1,420 cm^{-1} (carboxyl group; C=O stretching), which is characteristic of alginates (Imbs et al., 2016). An additional broad band at 3,359 cm^{-1} was assigned to hydrogen bonded O–H stretching vibrations, and the signal at 2,933 cm^{-1} was attributed to C–H stretching. More signals at 891 and 819 cm^{-1} were assigned to the α-L-gulopyranuronic asymmetric ring vibration and to the mannuronic acid residues, respectively (Fenoradosoa et al., 2010). In the ^{13}C NMR spectrum of the alginate fraction, there are thee regions consisting of C2-C5 (60-90 ppm), anomeric (C1, 90-110 ppm), and carboxyl (C6, 172-180 ppm) carbon signals. The major chemical shifts were detected at 178.04, 104.75, 103.95, 83.49, 80.60, 78.54, 74.09, 73.52, 72.69, 71.82, 67.47 ppm which could refer to G6 M6, G1, M1, G4, M4, M5, M3, M2, G3, G5, and G2, respectively (Salomonsen et al., 2009). For ^{1}H NMR, the characteristic anomeric proton signal of polymannuronic acid appeared at 4.71 ppm. The signals at 4.44 and 5.02 ppm were indicative for the presence of some guluronic acid residues (Chandía et al., 2004).

Fucoidan The FT-IR spectrum of fucoidan contained an intense absorption band at 1,261 cm^{-1} attributed to asymmetric O=S=O stretching vibration of sulphate esters with some contribution of COH, CC, and CO vibrations, which is a typical characteristic of sulphated polysaccharides (Synytsya et al., 2010). An additional sulphate absorption band at 826 and 850 cm^{-1} were attributed to C-O-S, secondary equatorial sulphate at C-2 or C-3 and axial sulphate at C-4, respectively (Kim et al., 2010). The ^{13}C NMR spectrum of fucoidan was complex. Results showed that C6 carbon signals of neutral sugar units were found at 18.62 ppm (CH_3 of α-L-fucopyranose units), and corresponding methyl carbon signals at 19.89 ppm were assigned to *O*-acetyl groups. The bands at 62.06 and 64.04 ppm were assigned to CH_2OH of β-D-galactopyranose units and CH_2OR of β-D-galactopyranose substituted at O6, respectively. The region 65-83 ppm consists of complex signals of C2-5 pyranoid ring carbons. Several resonance signals of anomeric (C1) carbons were found around 95-105 ppm, and a carbonyl carbon signal was observed at 177.79 ppm (Synytsya et al., 2010; Ermakova et al., 2011). The signals of ^{1}H NMR spectrum in the last region at 1.00 and 1.13 ppm were assigned to C6 methyl protons of L-fucopyranose, and a signal at around 2 ppm arose from CH_3 protons of *O*-acetyl groups. Signals in the α-anomeric were found at 5.0-5.6 ppm assigned mainly to CH protons of *O*-substituted carbons and H1 of α-L-fucopyranose residues (Bilan et al., 2004; Synytsya et al., 2010).

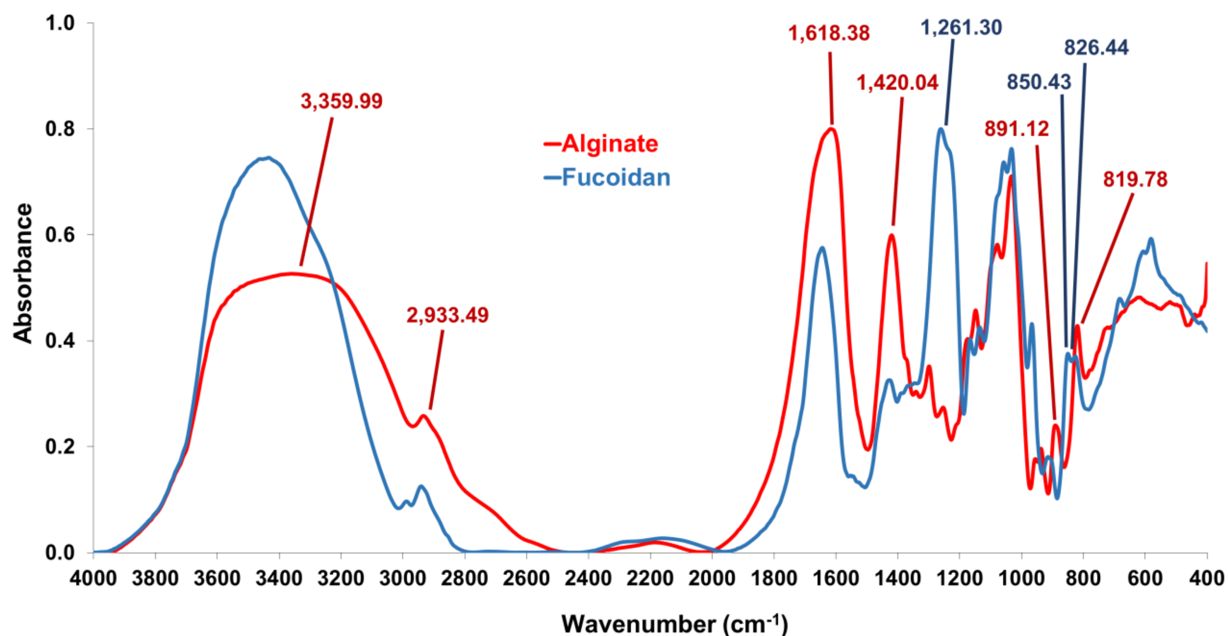
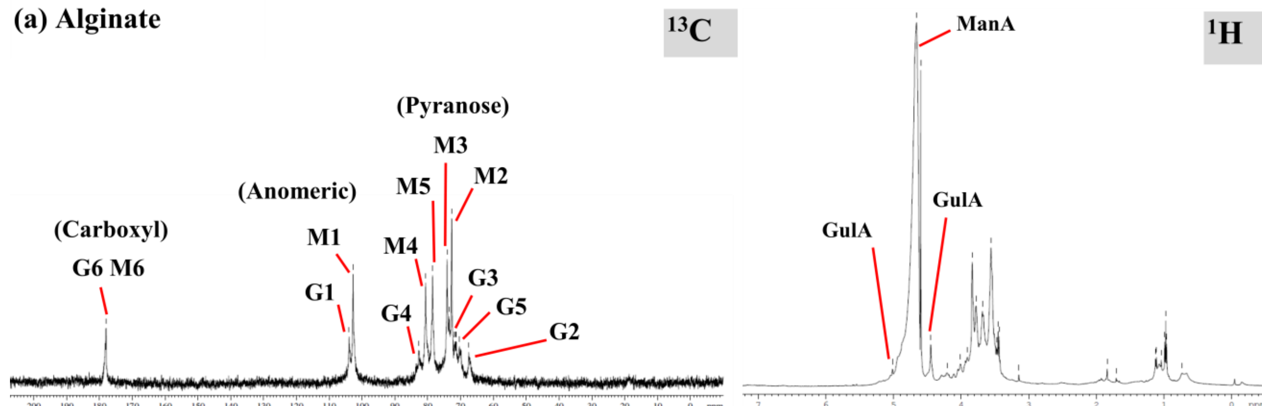


Figure 6.1 FT-IR spectra of alginate and fucoidan separated from the PF of seaweed *E. radiata*



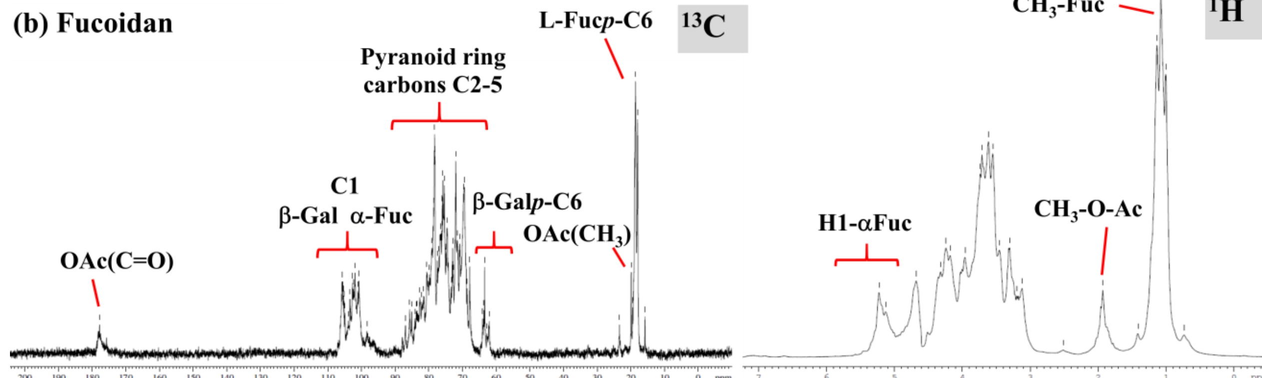


Figure 6.2 ^{13}C and ^{1}H NMR spectra of (a) Alginate and (b) Fucoidan separated from the PF of seaweed *E. radiata*

6.5 Discussion

In this study, brown seaweed *E. radiata* and its derived polysaccharides were examined as potential sources of prebiotics using a rat model. Rats were fed with diets supplemented with 5% of WS or PF (which we show here to be rich in fucoidan and alginate) for 1 wk and effects compared with those of a standard (control) diet. We show that the WS and/or the PF are able to undergo fermentation by gut microbes *in vivo*, resulting in the production of beneficial SCFA, stimulate an increase in digesta bulk, and alter some populations of microbes which can positively influence gut health.

The increases in mass or length of gut tissue and digesta of the large bowel in rats fed with PF diet are typical effects of dietary fibre on the gut (Eastwood, 1992). These effects could be explained by the high levels of dietary fibre and NNSP present in the PF. The PF diet had significant impacts on the cecum and colon of rats. In comparison to other studies, the 2-fold increase in total cecum weight of rats fed with this PF diet compared with the control group was higher than the increase in cecum weight of rats fed with 6% (w/w) of the prebiotics fructo-oligosaccharide (1.7-fold) and oligofructose (1.9-fold), but lower than xylooligosaccharides (2.4-fold), when fed for a period for 2 wks (Campbell et al., 1997). In addition, relative to the control group, the increase in colon weight (1.4-fold) and length (1.1-fold) of rats fed with this PF diet was comparable to the increase in colon weight and length (1.2-fold) of obese leptin-deficient mice fed with 10% (w/w) of the prebiotic oligofructose-supplemented diet for 5 wks (Everard et al., 2011). The increase in fecal moisture content in rats fed with PF diet was likely a sign of the elevated water holding capacity of the fibre and might be related to the increase in the growth of colonic mucosa (Sircar et al., 1983).

Significant improvements of SCFA production in rats fed with WS and PF diets compared to the control group were most likely a result of the fermentable fibres in the WS and PF. de Jesus Raposo et al. (2016) indicated that the fermentation of seaweed polysaccharides by beneficial bacteria have been shown to generate SCFA, which can have different physiological impacts within the gut. Butyric acid is the primary energy source of cells lining in the colon, and helps maintain colonic tissue integrity though stimulation of apoptosis in cells with high levels of DNA damage (Canani et al., 2011). Acetic acid can inhibit the growth of enteropathogenic bacteria (Fukuda et al., 2011), and propionic acid produced in the gut may influence hepatic cholesterol synthesis (Raman et al., 2016). In our study, the most abundant SCFA in rat cecal digesta were acetic acid followed by propionic acid and butyric acid, with low concentrations of the branched chain fatty acids. This result was similar to the SCFA profiles obtained from previous *in vitro* fermentations of the WS and PF (Charoensiddhi et al., 2016, 2017).

