Important Chemical Products from Macroalgae (*Ascophyllum nodosum*) Biorefinery by Assistance of Microwave Technology

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Abstract

The biorefinery is an important concept for the development of alternative routes to a range of interesting and important materials from renewable resources. It ensures that the resources are used fully and that all parts of them are valorized. The project will develop this concept, using macroalgae *Ascophyllum nodosum* as an example. The project focuses on valuable chemicals (e.g. fucoidan, alginate and sugars) and fuels (bioethanol and biochar) from macroalgae by assistance of microwave technology.

Microwave assisted extraction of fucoidan proves that fucoidan could be extracted in 5-30 min by microwave heating from 90 ^{o}C to 150 ^{o}C, and the highest yield (16.08%) was obtained at 120 ^{o}C, 15 min. The compositional characterization of fucoidan was carried out, and antioxidant activity test shows that fucoidan could potentially be a resource for natural antioxidant.

Microwave assisted extraction of alginate proves that alginate could be extracted successfully under low temperature (60-90 ^{o}C) - open vessel system and high temperature (100-140 ^{o}C) - closed vessel system, respectively. The results show that high temperature extraction had higher yield in less time compared with low temperature extraction, and M_w from both extraction were similar, 180 kDa – 220 KDa.

Microwave assisted hydrolysis of *Ascophyllum nodosum* shows that the optimal condition for saccharification was 0.4 M H_2SO_4, 3.13% (w/v) of biomass loading, reaction temperature at 150 ^{o}C for 1 min holding time, resulting in 127 mg/g monosaccharides of seaweed being released. An ethanol concentration of 5.57 g/L and a conversion efficiency of 60.7% were achieved after fermentation. More than 50% energy yield of alga residue was recovered after hydrolysis, and the energy densification ranged from 1.4 to 1.7, with HHVs from about 19 - 24 MJ/kg.

In addition, a step-by-step extraction and purification method was developed, in which fucoidan, alginate, sugar and biochar could be separated subsequently.

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Author's Declaration

Some of the results presented herein were obtained by, or in collaboration with other co-workers. They are all fully acknowledged in the list below along with their corresponding institution. All other results are the work of the author. This thesis has not been submitted previously for another degree at this, or any other university.

Piece of work	Chapter	Collaborator	Institution
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Molecular weight of alginate determination (GPC)	4	Raymond Sloan (Senior Technologist)	Biorenewables Development Centre (BDC)
Monosaccharide composition analysis of hydrolysis liquid (HPAEC)	5	Dr. Leonardo Gomez (Research fellow) Rachael Simister (Research technician)	Centre for Novel Agricultural Products (CNAP)- Department of Biology – University of York
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Molecular weight determination (GPC)	6	Jennifer Dyer (Research technician)	Department of Chemistry – University of York
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Chapter 1: Introduction

1.1 Biorefinery concept

Our high demand for and consumption of fossil fuels has caused several problems such as energy security, resource depletion and climate change, which are great challenges facing our societies. The annual world primary energy consumption in 2013 was 12730.4 million tonnes of oil equivalent, among which fossil fuels were about 11032.2 million tonnes, accounting for 86.7% of energy consumption with oil 32.9%, natural gas 23.7% and coal 30.1%, respectively(BP, 2014). High consumption of fossil fuels led to high emissions of greenhouse gases (GHG), such as CO_2, CH_4 and N_2O, which was proved to perturb the Earth's climate(Cherubini, 2010). Therefore, the biorefinery concept was spotlighted as a potential solution to replace or partially replace the current fossil based economy(Jung *et al.*, 2013).

It is defined that " biorefining is the sustainable processing of biomass into a spectrum of bio-based products and bioenergy"(IEA, 2009). Bio-based products include chemicals, materials, human food and animal feed, while bioenergy refers to fuels, power and/or heat. The biorefinery concept is analogous to today's petroleum refineries, which produce a number of different fuels, chemicals and platform molecules from petroleum. By producing multiple products, a biorefinery can take advantage of the natural complexity and differences in biomass components and intermediates and therefore maximize the value derived from the biomass. However, the natural complexity and differences in biomass components are also big challenges for the biorefinery, new technologies and industrial facilities for biomass processing need to be developed before the biorefinery can really compete with well-established petroleum refineries.