Putrefactive microbial products derived from protein fermentation include phenols and *p*-cresol. The accumulation of these compounds in the gut are linked to loss of gut integrity and function, and increased risk of bowel diseases (Windey et al., 2012). The rats fed with WS and PF diets tested in

this study produced significantly lower levels and concentrations of phenol and *p*-cresol in cecal digesta than the control. This might be associated with their polysaccharide content as it has been reported that the fermentation of alginate by gut microbes could suppress the formation of putative risk markers for colon cancer (Kuda et al., 2005). The WS diet was more effective in reducing phenol and *p*-cresol contents in rat cecum compared to the PF diet group. This result may be related to the 3-fold greater phlorotannin content in the WS. Other studies suggest dietary polyphenols can have such an effect. For instance, a decrease in phenol and *p*-cresol in feces were found in humans after consuming a grape seed extract supplemented diet for 2 wks (Yamakoshi et al., 2001). Similar results with the decrease in phenol and *p*-cresol concentrations in pig feces was observed after 2 wks feeding with a tea phenol-enriched diet (Hara et al., 1995). Further studies to understand the roles of seaweed phenolic compounds on protein fermentation are still required. However, a decrease in pH of the feces to acidic resulting from bacteria fermentation after phenolic intervention may decrease the concentration of putrefactive compounds as neutral or slightly alkaline are more optimal conditions for microbial proteases (Macfarlane et al., 1988). In addition, phenolic compounds are reported to inhibit the growth of proteolytic bacteria and reduce the expression of genes involved in the production of proteases (Mosele et al., 2015).

In this study, we used Q-PCR to monitor changes in populations of selected colonic bacteria populations in response to diets supplemented with WS and PF. An increase in the key butyric acid producer *F. prausnitzii* was observed in the cecum of rats fed with the PF diet compared to the control, concurrent with an increase in butyric acid production. This bacterium is of particular interest because in addition to its ability to generate butyrate it has been identified as having anti-inflammatory effects which may help protect against inflammatory bowel disease (Sokol et al., 2008; Miquel et al., 2013). The use of an enzyme-assisted extraction process has resulted in modification and/or breakdown of some high MW components, including polysaccharides, in the PF, which may improve the activity or bioavailability of lower MW components that promote the growth and activity of *F. prausnitzii*. A decrease in the cecal numbers of the potentially pathogenic *Enterococcus* was observed when rats were when fed the WS diet. Other *E. radiata* compounds present in WS but not the PF, particularly polyphenols, may have contributed to this effect, as polyphenols have previously been shown capable of regulating of pathogenic microbial activity (Eom et al., 2012). Feeding with WS resulted in a reduced ratio of the *Firmicutes*/*Bacteroidetes*. This may be beneficial to human health as it is associated with a decreased risk of obesity or excessive body weight (Ley et al., 2005; Ley et al., 2006). An increase in the number of *E. coli* was observed in the cecum of rats fed with the PF diet. Although *E. coli* are commonly known for their pathogenic potential, they can also have some benefits as a consequence of non-pathogenic strains outcompeting the pathogenic forms, as is thought to occur for the probiotic *E. coli* Nissle 1917 (Gerritsen et al., 2011). The abundance of some beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*, traditional markers of prebiosis, were not increased by diets in our study. Despite these bacteria not changing, a significant increase

in the beneficial activity of gut bacteria is nevertheless suggested by the increase in cecal SCFA. It is quite likely that the populations or activities of other beneficial bacteria were also increased, but these were not analysed here and would need to be examined in future studies.

A few studies have previously demonstrated gut health benefits (prebiotics effects) of alginates and fucoidans. Kuda et al. (2005) reported that rats fed with 2% (w/w) of a low MW (49 kDa) alginate-supplemented diet could increase cecum weight and total SCFA production, and decrease indole and *p*-cresol levels, when compared with the control group. In another rat study, a 2.5% (w/w) alginate oligosaccharide-supplemented diet stimulated the growth of fecal bifidobacteria by 13-fold and 4.7-fold when compared with a control diet and a diet supplemented with 5% fructo-oligosaccharides, respectively. In addition, the alginate supplemented diet resulted in a significant increase in *Lactobacillus* and decreases in *Enterobacteriaceae* and *Enterococcus* compared with the control group (Wang et al., 2006). Pigs fed with a 238 ppm of fucoidan-supplemented diet significantly increased their colonic *Lactobacillus* populations and total SCFA production (Lynch et al., 2010). Shang et al. (2016) also demonstrated that mice fed with fucoidan (100 mg/kg/day) increased the abundance of *Lactobacillus* and *Ruminococcaceae*, and decreased the number of pathogenic *Peptococcus* in cecum. In our study, when rats were fed with the PF diet we did not detect an increase in the abundance of *Bifidobacterium* or *Lactobacillus*, nor did we find a decrease in the potentially pathogenic bacteria we examined.

Alginate and fucoidan are major polysaccharides found in brown seaweeds (Holdt and Kraan, 2011) and were expected to be present in *E.radiata*. To better understand the contributions they may make to the effects of dietary PF on gut health in this study, alginate and fucoidan were each selectively isolated from this fraction to enable their characterisation. The PF contained primarily fucoidan (46%) and alginate (24%). The FT-IR spectrum was in complete agreement with the ^{13}C and ^{1}H spectra, indicating that mannuronic acid was predominant in the alginate. This result corresponded to the NMR spectra reported by Salomonsen et al. (2009) for commercial sodium alginate powders containing 65% mannuronate. Moreover, low viscosity was observed for this mannuronic acid-rich alginate, and the addition of calcium did not significantly promote gelation (data not shown). These observations further support the assertion that the alginate was dominated by mannuronic acid, as the polyguluronic acid (G) blocks can bind calcium ions between two chains more effectively than the polymannuronic acid (M) blocks, resulting in higher viscosity and stronger gel forming capabilities (Sari-Chmayssem et al., 2016). For fucoidan, the carbon signals of NMR were split into several peaks, which confirm the presence of several structural patterns of sugar units, depending on the substitution. It may indicate that this fucoidan is galactofucan, sulphated and acetylated at different positions of the galactose and fucose residues. It was also worth noting that the sulphate group in this fucoidan (24.6%) was relatively higher than that of other brown seaweed species, such as *Ascophyllum nodosum* (22.3%), *Fucus vesiculosus* (19.0%), and *Saccharina longicruris* (14.2%) analysed using the same technique (Rioux et al., 2007). The bioactivity of many fucoidans, such as

anticoagulant activity, is enhanced with increased sulphatation (Li et al., 2008), so the role of highly sulphated fucans on the prebiotic properties of these polysaccharides should be investigated further.

Further studies covering a different range of seaweed-derived polysaccharide components and structures are suggested in order to more fully understand which specific components in seaweeds are responsible for gut health effects, and whether polysaccharide mixtures could be tailored to optimise these health benefits. Also, the characterisation and development of low MW seaweed polysaccharides may facilitate dietary formulations which improve fermentability by gut microbes (Ramnani et al. 2012).

6.6 Conclusion

Our study has shown for the first time that the brown seaweed *E. radiata* and its polysaccharide fraction can improve different aspects of gut health *in vivo*. Relative to the control group, rats fed the WS diet produced significantly higher levels of SCFA and lower levels of phenol, *p*-cresol, and potentially pathogenic *Enterococcus*, whereas rats fed with the PF diet significantly increased their cecum and colon weights, cecal SCFA production, and the abundance of *F. prausnitzii*, a primary butyrate producer with anti-inflammatory actions. Sulphated fucan and mannuronic acid-rich alginate with approximate MW of 339.8 and 237.0 kDa respectively, are the predominant components of PF and hence most likely to be responsible for any benefits which we have described. In addition, the higher phlorotannin content in the WS compared to the PF might play a role in the inhibition of phenol and *p*-cresol production and pathogenic bacteria growth. These findings suggest that *E. radiata* and its polysaccharides have the potential to be used as a dietary supplement with gut health benefits in humans.

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7. PROCESS AND ECONOMIC FEASIBILITY FOR THE PRODUCTION OF FUNCTIONAL FOOD FROM THE BROWN ALGA *ECKLONIA RADIATA*

After developing enzyme-assisted extraction processes for the brown seaweed *E. radiata*, and confirming the antioxidant and prebiotic activities of the resulting extracts and fractions *in vitro* and *in vivo*, we wanted to explore the potential for commercialisation. Therefore, we performed a techno-economic analysis in order to compare the commercial feasibility of the various processes at industrial scale, for the production of seaweed-derived bioactive food ingredients, as documented in this chapter.

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Author contributions: SC designed all simulations, performed techno-economic analyses, analysed data, and wrote all primary contents. AL provided training on the SuperPro Designer 8.0® software. AL and PS provided the information and advice regarding industrial-scale processing. WZ provided advice on experimental directions as well as data analyses and results. WZ, CF, and MC provided suggestions for the improvement of process simulations and the scope of the manuscript. All of the co-authors assisted with the revision of the manuscript before journal submission.

Process and economic feasibility for the production of functional food from the brown alga *Ecklonia radiata*

Suvimol Charoensiddhi [a,b], Andrew Lorbeer [a,b], Chistopher M.M. Franco [a,b], Peng Su [a,b], Michael A. Conlon [c], Wei Zhang [a,b]

[a] Centre for Marine Bioproducts Development, School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia
[b] Department of Medical Biotechnology, School of Medicine, Flinders University, Bedford Park, South Australia 5042, Australia
[c] CSIRO Health and Biosecurity, Kintore Avenue, Adelaide, SA 5000, Australia

7.1 Abstract

This article provides a case-study for the simulated industrial-scale production of high-value functional food products from the brown seaweed *Ecklonia radiata*. Three process scenarios were assessed for their economic feasibility: Scenario 1: Enzyme-assisted production of a crude seaweed extract; Scenario 2: Scenario 1, followed by fractionation based on different molecular weights (MW); Scenario 3: Ethanolic extraction, followed by enzyme-assisted extraction and separation into high and low MW fractions. Scenario 2 demonstrated greater profitability, with a payback period of 1.6 years and a net present value (NPV) twice that of Scenario 1 (2.1 years and US$ 45.03 mil). Scenario 3 was not economically feasible, with a negative NPV and payback period which was three times longer than Scenario 1. To improve profitability, Scenario 4: Scenario 1, integrated with formulation of the extract into a juice-based beverage was assessed. This process was more profitable than Scenario 1, with a payback period and NPV of 1.1 years and US$ 89.43 mil, respectively.

Key words Bioprocess; Economic assessment; Phlorotannin; Polysaccharide; Process simulation; Seaweed

7.2 Introduction

Brown seaweeds (Phaeophyceae), in general, are considered important sources of bioactive compounds with a range of biological activities [1]. Brown seaweed-derived, bioactive ingredients include: polysaccharides (e.g. fucoidans, alginates and laminarins), polyphenols (phlorotannins) and carotenoids (fucoxanthins). In addition, they also contain polyunsaturated fatty acids (PUFAs), including omega-3 fatty acids, proteins and bioactive peptides [2]. These compounds are of increasing interest for their broad range of bioactivities, in particular antioxidant, prebiotic, neuro-protective, anti-bacterial, anti-inflammatory, immune modulation, anti-diabetic, anti-cancer and anti-coagulant properties [3,4,5], all with potential applications in the functional food and nutraceutical

industries. The brown seaweed *Ecklonia radiata* was chosen in this study as it is one of the most abundant seaweed species in South Australia, with significant quantity and potential for commercial exploitation. Despite this natural abundance, most brown seaweeds harvested locally are underutilised and processed primarily into fertilisers and animal feeds [6].

The efficient extraction of polysaccharides and other bioactive compounds from seaweeds can be impeded by the high degree of structural complexity of their cell walls [7]. Our previous study [8] demonstrated that the enzyme-assisted extraction intensified with microwaves was a more effective means of increasing the recovery of phlorotannins from *E. radiata*, when compared with conventional acidic extraction. The seaweed extracts obtained showed high potential for use as functional food ingredients with reported antioxidant activities of 740 μmol Trolox equivalents (TE)/g DW. This activity is comparable to green and black tea (761 μmol TE/g DW) [9], and greater than that of some fruits, vegetables, and medicinal plants commonly recognised as high antioxidant sources (e.g. 100-500 μmol TE/g DW) [10,11,12,13].

Aside from increasing the recovery of phlorotannins and antioxidant compounds, we also demonstrated enzymatic extraction to be an effective technique for producing brown seaweed polysaccharides with prebiotic potential [14]. Currently, the low efficiency of biomass utilisation and the large volumes of waste by-products are problems generally associated with the industrial processing of seaweed. In order to alleviate these problems and improve economic viability, integrated biorefinery processes have been proposed for the production of multiple products through the comprehensive utilisation of seaweed biomass [15,16,17,18,19,20,21,22]. In our research, a sequential extraction process, based on the biorefinery concept, was developed to produce different fractions enriched with different bioactive compounds from *E. radiata*. The process involved ethanolic extraction of a phlorotannin-enriched fraction, followed by the use of carbohydrate-hydrolytic enzymes and the fractionation of the extract according to molecular weight (MW). One of the products was a high MW polysaccharide-enriched fraction, which was observed to be resistant to human digestive enzymes, but readily fermentable by gut bacteria, resulting in the production of beneficial short chain fatty acids (SCFA) including butyric acid. Meanwhile, the phlorotannin-enriched fraction was also shown to influence the gut microbiota through the inhibition of the growth of potentially pathogenic bacteria *in vitro* [23]. During *in vivo* studies, rats fed with the high MW polysaccharide-supplemented diet showed significant improvements in their cecal digesta weight (1.4 vs control 0.6 g/100g body weight), total SCFA (209 vs control 160 μmol) and an abundance of the key butyric acid producer *Faecalibacterium prausnitzii*, accompanied by a decrease in potentially toxic phenol and *p*-cresol in the cecum [24]. These results suggested that the high MW polysaccharide components could potentially be consumed by humans as dietary fibres and prebiotics.

In this study, in order to understand the economic feasibility of such production processes at an industrial scale, a widely used industry process simulation software SuperPro Designer was used to assess and compare four possible seaweed value-added processes based on our previous studies. This approach has been used to assess the feasibility of biofuel and succinic acid production from seaweeds [25,26,27,28], functional food ingredients from fish protein hydrolysates [29], and production of bioactive compounds from other plant materials such as grape bagasse, turmeric, and ginseng [30,31,32,33,34]. However, there have been no reports on a techno-economic feasibility for the production of seaweed-derived bioactive compounds and functional food products. Therefore, the aim of this study was to conduct a comparative economic assessment of industrial-scale production processes for high value bioactive compounds and functional food products from the brown seaweed *E. radiata* as a case study. However, the outcomes of this assessment are likely to have much wider applicability, with potential to inform the economic feasibility of many other processes for the isolation and use of bioactive ingredients from other seaweeds that are increasingly seen as important sources of functional foods and nutraceuticals. The results from this study should help bring about the informed use and expansion of new platforms that enable seaweed utilisation for the growing demand of higher-value food and health products and thereby also support economic development.

7.3 Materials and methods

7.3.1 Simulation description

The simulations were all performed using the software SuperPro Designer 8.0® (Intelligen Inc., Scotch Plains, USA). All of the models used batch processes, with the baseline processing capacity of 2,000 kg of dried seaweed per batch for three extraction processes. Taking into account the limiting factor of the size of the functional juice market and the ability of the market to absorb the new product at the starting period, production scale was reduced to 500 kg of dried seaweed per batch for the process that incorporated seaweed extract into the production of a model beverage product. The annual operating time of 7,920 h per year was employed, which corresponds to 330 days per year of continuous 24 h per day shifts. The currency is in $US. *Ecklonia radiata* was selected as the raw material in this study based on our previous experimental studies [6,8,14,23,24]. It was assumed that seaweed used in all simulations was commercial dried Australian beach-cast seaweed for human consumption. The change of seaweed compositions due to different seasons was not taken into account in this study. The seaweed was rinsed in fresh water to remove any visible surface contaminants, and placed on mesh racks to dry. Then it was dried at 45-50°C to obtain a moisture content of approximately 10%.

7.3.2 Proposed industrial process scenarios

Three different process scenarios were designed and considered for the production of different high-value seaweed fractions as ingredients. Scenario 1: A single enzyme-assisted extraction step to produce a “Crude extract Fraction (CF)”, as well as a “Fibre-enriched Fraction (FF1)”, which corresponded to that biomass remaining after the enzymatic extraction; Scenario 2: The same process as Scenario 1, but with the CF subsequently being separated into two fractions based on their MWs, a “High MW Polysaccharide and Phlorotannin-enriched Fraction (HPPF)” and a “Low MW Polysaccharide and Phlorotannin-enriched Fraction (LPPF)”. Scenario 3: A sequential extraction process consisting of two steps: the first used ethanol to extract a “Phlorotannin-enriched Fraction (PF)”, with the residual material then being subjected to enzyme-assisted extraction to produce an extract that was separated into a “High MW Polysaccharide-enriched Fraction (HPF)” and a “Low MW Polysaccharide-enriched Fraction (LPF)”. Another product, the: “Fibre-enriched Fraction 2 (FF2)”, corresponded to the biomass which remained after the enzymatic extraction. To further improve the profitability of Scenario 1, Scenario 4 represented the same process as Scenario 1, but with the liquid CF being incorporated into a “Fruit Juice-based Beverage (FJB)”, as a functional ingredient, at 2% w/w (based on DW of CF).

7.3.2.1 Single extraction

The process layouts proposed for Scenarios 1 and 2 are presented in Fig. 7.1a,b.

CF: The dried and ground seaweed was dispersed in pH-adjusted tap water in the ratio 1:10 (w/v). The pH of the water was adjusted to 4.5 using 1M HCl prior to the addition of the seaweed, to achieve the optimum pH for Viscozyme® L (major activity: beta-glucanase, including the activity of xylanase, cellulase, and hemicellulase). The enzyme solution was added at 10% (v/w), and the enzymatic hydrolysis was performed at the optimal temperature of 50°C for 3 h under continuous mixing. The enzyme was then inactivated by boiling the sample at 100°C for 10 min. The extract was cooled and transferred through a plate and frame filter to separate the residual biomass. The filtrate was collected, adjusted to pH 7.0 using 1M NaOH, spray dried, and packed into aluminium bags (1 kg/ bag) and then carton boxes (24 bags/ box). Adjuvants such as maltodextrin and glucose syrup may be used to assist in the drying process of CF and also other products (HPPF, LPPF, PF, HPF, and LPF) if required. In this simulation, these adjuvants or additives were not considered to simplify the simulation.

FF1: The residue remaining after filtration was drum dried and packed into plastic bags (10 kg/ bag).

HPPF and LPPF: The liquid CF derived from the single extraction was subjected to ultrafiltration using a 30 kDa molecular weight cut-off (MWCO) membrane with a filtrate flux rate of 20 L/ (m2.h). Three ultrafiltration units, each with a membrane area of 70 m^2, were required to complete the process within 5 h to separate the HPPF and LPPF. Both fractions were then spray dried and packed into aluminium bags (1 kg/ bag) and carton boxes (24 bags/ box).

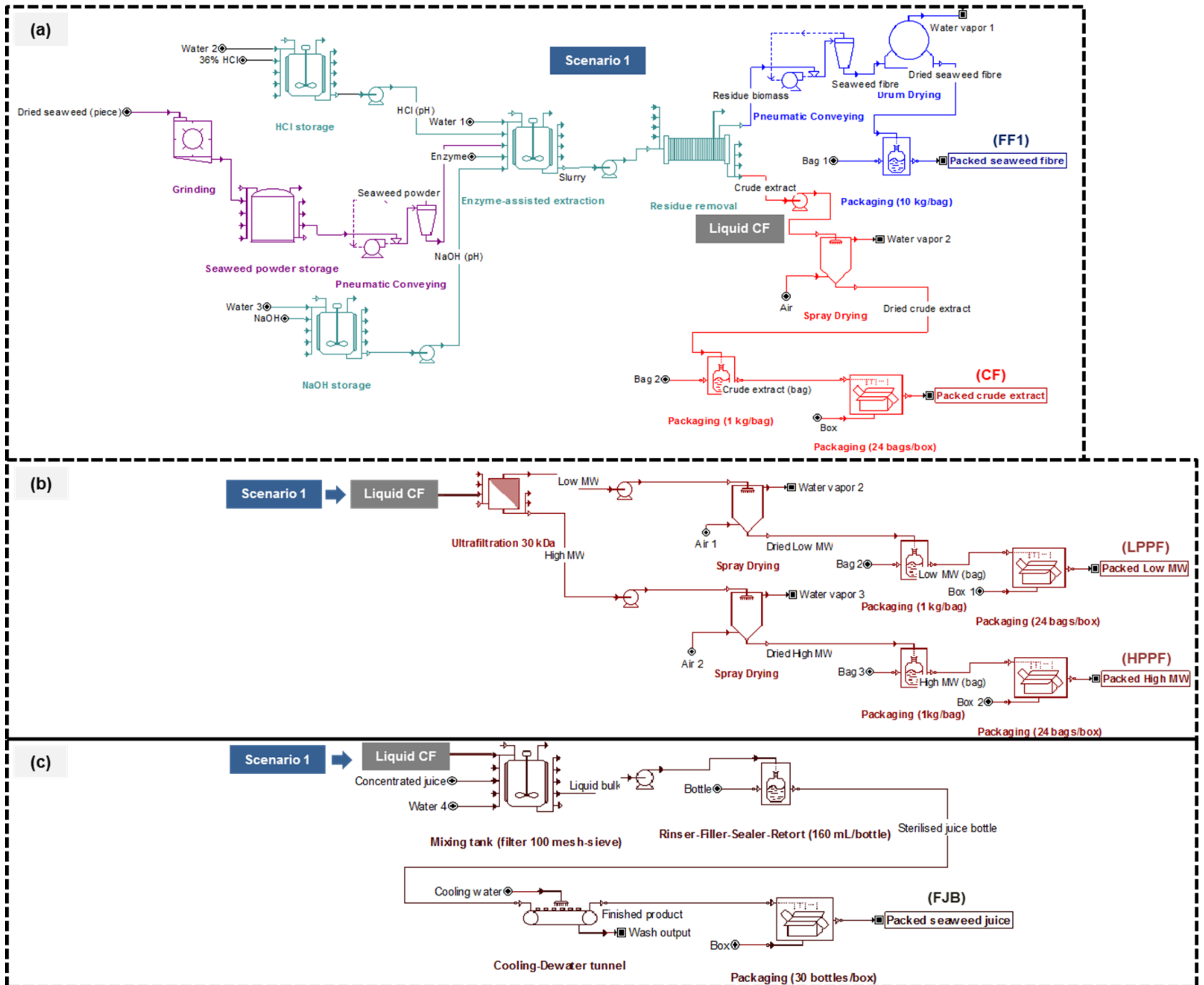


Figure 7.1 Process flow-chart of a single extraction process (a) Scenario 1: Crude extract Fraction (CF) and Fibre-enriched Fraction 1 (FF1) production, **(b) Scenario 2**: High MW Polysaccharide and Phlorotannin-enriched Fraction (HPPF), Low MW Polysaccharide and Phlorotannin-enriched Fraction (LPPF), and FF1 production, and **(c) Scenario 4: Seaweed and Fruit Juice-based Beverage** (FJB) product model and FF1 production

7.3.2.2 Sequential extraction

The process layout proposed for the sequential extraction process, Scenario 3, is presented in Fig. 7.2.

PF: The dried and ground seaweed was firstly extracted with 90% (v/v) ethanol at a ratio of 1:10 (w/v). The suspension was continuously mixed in a blending tank at room temperature for 3 h, and then transferred through a plate and frame filter in order to separate: (1) the extract and (2) the residual biomass. The ethanol in the extract was evaporated and recycled back into the process by condensation. Taking into account predicted losses of ethanol, mainly from the evaporation equipment, it was assumed that 90% of ethanol would be recovered and recycled in each cycle. The residual aqueous extract was spray-dried and packed into aluminium bags (1 kg/ bag) and carton boxes (24 bags/ box).

HPF and LPF: The residual biomass from the PF extraction step was dispersed in pH-adjusted water in the ratio 1:10 (w/v). The pH was adjusted to 4.5 using 1M HCl prior to the addition of the seaweed, to achieve the optimum pH of the Viscozyme® L. The enzyme solution was added at 10% (v/w), and the enzymatic hydrolysis was performed at the optimal temperature of 50°C for 3 h under continuous mixing. The enzyme was inactivated by boiling the slurry at 100°C for 10 min. The solution was cooled and transferred through a plate and frame filter to separate the extract and the residual biomass. The extract was adjusted to pH 7.0 using 1M NaOH, and subjected to ultrafiltration using a 30 kDa MWCO membrane to separate the HPF and LPF, with a filtrate flux rate of 20 L/ (m^2.h). Two ultrafiltration units, each with a membrane area of 74 m^2 were required to complete the process within 6 h. Then, both fractions were spray-dried and packed into aluminium bags (1 kg/ bag) and carton boxes (24 bags/ box).

FF2: The remaining residual biomass from the plate and frame filtration step of the HPF and LPF process was dried by drum drying and packed into plastic bags (10 kg/ bag).