1.2 Green chemistry

Since the late 1960s, people began to realize the extent of environmental problems, and some early environmental legislation was introduced. The phrase "Green

Chemistry" was first coined by staff of the United States Environmental Protection Agency (EPA) Office of Pollution Prevention and Toxins in the early 1990s(ACS). The US EPA defines that "Green chemistry is the design of chemical products and processes that reduce or eliminate the use or generation of hazardous substances. Green chemistry applies across the life cycle of a chemical product, including its design, manufacture, use, and ultimate disposal"(USEPA). To help scientists and engineers to think about how chemistry and chemical engineering can be done to protect and benefit the people and society, different green chemistry principles have been proposed over the years, and most well known one is the "12 principles of Green Chemistry" developed by Paul Anastas and John Warner(Anastas & Warner, 1998):

1. **Prevention.** It is better to prevent waste than to treat or clean up waste after it has been created.
2. **Atom Economy.** Synthetic methods should be designed to maximize the incorporation of all materials used in the process into the final product
3. **Less Hazardous Chemical Syntheses.** Wherever practicable, synthetic method should be designed to use and generate substances that possess little or no toxicity to human health and the environment.
4. **Designing Safer Chemicals.** Chemical products should be designed to affect their desired function while minimizing their toxicity.
5. **Safer Solvents and Auxiliaries.** The use of auxiliary substances (e.g., solvents, separation agents, etc.) should be made unnecessary wherever possible and innocuous when used.
6. **Design for Energy Efficiency.** Energy requirements of chemical processes should be recognized for their environmental and economic impacts and should be minimized. If possible, synthetic methods should be conducted at ambient temperature and pressure.
7. **Use of Renewable Feedstocks.** A raw material or feedstock should be renewable rather than depleting whenever technically and economically

practicable.

8. **Reduce Derivatives.** Unnecessary derivatization (use of blocking groups, protection/ deprotection, temporary modification of physical/chemical processes) should be minimized or avoided if possible, because such steps require additional reagents and can generate waste.
9. **Catalysis.** Catalytic reagents (as selective as possible) are superior to stoichiometric reagents.
10. **Design for Degradation.** Chemical products should be designed so that at the end of their function they break down into innocuous degradation products and do not persist in the environment.
11. **Real-time analysis for Pollution Prevention.** Analytical methodologies need to be further developed to allow for real-time, in-process monitoring and control prior to the formation of hazardous substances.
12. **Inherently Safer Chemistry for Accident Prevention.** Substances and the form of a substance used in a chemical process should be chosen to minimize the potential for chemical accidents, including releases, explosions, and fires.

By developing sustainable technology and greener products, green chemistry not only benefits human health, environment, but also contributes to economy and business. Pike Research reported "the use of green chemistry in a range of industrial activities will grow rapidly in the coming decade, offering significant direct cost savings as well as indirect savings in the form of avoiding liability for environmental and social impacts. The total amount saved, the cleantech market intelligence firm forecasts, will reach $65.5 billion by 2020"(PikeResearch, 2011).

1.3 Macroalgae backgrounds

1.3.1. Taxonomical classification

Algae are all those photosynthetic, eukaryotic organisms, which are thalloid, having no true roots, stems and leaves(Prescott, 1969). They can range from the microscopic (microalgae) that only has 0.5 micron unicell in diameter, to large seaweed (macroalgae) that maybe more than one hundred feet in length, such as the giant kelp *Macrocystis pyrifera* (Figure 1.1). In this work, we focus on macroalgae.