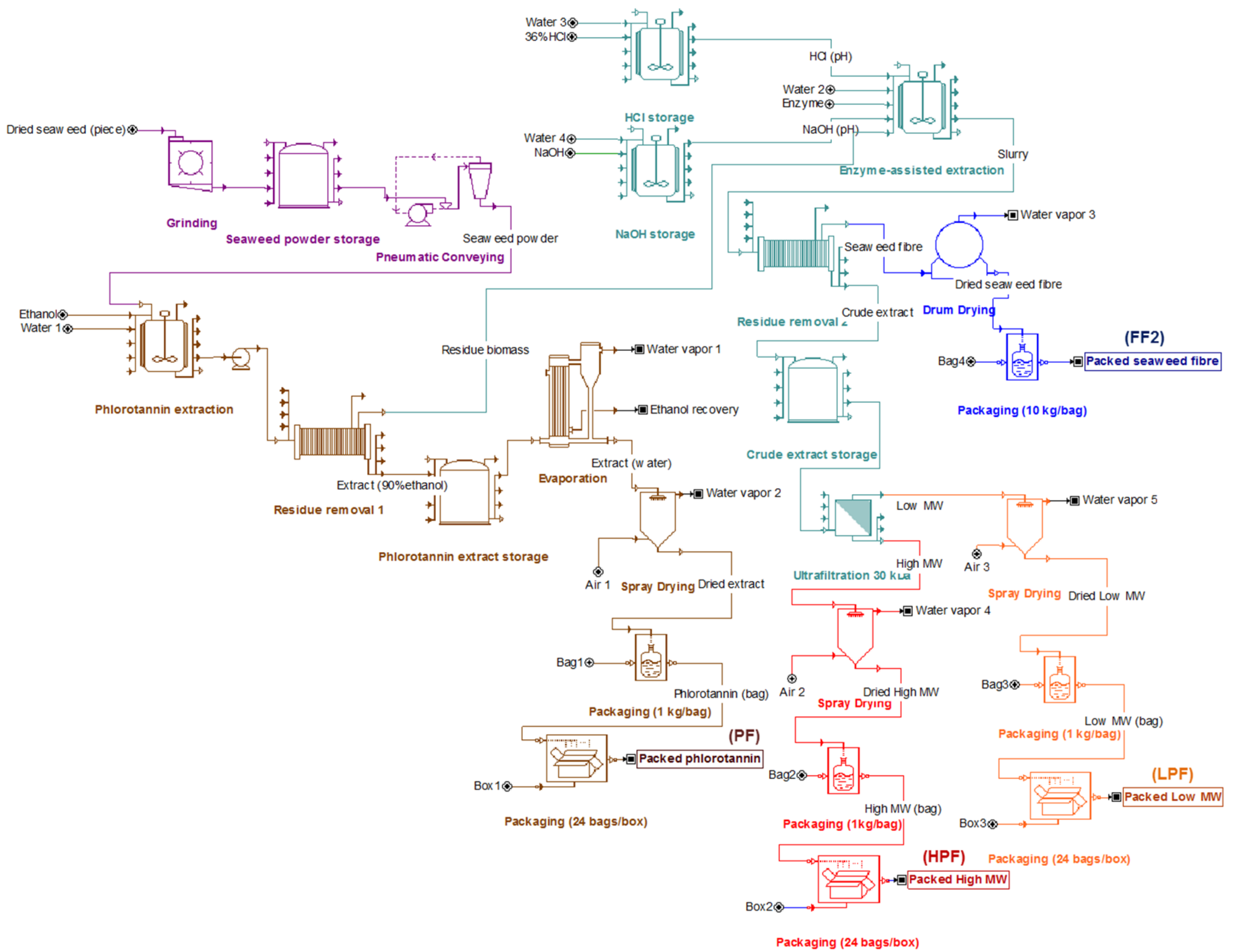


Figure 7.2 Process flow-chart of a sequential biorefinery extraction of seaweed for Scenario 3: Phlorotannin-enriched Fraction (PF), High MW Polysaccharide-enriched Fraction (HPF), Low MW Polysaccharide-enriched Fraction (LPF), and Fibre-enriched Fraction 2 (FF2) production

7.3.2.3 Production of FJB using crude seaweed extract

In this simulation (Scenario 4), fruit juices were selected as a model beverage delivery system for seaweed functional ingredients, as they are produced in almost every country in the world and its availability is remarkable [35]. In addition, the global market for juice was valued at US$ 129 bil in 2016, and it was expected to keep growing and driving market value up to US$ 151 bil by the end of 2020, as compared with other well-known beverage products such as ready-to-drink coffee (US$ 21 bil in 2016 and US$ 25 bil in 2020) and tea (US$ 63 bil in 2016 and US$ 76 bil in 2020) [36].

Briefly, the liquid CF derived from the single extraction (Scenario 1) was directly incorporated into the juice concentrate, without prior drying or concentration, along with water as required, in order to achieve a seaweed ingredient concentration of 2% w/w (calculated based on DW of CF and wet weight of juice), as well as an appropriately diluted fruit juice. After mixing, the formulated juice was passed through the 100-mesh sieve filter, transferred into 160 mL bottles (filling speed 400 bottles/min), capped, sterilised at 90°C for 10 min by hot water rotary retort, cooled to <45°C, and packed into carton boxes (30 bottles/ box) (Fig. 7.1c). The selected concentration of seaweed extract at 2% was applied to this juice prototype product as our earlier studies [14,23,24] and other studies [37,38,39] indicated that supplementation with 0.5-5% DW of seaweeds and their extracts could provide gut health benefits *in vitro* and in a rodent model *in vivo*. The sensory evaluation of this seaweed juice prototype (produced at lab scale in a pilot study) was also deemed acceptable when tested by panellists (n=20) (data not shown).

7.3.3 Estimation of capital costs

It was assumed that the processing facility will be built in Australia as this would reduce the cost of transportation given that is also where seaweed collection would take place. The total capital investment (TCI) is the fixed costs associated with a process, and includes the direct fixed capital (DFC), working capital, and start-up costs. The DFC is the sum of direct, indirect, and miscellaneous costs that are related to plant capital investment. The total plant direct costs (TPDC) include items such as the cost of equipment, installation, process piping, instrumentation, and buildings. The total plant indirect costs (TPIC) include the cost of engineering and construction. Additional miscellaneous costs include the contractor's fee and contingency (CFC). The default costings in the software were used for capital (which were automatically adjusted according to the year of commencement, which was 2016), with a construction period, startup period and project lifetime of 30 months, 4 months and 15 years, respectively. The size and cost of the equipment used in each process was scaled according to the processing capacity required. Some of the equipment were shared between different steps in each process in order to reduce capital expenditure, but only in instances where savings were not then negated by prolonging of the batch cycle time.

7.3.4 Estimation of operating costs

The annual operating cost (AOC) includes those related to the demand for a number of resources, particularly raw materials, labour-dependent, facility-dependent, utilities, and consumables. Utility costs were set at figures reflective of the intended location, with electricity charged at $0.104 per kWh and water at $2.42 per kiloliter, and an average labour rate of $69 per hour was set by taking specific types of operators and quality assurance into account. Raw materials and food grade chemicals were costed according to quotes received from local commercial-in-confidence suppliers. The unit value of the various materials and chemicals is shown in Table 7.1. With regard to the seaweed raw material, the purchasing price of $15 per kg was applied, which was roughly equal to the price of commercial dried Australian beach-cast seaweed for human consumption, based on the local industry advice. No waste disposal costs were applied in the simulations as the solid waste generated in the extraction process could be sold as seaweed fibre products.

Table 7.1 Estimated values of materials and product revenues used in this baseline simulation

Materials		Value
Dried seaweed		$15/kg
36% Hydrochloric acid		$0.38/kg
Sodium hydroxide		$0.66/kg
Enzyme		$20/kg
Ethanol		$0.94/kg
Concentrated juice		$4/kg
Aluminum bag (size 1 kg)		$0.11/bag
Plastic bag (size 10 kg)		$0.23/bag
High-density polyethylene (HDPE) bottle (size 160 mL)		$0.04/bottle
Carton box (for 24 bags of 1 kg bag)		$0.11/box
Carton box (for 30 bottles of 160 mL bottle)		$0.18/box
Products	**Packaging size**	**Value**
CF PF LPF LPPF	1 kg/bag, 24 bags/box	$30/kg ($720/box)
HPF HPPF	1 kg/bag, 24 bags/box	$80/kg ($1,920/box)
FF1 FF2	10 kg/bag	$15/kg ($150/10 kg)
FJB	160 mL/bottle (30 bottles/box)	$0.8/bottle ($24/box)

The abbreviations used for the different seaweed fractions are the same as in Fig. 7.1 and 7.2.

7.3.5 Revenues/ Credits

The selling prices of the products derived from single and sequential extractions are presented in Table 7.1. These prices were estimated based on their nutritional composition and advice obtained from commercial-in-confidence functional food and nutraceutical suppliers. da Costa [40] reported that consumers were willing to purchase and pay a premium price for nutraceuticals and functional foods, particularly when the functional property was added to plant-derived foods. Therefore, relatively high market prices could be expected for seaweed fractions and functional fruit juice-based beverages.

7.3.6 Indicators of economic performance in processing

Four main profitability measures were utilised: gross margin, payback time, net present value (NPV), and return on investment (ROI). The gross margin is the percentage of annual revenues that become gross profit; the payback time is the time needed for the TCI to be balanced by cumulative annual net profits; the NPV is the total value of future net cash flows during the lifetime of the project (discounted to reflect the value of money at the present year, using the default interest rate of 7%); and the ROI is the annual percentage return on the TCI. Vardanega et al. [34] reported that projects with NPV>0 and a payback time between two to five years were considered feasible. Therefore, the criteria of economic viability in this study were to achieve an NPV higher than $0 and a payback time ≤5 years. When comparing multiple projects, the one with the highest NPV would generally be considered the most financially attractive, assuming the TCI was considered affordable.

7.3.7 Proximate composition analyses

Moisture, protein (Kjeldahl), total fat (Mojonnier or Soxhlet extraction), ash (ignition at 550°C), and carbohydrate (estimated by subtracting moisture, fat, protein, ash, and phlorotannin from the whole weight) contents of the seaweed fractions were analysed by standard methods of the National Measurement Institute (results expressed as g/100g of DW). Total phlorotannins of dried seaweed fractions FF1 and FF2 were extracted using 70% acetone prior to analysis. All dried seaweed samples were analysed for their phlorotannin contents by Folin Ciocalteu's phenol reagent (results expressed as g phloroglucinol equivalent; PGE/100g of DW) [41,42].

7.4 Results and Discussion

7.4.1 Nutritional composition and biological properties of seaweed fractions

The simulation of the yield and operating costs of the seaweed fractions derived from different process scenarios were based on the laboratory experimental data shown in Table 7.2. Differences in the % yield of each seaweed fraction were observed. Apart from the residue (FF1 and FF2), CF obtained from Scenarios 1 and 2 showed the highest yield, while the lowest yield was found in the HPF derived from Scenario 3. Carbohydrates were the major component, followed by ash, for all seaweed fractions. PF contained the highest phlorotannin content, approximately 5 to 20-fold higher than that of all other fractions. It was noticed that the total yield of all products was over 100% of the starting seaweed materials as other ingredients such as acid/base and enzyme were also added during the process. The mass balance is absolutely warranted as the basis for such simulation. The different potential functional properties of each seaweed fraction obtained from our previous studies and published reports were taken into account when assigning product prices. As expected, the HPPF and LPPF, which were directly fractionated from CF without the separation of phlorotannin, contained a higher phlorotannin content, as compared to HPF and LPF. Aside from the potential health benefits of the PF, as shown in Table 7.2, phlorotannins can have effects against cancer,

allergies and viruses [43]. Therefore, the additional phlorotannin component in both fractions was expected to contribute to the potential health benefits of the products, so their prices were assigned as the same as the HPF and LPF. Apart from their dietary potential, FF1 and FF2 may also be used as substrates for the further recovery of valuable components. The enzyme-assisted extraction at pH 4.5 required a dilute acid pre-treatment, which allowed for the subsequent extraction of alginate, with reduced contaminants and greater ease of extraction, as compared with the use of whole seaweed biomass [20].

Table 7.2 Composition and potential functional properties of seaweed fractions

	CF	PF	HPF	HPPF	LPF	LPPF	FF1	FF2
Yield (g/100g dried seaweed)	40.16	15.75	9.51	12.30	25.27	28.97	60.70	60.52
% Content of fraction (g/100g dried fraction)								
Fat	<0.2	12.1	<0.2		<0.2		0.5	0.2
Protein	4.0	2.6	6.1		2.4		9.3	9.5
Mineral	32.7	34.9	26.7	N/A	21.8	N/A	18.2	18.2
Carbohydrate	57.6	22.7	65.6		71.2		66.4	70.3
Phlorotannin[a]	5.4	28.3	1.4	3.2	4.8	7.4	5.6	2.7
Functional properties/ Active ingredients	- Antioxidant activities (Charoensiddhi et al., 2015)	- Phenolic compounds - Prebiotic activities (Charoensiddhi et al., 2017a) - Neuroprotective potential (Our previous study; data not shown)	- Dietary fibre - Prebiotic activities (Charoensiddhi et al., 2017a,b)		- Antioxidant activities - Collagen production (Our previous study; data not shown)		Dietary fibre	

[a]g PGE/100 g dried fraction; N/A = data were not analysed
The abbreviations used for the different seaweed fractions are the same as in Fig. 7.1 and 7.2.

7.4.2 Batch-processing information

Table 7.3 shows the batch time, cycle time, and number of batches achievable per year for the various processes, when operating at full-time capacity. Generally, the cycle time (the shortest possible time between the commencements of consecutive batches) is shorter than the batch time (the duration of each batch, from start to finish), as a new batch can often be started before the previous batch is completed. Therefore, the cycle time (not the batch time) dictates the total number of batches per year. Results showed that Scenario 3 had the longest batch time and cycle time, and therefore the lowest total number of batches per year. Evaporation and ultrafiltration were time-consuming steps, and responsible for a large proportion of the total cycle time. In contrast, the

shortest batch time and cycle time was obtained from Scenario 1 due to a simpler process and the distribution of the short-duration operational units, resulting in the high number of total batches per year. Although the batch time of Scenario 2 was longer than that of Scenario 1, the same cycle time and total number of batches per year were achieved. The higher annual labour hours were observed in Scenarios 2 and 3, as compared to Scenario 1, due to the additional number of unit operations (requiring more labour) in processes to produce different MW polysaccharide fractions and the phlorotannin-enriched fraction (only in Scenario 3).

Table 7.3 Scheduling information for the different process scenarios, when operating at full-time capacity

Process	Batch time (h)	Cycle time (h)	Batches/yr	Productivity (kg/batch)	Annual labour hours
Scenario 1	19.5	7.5	1,054	CF 1,162.13 FF1 1,208.33	42,189
Scenario 2	23.2	7.5	1,053	HPPF 213.88 LPPF 948.24 FF1 1,208.33	54,200
Scenario 3	28.08	12	658	PF 399.20 HPF 187.04 LPF 750.20 FF2 983.63	56,935
Scenario 4	22.92	10	790	FJB 14,926.43 FF1 302.08	109,371

The abbreviations used for the different seaweed fractions are the same as in Fig. 7.1 and 7.2.

7.4.3 Fixed capital and operating costs

As shown in Fig. 7.3a, the sequential extraction process Scenario 3 required fixed capital investments of 1.9 and 1.4-fold higher than the single extractions of Scenarios 1 and 2, respectively. This was because more equipment, installation, and buildings were required, and no major equipment could be shared during this process without substantially increasing the cycle time (which would have more deleterious economic effects). The DFCs of process Scenarios 1 and 2 were slightly different, due to the addition of ultrafiltration and packing processes for the HPPF and LPPF products in Scenario 2.

As shown in Fig. 7.3b, the cost of raw materials represented the major operating costs for all process scenarios, accounting for approximately 80% of the total. Seaweed was the biggest raw material cost for all scenarios, accounting for 85.6% in Scenarios 1 and 2 and 63.2% in Scenario 3. The labour cost was slightly different in Scenarios 1-3, from 6.7 to 9.6%, but the facility costs were approximately twice as high for Scenario 3, simply because more equipment and machinery were required to produce the multiple products. Scenarios 2 and 3 included a consumable cost for the

membrane used in the ultrafiltration step. Relatively low operating costs were observed for Scenario 3, as the longer cycle time resulted in fewer production batches per year, which in turn necessitated a lower raw material consumption.

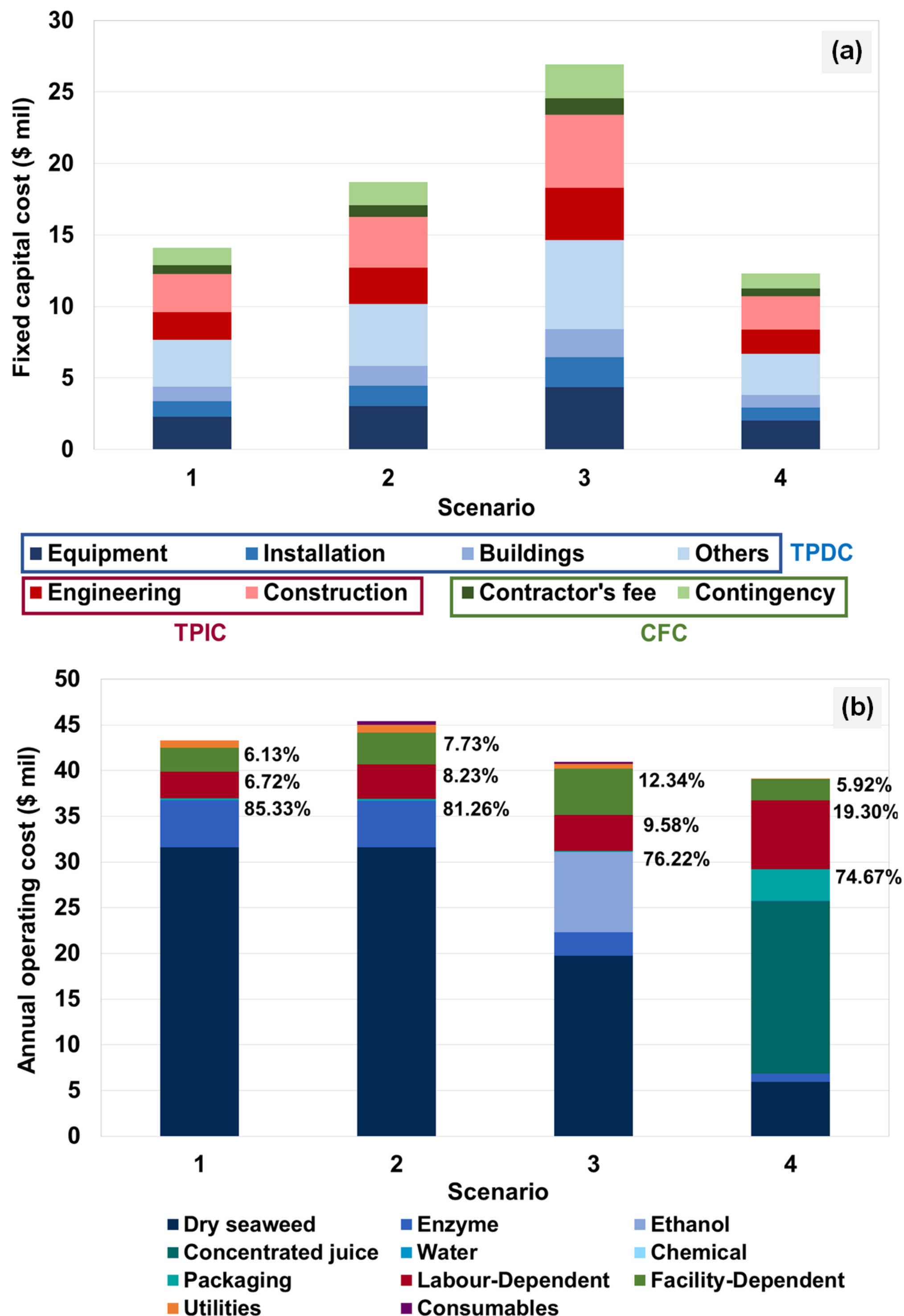


Fig. 7.3 (a) Fixed capital cost; Total Plant Direct Cost (TPDC); Total Plant Indirect Cost (TPIC); Contractor's Fee & Contingency (CFC) and **(b) Annual operating cost** (AOC) of the different process scenarios, when operating at full-time capacity

7.4.4 Economic evaluation of the selected scenarios

Table 7.4 summarises a range of economic indicators for the different process scenarios when operating at full-time capacity. Scenarios 1 and 2 were predicted to perform better than Scenario 3 in all indicators, and they both achieved an NPV>0 and a payback time ≤2 years, demonstrating their commercial feasibility. In contrast, Scenario 3 had a negative NPV and a payback time >5 years. Compared to Scenario 1, Scenario 2 had substantially better values for all indicators of process economic performance, with an NPV approximately 2-fold higher ($81.88 vs $45.03 mil), accompanied by a TCI that was only 1.3-fold higher. Taken together, these findings suggested that, in economic terms, the preliminary ethanolic extraction of phlorotannins was not a cost-effective inclusion in the proposed industrial process, whereas the post-extraction fractionation of high and low MW polysaccharide and phlorotannin-enriched products using ultrafiltration could be a potential industry process which increased the value of crude seaweed extract due to the additional revenue streams, with more defined higher-value end products.

Table 7.4 Economic performance of the different process scenarios, when operating at full-time capacity

Economic performance	Scenario 1	Scenario 2	Scenario 3	Scenario 4
TCI ($ mil)	18.52	23.41	31.51	16.28
Operating cost ($ mil/year)	43.32	45.44	40.99	39.10
Revenues ($ mil/year)	55.85	67.06	42.24	62.54
Net profit ($ mil)	8.86	14.75	5.04	15.23
Gross margin (%)	22.44	32.24	9.78	37.48
ROI (%)	47.85	63.01	15.99	93.57
Payback time (year)	2.09	1.59	6.26	1.07
NPV ($ mil)	45.03	81.88	-8.26	89.43

TCI represents total capital investment, ROI represents return on investment, and NPV represents net present value.

7.4.5 Economic performance of seaweed and fruit juice-based beverage product model

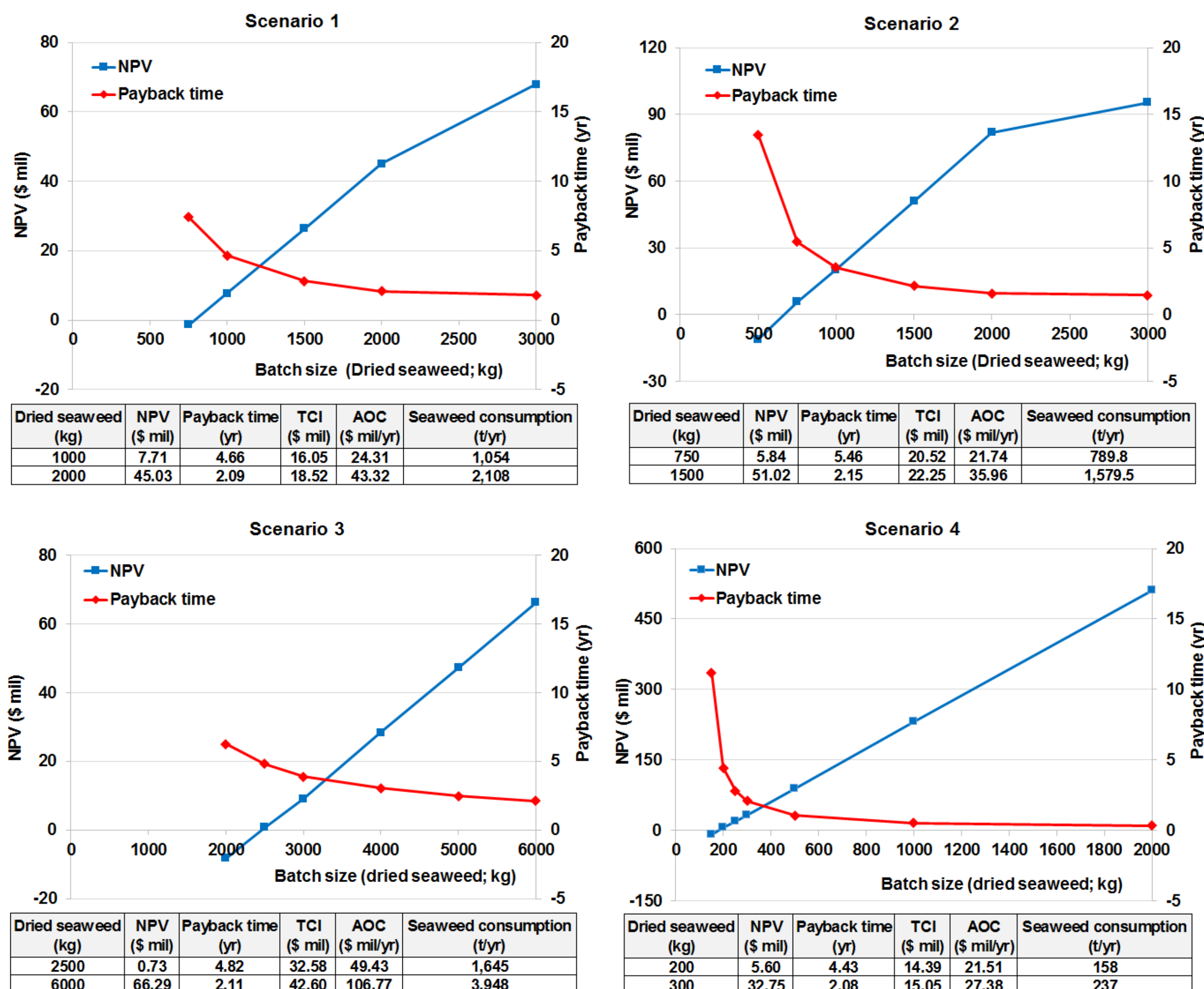
In order to demonstrate the economic potential, commercial feasibility, and profitability of the industrial production of bioactive health supplements from brown seaweed functional fractions, fruit juice was selected as a model beverage delivery system for seaweed functional ingredients. As shown in Scenario 1, the single extraction of CF appeared to be a relatively simple and economically feasible process, with lower capital and operational costs, as compared to Scenario 2. However, the drying step of CF was both energy intensive and time consuming. Therefore, Scenario 4 was considered, involving the integrated incorporation of the liquid CF (produced using the single extraction of Scenario 1) into a fruit juice-based beverage at 2% w/w (DW of CF).