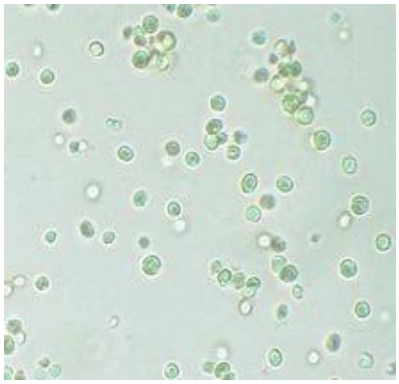
a)  b) 

Figure 1.1 Picture of algae (a) *Nannochloropsis* viewed under a light microscope (b) Giant kelp *Macrocystis pyrifera*(Guiry, 2000)

Macroalgae, also called seaweed, generally live attached to rock or other hard substrata in coastal areas. In the primary classification, the macroalgae are divided to three different groups on the basis of the thallus colour: the green algae or Chlorophyceae, the red algae or Rhodophyceae, and the brown algae or Phaeophyceae. There are a number of other groups, but these are not of important economic use(Chapman, 1970).

For green algae, the green colour is from chlorophyll a and b in the same proportions as the land plants; beta-carotene (a yellow pigment); and various characteristic xanthophylls (yellowish or brownish pigments). There are about 4500 species of green algae, among which about 3050 species are freshwater-favorable and 1500 species

marine.

The red algae are the most diverse group, and the colour is from the pigments phycoerythrin and phycocyanin, which mask the other pigments; Chlorophyll a (no Chlorophyll b), beta-carotene and a number of unique xanthophylls. There are about 6500 species of red algae, the majority of which are marine. Red algae are found in the intertidal and subtidal zones of the sea at depths up to 40 m or occasionally as deep as 250 m.

For brown algae, the colour is from the dominance of the xanthophyll pigment fucoxanthin, which masks the other pigments, Chlorophyll a and c (there is no Chlorophyll b), beta-carotene and other xanthophylls. There are about 1800 species of brown algae, and most are marine species. In general, most brown algae species are found in cold water, and the majority of brown algae biomass worldwide comes from a relatively small number of species in the orders Laminariales and Fucales(Guiry, 2000)

1.3.2 Chemical compositions of macroalgae

It is necessary to understand the composition of biomass feedstocks as the potential products that can be derived from biomass depend on its chemical content. Unlike terrestrial plants, which consist of cellulose, hemicellulose and lignin, the composition of macroalgae is more complex. In addition to carbohydrate content, macroalgae also contains protein and lipid (Table1.1). Generally, the carbohydrate of red algae is about 40% - 70% dry wt., which is higher than those of brown and green algae (30% - 60% dry wt.). Macroalgae contains a higher content of ash compared with terrestrial biomass, and brown algae have a higher value (20%–40% dry wt.) than other seaweed species(Ross *et al.*, 2008). The protein and lipid content of macroalgae are 5%-20% dry wt. and 1% - 5% dry wt., respectively, whereas most microalgae have high contents of protein (20%–50% dry wt.) and lipid (10% -30% dry wt.), but low content of carbohydrate (<20% dry wt.). The chemical composition of macroalgae strongly

depends on several environmental factors such as water temperature, salinity, light and nutrients(Marinho-Soriano *et al.*, 2006). It is well known that macroalgae is lignin free or contains little lignin, compared to terrestrial plants. This is advantageous in processing as macroalgae can be depolymerised relatively easily, avoiding difficult processes such as lignin removals and detoxification of lignin derived enzyme-inhibiting compounds (Meinita, Hong, *et al.*, 2012b).