The scheduling information, fixed capital cost, and annual operating cost (Table 7.3 and Fig. 7.3) of Scenario 4 could not be directly compared to other scenarios due to the smaller processing scale used. However, this process scenario was more labour intensive, mainly due to the additional packing requirements, with labour costs accounting for 19.3% of the total annual operation costs (as compared to 6.7-9.6% in Scenarios 1-3) (Fig. 7.3b). Raw materials were the biggest contributor to the AOC, accounting for 74.7%, with the concentrated juice representing the main cost (64.6%) followed by the dried seaweed (20.3%) (Fig. 7.3b). As shown in Table 4, the production of a value-added beverage from the crude seaweed extract appeared to be the most profitable outcome among the four scenarios simulated here, and appeared to be an attractive investment due to the low payback time and high NPV of 1.07 years and $89.43 mil, respectively, with a TCI of $16.28 mil at one quarter of the processing scale of the other three scenarios.

7.4.6 Sensitivity analysis of production scale on economic viability

It has been assumed thus far that the four scenarios would operate at a maximum production capacity of 7,920 hours per year, in order to assess their viability in a scenario of plentiful seaweed raw material availability. However, it must be considered that in a real-world scenario, the availability of seaweed biomass could be the production-limiting factor, especially considering the majority of commercial seaweeds produced in Australia are supplied by locally harvested beach-cast wrack [6,44]. Therefore, in order to assess the sensitivity of the four process scenarios to circumstances of limited biomass, the lowest amount of seaweed per batch at which each process remained economically viable was determined. The data from this analysis are presented in Fig. 7.4. For Scenarios 1, 2, and 4, which appeared to be economically viable at full-time production capacity, the scale of production was reduced to the lowest point at which a payback time of less than 5 years could still be achieved, by reducing the batch size and holding the operating hours constant at 7,920 hours per annum. The results showed that the acceptable payback time was still achieved when the batch size was decreased from 2,000 to 1,000 kg/batch for Scenario 1, from 2,000 to 750 kg/batch for Scenario 2, and from 500 to 200 kg/batch for Scenario 4. These scenarios translated to annual seaweed consumptions of 1,054 t/year for Scenario 1, 789.8 t/year for Scenario 2, and 158 t/year for Scenario 4. For Scenario 3, which did not appear to be economically feasible at the originally simulated scale of 2,000 kg/batch, an acceptable payback time of 5 years was achieved when the batch size was increased from 2,000 to 2,500 kg/batch. This translated to annual seaweed consumption of the maximum 1,645 t/year. The increase of batch size necessitated an increase in TCI, as more and larger units of equipment were required. The AOC was also increased due to the increase in annual expenditure on raw materials. However, the economic performance was improved with larger scale production as this reduced the costs per unit of production through economies of scale. According to their relative economic performances and annual seaweed consumption, the

process scenarios most likely to be profitable under circumstances of limited biomass availability were Scenarios 2 and 4.



Scenario 1

Dried seaweed (kg)	NPV ($ mil)	Payback time (yr)	TCI ($ mil)	AOC ($ mil/yr)	Seaweed consumption (t/yr)
1000	7.71	4.66	16.05	24.31	1,054
2000	45.03	2.09	18.52	43.32	2,108

Scenario 2

Dried seaweed (kg)	NPV ($ mil)	Payback time (yr)	TCI ($ mil)	AOC ($ mil/yr)	Seaweed consumption (t/yr)
750	5.84	5.46	20.52	21.74	789.8
1500	51.02	2.15	22.25	35.96	1,579.5

Scenario 3

Dried seaweed (kg)	NPV ($ mil)	Payback time (yr)	TCI ($ mil)	AOC ($ mil/yr)	Seaweed consumption (t/yr)
2500	0.73	4.82	32.58	49.43	1,645
6000	66.29	2.11	42.60	106.77	3,948

Scenario 4

Dried seaweed (kg)	NPV ($ mil)	Payback time (yr)	TCI ($ mil)	AOC ($ mil/yr)	Seaweed consumption (t/yr)
200	5.60	4.43	14.39	21.51	158
300	32.75	2.08	15.05	27.38	237

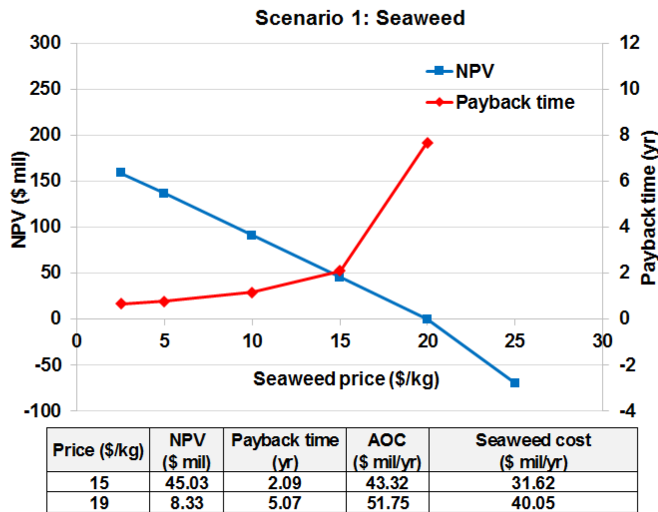
Fig. 7.4 Sensitivity analysis of production scale on economic viability of different process scenarios and their annual seaweed consumption

7.4.7 Sensitivity analysis of the price variations of both raw materials and products

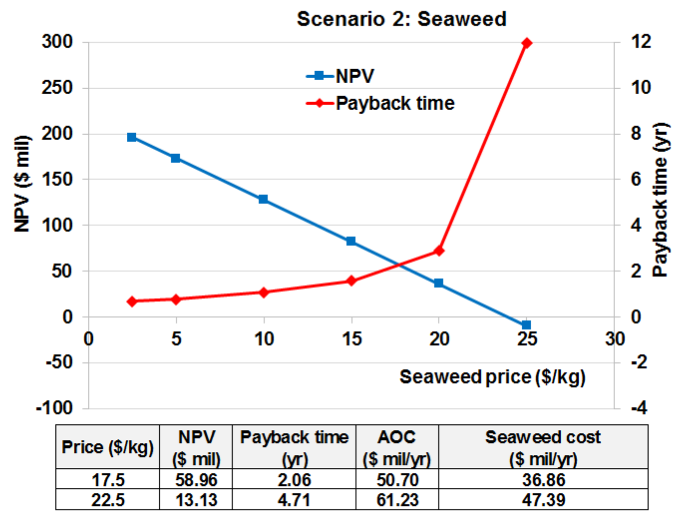
The costs of raw materials and selling prices of the products could vary due to the market demand, and have considerable impacts on the overall economic feasibility of the projects. Seaweed and concentrated juice were selected for evaluation as they are the main costs contributing to total raw material costs. Other bioactive compounds present in the seaweed extract ingredients, aside from the properties we reported, such as minerals, vitamins, and fucoxanthin [45] may also contribute to the selling price of the seaweed ingredient products. Moreover, variability in market demand may cause fluctuations in the selling prices. In order to evaluate how profitability will change in response to the changes in raw materials costs and product prices, the sensitivity analysis of the baseline

simulation was assessed by varying current market prices (defined in Table 7.1) and potential cost variations suggested by commercial-in-confidence seaweed raw material and functional food suppliers. Each price was individually changed to higher and lower values, while all other prices were held constant at the current market prices.

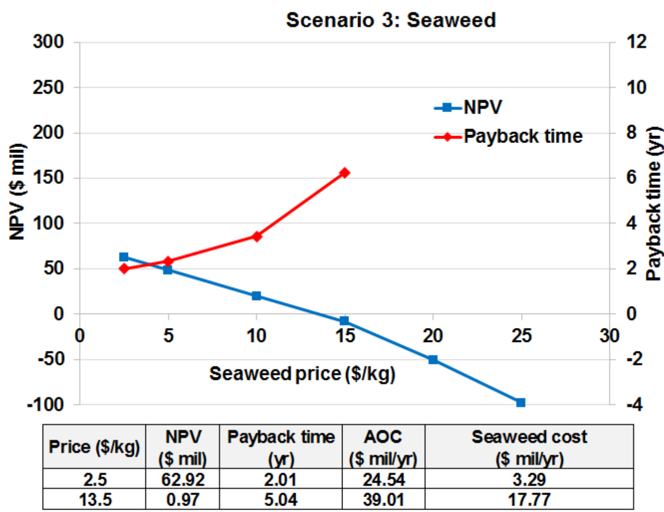
As shown in Fig. 7.5, in Scenarios 1 and 2, the seaweed raw material accounted for a high proportion (85.6%) of the total operating costs. To achieve an acceptable payback time of ≤5 years, the price of seaweed in Scenario 1 could be increased from $15/kg to $19/kg (NPV $8.33 mil), causing an increase in AOC and total annual seaweed costs of approximately 1.2-fold. Meanwhile, the price of seaweed in Scenario 2 could be increased from $15/kg to $22.5/kg, while remaining within the acceptable range of NPV ($13.13 mil) and payback time (4.71 years). In contrast, a decrease in the price of seaweed from $15/kg to $13.5/kg was required in Scenario 3 in order to achieve an acceptable payback time of 5 years. Under these circumstances, the AOC and total annual seaweed costs in Scenario 3 decreased approximately 1.1-fold. Variations in the seaweed feedstock price had less of an impact on the overall economic feasibility of Scenario 4, in which it could be increased up to $64/kg while still achieving a payback time of 4.98 years, as it accounted for a relatively low proportion of the total raw material costs (20.3%). The price of concentrated juice, on the other hand, had a greater impact on economic viability, as it accounted for approximately 64.6% of the total raw material costs in the baseline simulation. The price of concentrated juice could be increased from $4/kg up to $8/kg, with a resulting NPV of $8.79 mil and a payback time of 4.60 years. The lowest possible price of concentrated juice in the market was also simulated at $2/kg. In this scenario, the concentrated juice accounted for 47.8% of the total raw material costs, and the NPV was increased to $130.5 mil, while the payback time was reduced to 0.74 years. In addition, the wholesale price of other brown seaweeds used for food in the market can vary between US$ 7.5-10/kg DW, whereas seaweed materials used for alginate extraction range from US$ 0.15-0.5/kg DW [46]. From the sensitivity analysis presented in Fig. 7.5, the lowest possible price of seaweed was also simulated at $2.5/kg. In this case, the NPV of Scenario 1-4 was increased to $159.06, 195.77, 62.92, and 110.92 mil, respectively, while the payback time was reduced to 0.65, 0.69, 2.01, and 0.87 years, respectively, demonstrating the great economic potential to apply these processes to other commercial brown seaweeds.



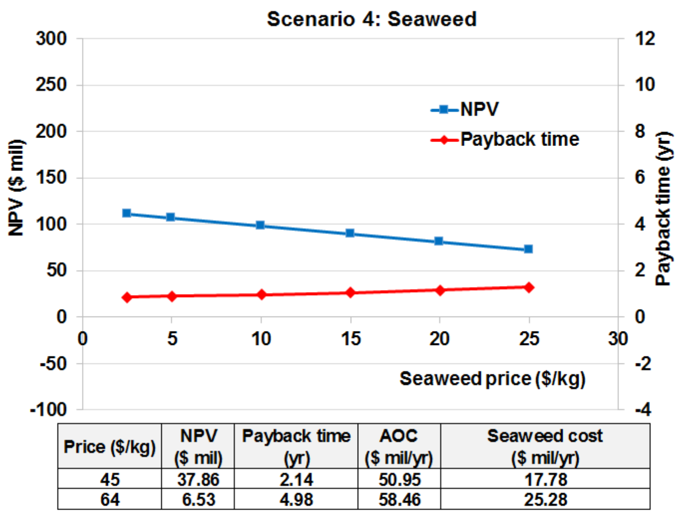
Price ($/kg)	NPV ($ mil)	Payback time (yr)	AOC ($ mil/yr)	Seaweed cost ($ mil/yr)
15	45.03	2.09	43.32	31.62
19	8.33	5.07	51.75	40.05



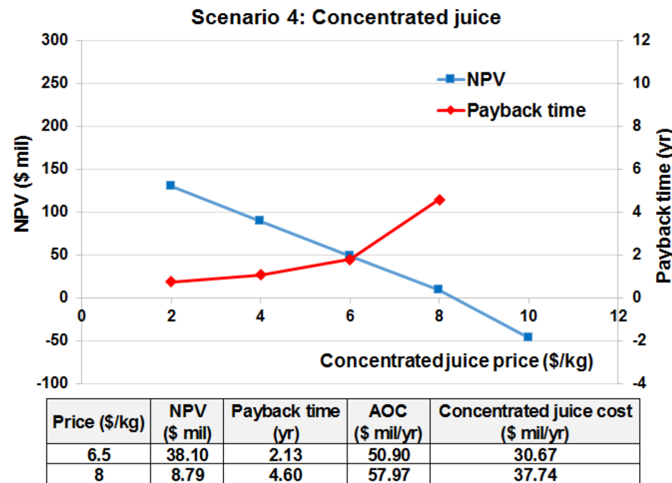
Price ($/kg)	NPV ($ mil)	Payback time (yr)	AOC ($ mil/yr)	Seaweed cost ($ mil/yr)
17.5	58.96	2.06	50.70	36.86
22.5	13.13	4.71	61.23	47.39



Price ($/kg)	NPV ($ mil)	Payback time (yr)	AOC ($ mil/yr)	Seaweed cost ($ mil/yr)
2.5	62.92	2.01	24.54	3.29
13.5	0.97	5.04	39.01	17.77



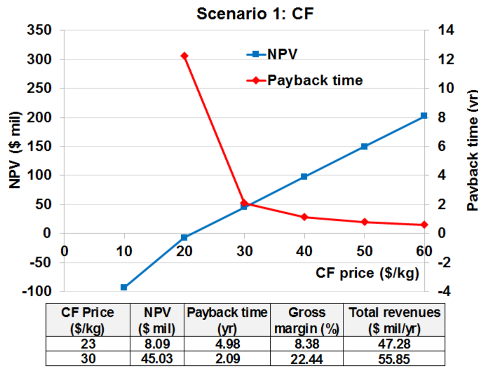
Price ($/kg)	NPV ($ mil)	Payback time (yr)	AOC ($ mil/yr)	Seaweed cost ($ mil/yr)
45	37.86	2.14	50.95	17.78
64	6.53	4.98	58.46	25.28



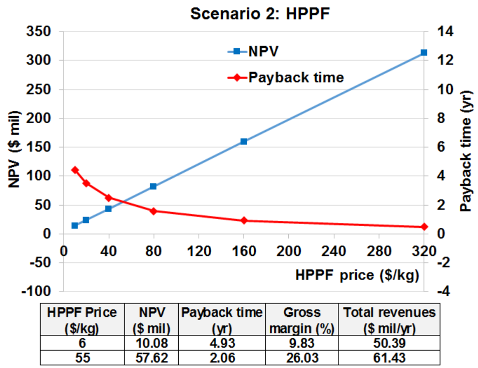
Price ($/kg)	NPV ($ mil)	Payback time (yr)	AOC ($ mil/yr)	Concentrated juice cost ($ mil/yr)
6.5	38.10	2.13	50.90	30.67
8	8.79	4.60	57.97	37.74

Fig. 7.5 Sensitivity analysis of price fluctuations of key raw materials in each process scenario on project economic viability

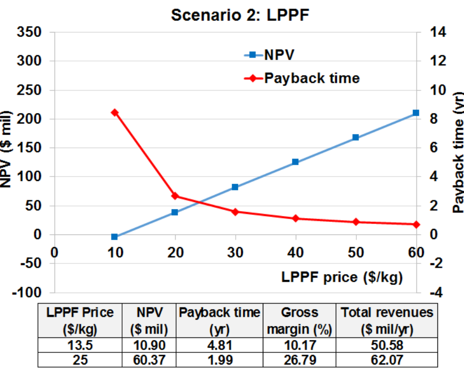
Fig. 7.6 summarises the possible price fluctuations of products used in the sensitivity analysis of each process scenario. The price of CF in Scenario 1 could be reduced from $30/kg to $23/kg to achieve a NPV of $8.09 mil and a payback time of 4.98 years, with a decrease in %gross margin and total annual revenues of approximately 2.7 and 1.2-fold, respectively. If the price of CF increased to $60/kg, a NPV of $202.08 mil and a payback time of 0.6 years could be achieved with a 2.4 and 1.7-fold increase in the %gross margin and total annual revenues, respectively. Likewise, Scenario 2 could still achieve the acceptable range of NPV>0 and payback time of 5 years if the selling prices of HPPF and LPPF fell from $80/kg and $30/kg to $6/kg and $13.5/kg, respectively. Accordingly, the %gross margin and total revenues would be reduced by approximately 3.2 and 1.3-fold. On the other hand, the price of HPPF and LPPF could potentially increase to $320/kg and $60/kg, respectively, which individually could raise the %gross margin and total annual revenues by at least 1.5-fold. In contrast, the economic feasibility was achieved for Scenario 3 only in the case of an increase in the product prices. The prices of either PF, HPF, or LPF would need to increase from $30/kg to $37.5/kg, $80/kg to $96/kg, and $30/kg to $34/kg, respectively, in order to achieve the maximum acceptable payback time of 5 years. In Scenario 4, production of FJB was predicted to achieve the acceptable NPV ($4.86 mil) and payback time (4.94 years) even if the selling price was reduced to $0.53/bottle. On the other hand, with high market demand for FJB, the price of this product could possibly increase to $1.2/bottle, in which case the payback time would reduce to 0.49 years, accompanied by an increase of approximately 1.5-fold in %gross margin and total annual revenues.



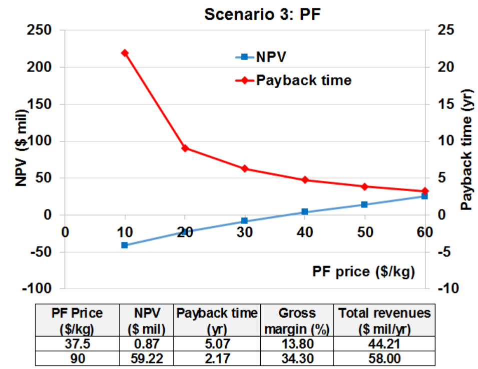
CF Price ($/kg)	NPV ($ mil)	Payback time (yr)	Gross margin (%)	Total revenues ($ mil/yr)
23	8.09	4.98	8.38	47.28
30	45.03	2.09	22.44	55.85



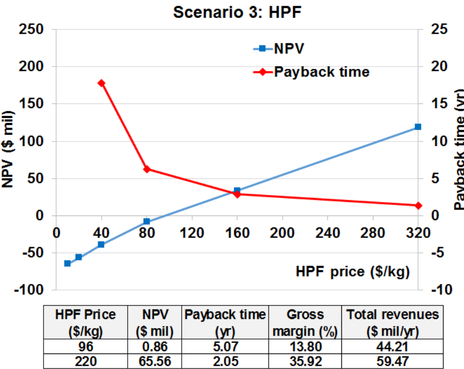
HPPF Price ($/kg)	NPV ($ mil)	Payback time (yr)	Gross margin (%)	Total revenues ($ mil/yr)
6	10.08	4.93	9.83	50.39
55	57.62	2.06	26.03	61.43



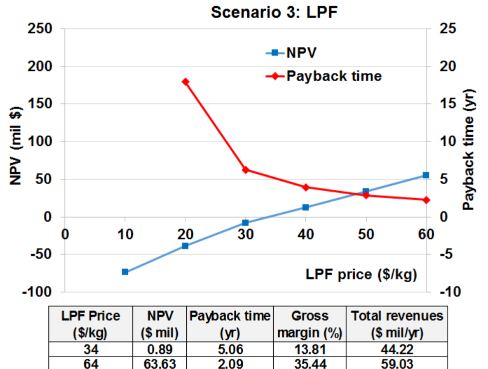
LPPF Price ($/kg)	NPV ($ mil)	Payback time (yr)	Gross margin (%)	Total revenues ($ mil/yr)
13.5	10.90	4.81	10.17	50.58
25	60.37	1.99	26.79	62.07



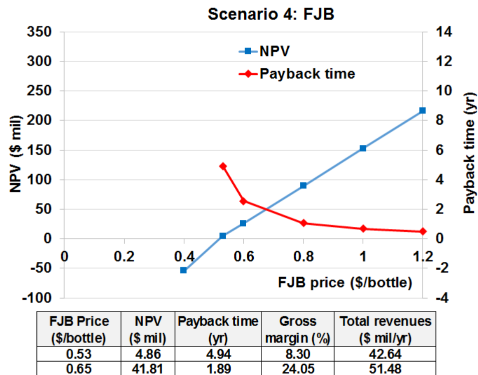
PF Price ($/kg)	NPV ($ mil)	Payback time (yr)	Gross margin (%)	Total revenues ($ mil/yr)
37.5	0.87	5.07	13.80	44.21
90	59.22	2.17	34.30	58.00



HPF Price ($/kg)	NPV ($ mil)	Payback time (yr)	Gross margin (%)	Total revenues ($ mil/yr)
96	0.86	5.07	13.80	44.21
220	65.56	2.05	35.92	59.47



LPF Price ($/kg)	NPV ($ mil)	Payback time (yr)	Gross margin (%)	Total revenues ($ mil/yr)
34	0.89	5.06	13.81	44.22
64	63.63	2.09	35.44	59.03



FJB Price ($/bottle)	NPV ($ mil)	Payback time (yr)	Gross margin (%)	Total revenues ($ mil/yr)
0.53	4.86	4.94	8.30	42.64
0.65	41.81	1.89	24.05	51.48

Fig. 7.6 Sensitivity analysis of price fluctuations of products in each process scenario on project economic viability

In addition, the price fluctuations of FF1 and FF2 were also evaluated. The prices of these products were already set at the highest predictable market prices (15$/kg) in the original simulations. In Scenarios 1 and 2, the prices of FF1 could be reduced to $8.5/kg and $2/kg to approximately achieve the maximum acceptable payback time (4.75 and 4.85 years, respectively). Meanwhile, the maximum acceptable payback time of 5 years could only be achieved in Scenario 3 if the price of FF2 was raised to $18/kg. Price fluctuations in FF1 derived from Scenario 4, on the other hand, showed little impact on overall economic performance, as that product accounted for a much lower proportion of total revenues.