Table1.1 Composition of macroalgae and microalgae species

Species	Classification	Carbohydrate (%)	Protein (%)	Lipid (%)	Ash (%)	References
Gelidium amansii	Red algae	77.2	13.1	1.1	8.6	(N.-J. Kim *et al.*, 2011)
Gelidium amansii		74.4 (fiber 11.6)	18.3	0.0	7.4	(J. S. Jang *et al.*, 2012)
***Gracilaria tenuistipitata*[a]**		67.1 (fiber 5.6)	6.86	0.29	25.7	(Chirapart *et al.*, 2014)
***Gracilaria salicornia*[a]**		63.8 (fiber 10.6)	18.7	1.9	15.5	(Chirapart *et al.*, 2014)
Gracilaria cevicornis		68.8 (fiber 5.7)	19.7	0.4	10.5	(Marinho-Soriano *et al.*, 2006)
Sargassum fulvellum	Brown algae	39.6	13.0	1.4	46.0	(N.-J. Kim *et al.*, 2011)
Sargassum fulvellum		44.5 (fiber 3.5)	19.9	0.5	35.1	(J. S. Jang *et al.*, 2012)
Saccharina japonica		66.0 (fiber 6.3)	10.6	1.6	21.8	(J. S. Jang *et al.*, 2012)
Undaria pinnatifida		52.0 (fiber 3.6)	18.3	1.8	28.0	(J. S. Jang *et al.*, 2012)
Hizikia fusiforme		59.0 (fiber 4.2)	13.9	0.4	26.6	(J. S. Jang *et al.*, 2012)
Laminaria japonica		51.9	14.8	1.8	31.5	(N.-J. Kim *et al.*, 2011)
Ulva lactuca	Green algae	54.3	20.6	6.2	18.9	(N.-J. Kim *et al.*, 2011)
***Ulva intestinalis*[a]**		63.3 (fiber 6.8)	12.2	0.7	23.8	(Chirapart *et al.*, 2014)
***Rhizoclonium riparium*[a]**		45.0 (fiber 12.9)	13.9	0.3	40.8	(Chirapart *et al.*, 2014)

Enteromorpha linza		37.4 (fiber 2.4)	31.6	1.8	29.2	(J. S. Jang *et al.*, 2012)
F. pinnata	Microalgae	5.8	25.5	14.9	34.6	(Renaud *et al.*, 1999)
Rhodomonas sp.		8.6	29.1	18.7	17.2	
Nephroselmis sp.		14.3	32.3	10.5	10.9	

[a] Calculated from wet base

Macroalgae have high carbohydrate contents, which is potential biochemical feedstock for production of chemicals and biofuels. However, there are some unique polysaccharides in the macroalgae that are different to those in lignocellulosic biomass. Therefore, an understanding of carbohydrate composition of macroalgae is necessary (Table1.2). The structures of some major polysaccharides in macroalgae are shown in Figure 1.2(Wei *et al.*, 2013).

Table1.2 Carbohydrate composition of macroalgae, microalgae and lignocellulosic biomass (Jung *et al.*, 2013; Roesijadi *et al.*, 2010)

Macroalgae			***Microalgae***	***Lignocellulosic biomass***
Green algae	**Red algae**	**Brown algae**	***Polysaccharides***	
Polysaccharides	***Polysaccharides***	***Polysaccharides***		
Starch Cellulose Ulvan	Carrageenan Agar Cellulose Lignin	Laminarin Mannitol Alginate Fucoidan Cellulose	Starch	
Monosaccharides	***Monosaccharides***	***Monosaccharides***	***Monosaccharides***	Cellulose Hemicellulose Lignin
Glucose Mannose Rhamnose Xylose Uronic acid Glucuronic acid	Glucose Galactose Agarose	Glucose Rhamnose [a] Galactose Fucose Xylose Mannose [a] Uronic acid Glucuronic acid Mannuronic acid	Arabinose Fucose Galactose Glucose Mannose Rhamnose Ribose Xylose	