7.5 Conclusion

The industrial process simulation and economic analyses demonstrated potential commercial feasibility and profitability for the industrial production of bioactive health supplements from the brown seaweed *E. radiata*. Of the four processing scenarios simulated, the fractionation of crude extract (Scenario 2) and the production of value-added product (Scenario 4) were predicted to have better overall economic performances. Both processes would also be fairly robust economically in the face of unforeseen economic circumstances, as indicated by the sensitivity analysis. In contrast, the sequential extraction process (Scenario 3) required higher capital investment, particularly with regard to the fixed capital cost, such that the economic viability of its production in a small to medium enterprise appeared to be low. Beyond the scope of this study, which was simply to evaluate the economic performance of four production strategies, details of other operating costs of a project such as transportation, product registration and regulation, as well as advertising and marketing would also have an effect on the real life economic performance. In addition, the economic situation of a fruit juice-based beverage product model is dependent on the demands for functional beverages in the market, so other food prototypes, which can offer more opportunities and a greater impact in the market, should be considered for further study. Importantly, tests are also required to confirm the bioavailability and stability of the seaweed-derived bioactive compounds when incorporated into food products, and to ensure compliance with standard food and nutraceutical safety regulations.

7.6 Acknowledgements

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8. CONCLUSIONS

8.1 Summary of key findings

There is a growing recognition that seaweeds are important sources of bioactive compounds with a variety of functions that can, or could potentially, be used to impact health. Although seaweeds in South Australia have high levels of diversity and endemism, the harvested seaweed in this region is still limited to use for low-value agricultural products such as fertiliser and animal feed. These seaweeds have not been studied for their potential to be used in natural health products, therefore presenting a significant opportunity to discover new bioactive ingredients and scientific evidence to support the development of functional food and nutraceutical products in the market. The aim of this project was to produce bioactive compounds from the brown seaweed, *E. radiata*, using enzyme-assisted processes, and assess their potential use as functional ingredients in nutritional applications. This seaweed was selected due to its abundance along coasts of South Australia and because it contains relatively high proportions of components of potential interest, including approximately 70% carbohydrates and 5% phlorotannins on a dry weight basis. In order to achieve this aim, extracts and fractions with varying chemical compositions were produced using advanced processes, and their biological properties, particularly prebiotic and antioxidant activities, were investigated using *in vitro* and *in vivo* systems. Based on the data obtained, several of the processes were then simulated at industry scale, and their commercial feasibility was evaluated. The key findings achieved from this work are expected to bridge the gaps between small scale laboratory studies and commercialisation, and help push forward the development of a marine-based industry of functional food and nutraceuticals in South Australia and other regions around the globe.

A detailed literature review was conducted which revealed that seaweeds are a valuable source of bioactive compounds, especially with regard to their polysaccharides and phenolic compounds. In addition, it was noted that enzymatic processing is a promising technology to assist with the efficient extraction and structural modification of value-added bioactive compounds from seaweeds.

Our first study focused on the integration of enzymatic and microwave-assisted extraction for the production of extracts rich in phenolic compounds. The results demonstrated that when using commercial carbohydrate-hydrolytic enzymes (Viscozyme® L) intensified with microwaves, relatively short treatments of 5 to 30 min could produce extracts with high phlorotannin content and antioxidant activities. Given their reported benefits for gut health, the production of seaweed-derived polysaccharides using enzyme-assisted extraction processes was also investigated. The type of enzyme used and the pH were shown to have minor impacts on the total sugar yield, but each affected the sugar composition and MW profile of the carbohydrate extracts differently. Acidic extraction yielded lower MW components compared to neutral and alkali conditions, while the inclusion of hydrolytic enzymes reduced the MW of the extracted polysaccharides by 20–50%,

compared with extraction using pH-adjusted water-only. Meanwhile, high concentrations of buffer salts were found to inhibit polysaccharide extraction.

The prebiotic potential of the brown seaweed extracts were then evaluated using an *in vitro* human gut simulation model, involving anaerobic fermentation by human fecal inoculants. Extracts prepared using carbohydrate hydrolytic enzymes were shown to undergo fermentation as evidenced by the production of short chain fatty acids (SCFA) and the growth of beneficial gut microbes which have been thoroughly investigated for their specific gut health benefits such as *Bifidobacterium* and *Lactobacillus* compared with the control. Fractions of these extracts with specific chemical properties were then analysed in order to identify the active components, and it was observed that the high MW polysaccharide-enriched fractions showed the most promise as prebiotics. These fractions were resistant to human digestive enzymes of the small intestine, (suggesting they would reach the large bowel *in vivo*) and readily fermentable by gut bacteria, producing beneficial SCFA, including butyric acid.

Given the promising results obtained from *in vitro* experiments, *E. radiata* biomass and the high MW polysaccharide-enriched fraction were further studied *in vivo*, using a rat model. Both whole seaweed and the polysaccharide fraction showed the potential to improve gut health in different ways. Relative to the control group, rats fed with a diet supplemented with 5% (w/w) of whole seaweed produced significantly higher levels of SCFA, accompanied by lower levels of phenol, *p*-cresol, and the potentially pathogenic bacteria *Enterococcus*. Meanwhile, rats fed with a diet supplemented with 5% (w/w) of the high MW polysaccharide fraction had significantly higher cecal tissue and digesta weights (equivalent to the stool bulking effect in humans), higher cecal SCFA production, and a higher abundance of the key butyrate producer *F. prausnitzii* in the cecum.

These findings suggested for the first time that the brown seaweed *E. radiata* and its extracts and fractions have the potential to be used as natural antioxidant ingredients and dietary supplements with gut health benefits in humans. Therefore, four processes that could be used for their production were simulated at industry-scale using Superpro Designer v8.0, in order to evaluate their commercial feasibility. The simulated processes included:

- Process 1: the enzyme-assisted production of seaweed crude extract
- Process 2: the enzyme-assisted production of seaweed crude extract, followed by fractionation using ultrafiltration, for the production of high and low MW polysaccharide and pholorotannin-enriched fractions
- Process 3: the ethanolic extraction of a phlorotannin-enriched fraction, followed by the sequential enzyme-assisted extraction, and separation into high and low MW polysaccharide-enriched fractions

- Process 4: the enzyme-assisted production of seaweed crude extract, followed by incorporation into a fruit juice-based beverage at a concentration of 2% (w/w dry weight of extract basis)

The results demonstrated that processes 2 and 4 had better overall economic performances compared with process 1, in terms of higher NPV and lower payback time. Both processes would also be fairly economically robust in the face of unforeseen economic changes, as indicated by a sensitivity analysis, with regard to production scale, raw material cost, and product selling price. In contrast, process 3 required high investment costs, particularly with regard to fixed capital costs, so the economic viability of its production in a small to medium enterprise appeared to be low, as an acceptable NPV (>$0) and payback time (<5 years) was only achieved with large-scale production (minimum 1,645 t of dried seaweed raw material per year). While process 4 demonstrated significant advantages by incorporating a final product formulation, with a much small scale of 200 kg of dried seaweed raw material per batch (158 t per year) to achieve a payback time <5 years.

8.2 Future directions

The key findings from this study indicate that there is potential for the commercial development of seaweed-derived bioactive compounds from *E. radiata* for use as functional ingredients in food and nutraceuticals. However, further studies are required to improve the efficiency of extraction processes, demonstrate health benefits in human, and produce high quality products.

A number of publications have demonstrated various advantages of enzymatic processes, particularly with regard to improving extraction yields, enhancing biological activities, and reducing the costs and time of production. However, in our study, the overall hydrolytic efficiency of the enzymes for carbohydrate extractions appeared to be relatively low. The activity of commercially available hydrolytic enzymes could be hindered by their access to specific substrates being obstructed by the seaweed cell wall mucilage. Therefore, enzymes such as alginate lyase and fucoidanase, which have activities specific to seaweed structures, should be further investigated in instances where one might study the effects of these substrates (alginate and fucoidan, respectively) with shorter MWs or where these substrates need not be preserved. In addition, other chemical and physical methods of enzyme intensification, such as ultrasound and ultra-high pressure conditions, should be considered for the improvement of extraction efficiency. Furthermore, the scaled up industrial processes should be designed in a way that supports the use of enzymes, as some limitations associated with costs and enzyme stability, for instance, may otherwise arise (Puri et al., 2012).

Aside from *E. radiata*, it might be worth investigating the use of other abundant seaweed species and processing strategies for the extraction of phlorotannins and polysaccharides. For instance, Sánchez-Camargo et al. (2016) demonstrated that pressurized liquids performed better than enzymatic treatments for the recovery of phlorotannins from the seaweed *Sargassum muticum*. Also, it would be beneficial to purify, characterise, and assay the extracted phlorotannins and polysaccharides in order to gain an understanding of which components were responsible for the antioxidant activities observed in the seaweed extracts.

With regard to the prebiotic activities, results from all studies demonstrated the prebiotic potential and gut health benefits of the brown seaweed *E. radiata*. However, further studies are still required to gain a clear understanding of the factors influencing the gut microbe composition and fermentation. Firstly, it would be interesting to assess the impacts of the extracts on the whole gut microbiome, going beyond the narrow range of targets (i.e. key species and fermentation products) examined here. Perhaps, populations of other beneficial bacteria may increase in number, but these were not analysed here. Also, an increase in the number of *E. coli* was observed with the seaweed samples during both the *in vivo* and *in vitro* studies. Although some strains of *E. coli* are known for their pathogenic potential in the gut, others have some benefits, mainly as a consequence of non-pathogenic strains outcompeting the pathogenic forms, as is thought to occur for the probiotic *E. coli* Nissle 1917 (Iannitti and Palmieri, 2010; Gerritsen et al., 2011). Therefore, the impacts of the seaweed products on pathogenic strains and other target bacteria would need to be tested in future studies.

It is also interesting that the phlorotannin-enriched fraction of the seaweed extract influenced the gut microbiota populations, as evidenced by inhibition of the growth of certain pathogenic bacteria *in vitro*. The same results were observed in an *in vivo* study, as rats fed with whole seaweed-supplemented diet showed the inhibition of pathogenic bacteria as well as phenol and *p*-cresol production in the cecum. As a whole seaweed contained higher phlorotannin content than the polysaccharide fraction, this implied that phlorotannins might have played a role in the effects observed with the whole seaweed. However, more purified phlorotannin fraction and further studies would be required in order to understand the effects of seaweed phenolic compounds on bacteria inhibition and protein fermentation.

From the results of the polysaccharide characterisation studies, the sulphated fucans and mannuronic acid-rich alginate may have been the major components contributing to the prebiotic and dietary fibre properties observed for the polysaccharide fractions during the *in vivo* study. However, it is still difficult to conclude from this study how the dominant components of the polysaccharide could be altered in order to better influence the gut microbe composition and fermentation. Further studies covering a different range of polysaccharide components and structures are suggested in order to better understand which specific components are responsible

for the prebiotic activities, and whether the polysaccharide mixture could further improve gut health benefits. It has been demonstrated that the development of low MW seaweed extracts containing oligosaccharide using more specific enzymes may improve the efficiency for fermentability by gut microbes. Ramnani et al. (2012) indicated that low MW polysaccharides obtained from alginate and agar and exhibited potential as novel sources of prebiotics.

In addition, lower dosages (<5% w/w) of whole seaweed and polysaccharide supplementation in diets should also be further studied in order to expand the breadth of product applications in the functional food market. Also, the prebiotic effects of these seaweed-derived supplements in the context of different diet models (e.g. high-fat diets) could also be assessed. Precedent for such studies has been provided by Kumar et al. (2015a,b), who showed that certain green algae and their extracts can attenuate metabolic syndromes in rats fed with high fat and high carbohydrate diets. Most importantly, further investigation of the gut health benefits in an *in vivo* human model would be interesting to assess in order to confirm the results obtained by the *in vitro* and *in vivo* animal studies and enhance the potential for their use as dietary supplement in humans.

For the process and techno-economic feasibility study, the optimised conditions and processing parameters from pilot-scale testing could be one of the potential improvement to use these data for economic analysis in order to obtain better understanding for commercial perspectives. In addition, other food prototypes, aside from fruit juice-based beverages, which have a high impact in the market, should be considered. Importantly, tests are also required to confirm the bioavailability and stability of the seaweed-derived bioactive compounds when incorporated into food products, and their safety have to comply with standard regulations for functional food and nutraceutical ingredients.

8.3 References

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