[a] Observed in this work

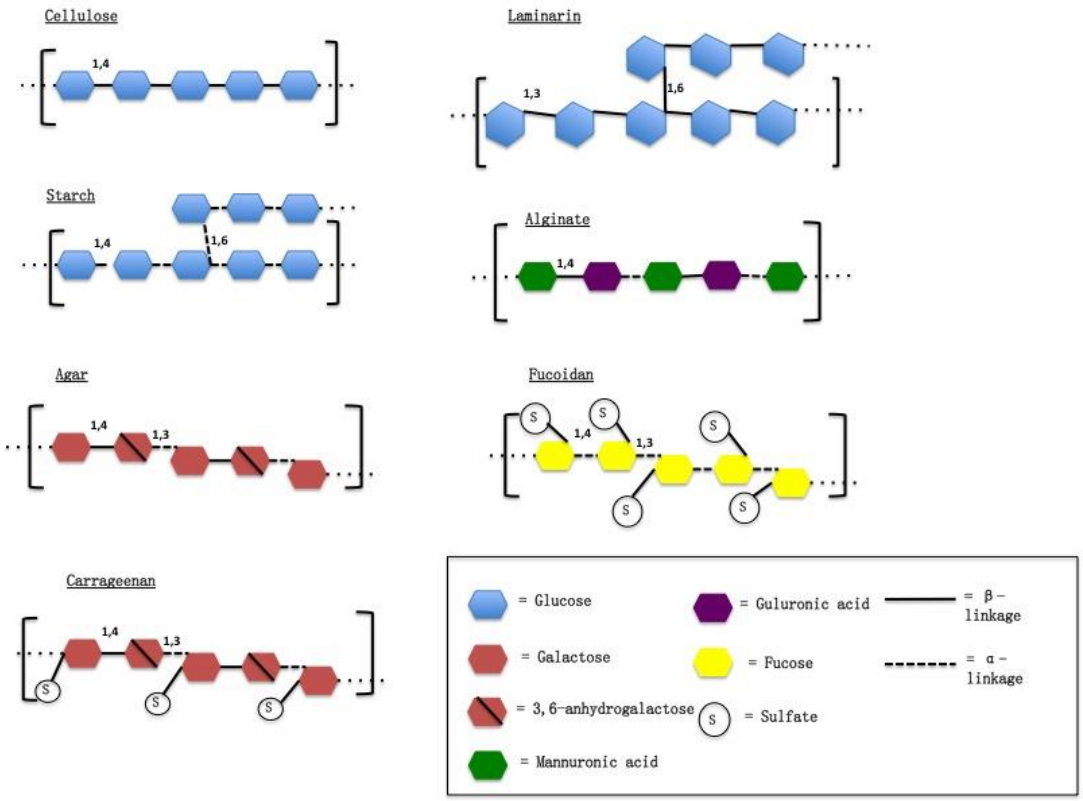


Figure 1.2 Structures of major polysaccharides in macroalgae(Wei *et al.*, 2013)

1.3.2.1 Green algae

The major polysaccharides in green algae are starch, cellulose and ulvan. It is reported that higher green plants (particularly herbaceous plant) has evolved from green algae, as the chemical composition of green algae is similar to land plants, in particular starch and cellulose(Lewis & McCourt, 2004). However, this is still controversial.

Cellulose is a polysaccharide consisting of a linear chain of several hundred to more than 10 000 β-1,4-linked D-glucose units. It is the structural component of the primary cell wall of green plants and many forms of algae(Wei *et al.*, 2013).

Starch is a linear or branched molecule, consisting of D-glucose units joined by α-1,4 and α-1,6 linkages. It is a storage polysaccharide found in green macroalgae(Wei *et al.*, 2013).

Ulvan is a unique polysaccharide in green algae, mainly composed of sulfate, rhamnose, xylose and glucuronic acid. It has been reported to display several physicochemical and biological features of potential interest for food, chemical and pharmaceutical applications(Lahaye & Robic, 2007).

1.3.2.2 Red algae

The storage polysaccharide of red algae is floridean starch, which is an α-1,4-glucosidic linked glucose homopolymer, and true starch like that of higher plants and green algae is absent. The major polysaccharides in cell wall of red algae are carrageenan (up to 75% dry wt.) and agar (up to 52%), which are the most important commercial polysaccharides for red algae(Jung *et al.*, 2013).

Carrageenan is a sulfated polysaccharide molecule consisting of alternate units of β-D-galactose and α-D-galactose. The main applications for carrageenan are in the food industry as additives. Based on the chemical structure and gel-forming abilities, commercial carageenans can be classified as lambda (λ), kappa (κ), or iota (ι)(Wei *et al.*, 2013).

Agar is a linear polymer of altering 3-linked β-D-galactopyranosyl and 4-linked 3,6-anhydro-α-L-galactopyranosyl subunits, and is commonly used as a gelling compound in food products and in culture mediums for research or industry.

1.3.2.3 Brown algae

Compared with red and green algae, the carbohydrate composition of brown algae is more complex, mainly including Laminarin, alginate, fucoidan, mannitol and cellulose (Figure 1.3).

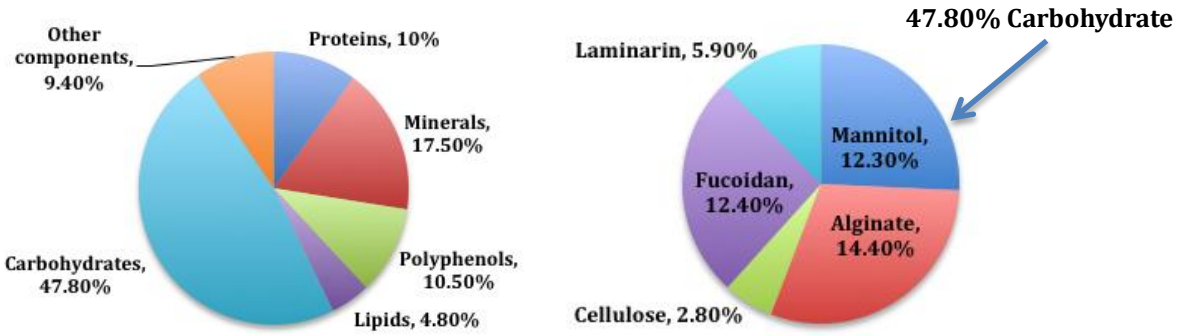


Figure 1.3 Overview of the average composition of *Fucus vesiculosus*(Hahn *et al.*, 2012)

Laminarin is the main storage polysaccharide isolated from brown macroalgae, consisting mostly of linear β-1,3-linked glucose units with small amounts of β-1,6-linkages .

Mannitol, a sugar alcohol, is also a major polysaccharide in brown seaweed. It can be converted to fructose by mannitol dehydrogenase for ethanol production(Horn *et al.*, 2000b).

Alginate is a linear polymer consisting of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) in varying sequences. It is present in cell wall of brown algae mainly in form of calcium form(Chee *et al.*, 2011). Alginates are widely used in the pharmaceutical, cosmetic and food industries as gelling agent or thickening agent(Hernandez-Carmona *et al.*, 1999).

Fucoidan is a heterogeneous polysaccharide in brown macroalgae, consisting primarily of α-(1→3)- and (1→4)-linked-L-fucose residues, that may be organized in stretches of (1→3)-α-fucan or of alternating α-(1→3)- and α-(1→4)-bonded L-fucose residues. The L-fucose residues may be substituted with sulfate (SO_3) on the C-2 or C-4 (rarely on C-3)(Ale *et al.*, 2011). Besides fucose, fucoidan also contains some other monosaccharides such as mannose, rhamnose, galactose, glucose, xylose and uronic acids(J. Wang *et al.*, 2008). Many factors such as seaweed species, geographic location, harvest season, anatomical regions and even extraction procedure have significant

influence on the polysaccharides compositions and structures(Foley *et al.*, 2011; Honya *et al.*, 1999; Mak *et al.*, 2013; Rodriguez-Jasso *et al.*, 2011). There has been intensive study on fucoidan due to its bioactivities in pharmaceutical applications(Ale *et al.*, 2011).

1.3.3 Cultivation and harvest

Macroalgae have higher photosynthetic ability than terrestrial plants, therefore can synthesize more biomass needed for biorefinery. Table1.3 compares the productivities of macroalgae and several lignocellulosic feedstocks. As the productivities of macroalgae are much higher than those of lignocellulosic biomass, it is promising to cultivate macroalgae biomass for biorefinery processes.

Table1.3 Comparisons of productivities of macroalgae and lignocellulosic biomass(Yanagisawa *et al.*, 2013)

Biomass		Productivity [dry g/(m²•year)
Macroalgae	Green algae	7,100
	Brown algae	3,300-11,300
	Red algae	3,300-11,300
Lignocellulosic biomass	Switchgrass	560-2,240
	Corn stover	180-790
	Eucalyptus	1,000-2,000
	Poplar	300-612.5
	Willow	46-2,700

Current, there are essentially two ways to obtain macroalgae biomass: wild harvesting and cultivation. For the worldwide total production of macroalgae, wild stock was only about 7%, while the cultivation accounted for 93% in 2006(Murphy *et al.*, 2013). Table1.4 shows the top ten countries for harvesting wild stocks and aquaculture respectively. As can be seen, production of wild macroalgae stocks are world wide distributed while the cultivations of macroalgae biomass are mainly in Asia with China

accounting for 72% of global annual production. The five major macroalgae genuses cultivated are *Laminaria*, *Undaria*, *Porphyra*, *Euchema* and *Gracilaria*(Murphy *et al.*, 2013*)*.

Table1.4 World production (wet metric ton) of wild stock harvest and cultured macroalgae in 2006 by country(Roesijadi *et al.*, 2010)

Harvests of wild stock			Aquaculture		
Source	**Production (metric ton)**	**% of total**	**Source**	**Production (metric ton)**	**% of total**
World total	**1,143,273**	**100**	**World total**	**15,075,612**	**100**
China	323,810	28.32	**China**	10,867,410	72.09
Chile	305,748	26.74	**Philippines**	1,468,905	9.74
Norway	145,429	12.72	**Indonesia**	910,636	6.04
Japan	113,665	9.94	**Republic of Korea**	765,595	5.08
Russian Fed	65,554	5.73	**Japan**	490,062	3.25
Ireland	29,500	2.58	**Korea DPRK**	444,300	2.95
Mexico	27,000	2.36	**Chile**	33,586	0.22
Iceland	20,964	1.83	**Malaysia**	30,000	0.20
France	19,160	1.68	**Vietnam**	30,000	0.20
Australia	15,504	1.36	**Cambodia**	16,000	0.11
Other	77,057	6.74	**Other**	18,091	0.12

1.3.4 Products from macroalgae

Currently, the commercial products from macroalgae include human food, algal hydrocolloids, fertilizers, animal feed and therapeutic materials (Table1. 5). The biggest macroalgae industry is human food, which was about $5 billion in 2003, accounts for 83%-90% of the total macroalgae market(Roesijadi *et al.*, 2010). Asia is the major market for human consumption of macroalgae, and in recent years seaweed meals have been strongly introduced into Europe. The most common edible seaweed includes nori (*Porphyra spp.*, red algae), aonori (*Monostroma spp.* and *Enteromorpha spp.*, green algae), kombu (*Laminaria japonica*, brown algae), wakame (*Undaria*

pinnatifida, brown algae)(Dennis J. McHugh, 2003).

Table1. 5 Estimated global value of seaweed products per annum as reported 2003(Dennis J. McHugh, 2003)

Products	***Value***
Human Food (Nori, aonori, kombu, wakame, etc.)	$5 billion
Algal hydrocolloids	
Agar (Food ingredient, pharmaceutical, biological/microbiological)	$132 million
Carrageenan (food additive, pet food, toothpaste)	$213 million
Alginate (Textile printing, food additive, pharmaceutical, medical)	$240 million
Other uses of seaweed	
Fertilizers and conditioners	$5 million
Animal Feed	$5 million
Therapeutic materials	
Total	$5.5-6 billion

As described above, there are a variety of polysaccharides in macroalgae; among which, agar and carrageenan from red seaweed, and alginate from brown seaweed are commercially important hydrocolloids, accounting for about $500 million on an annual basis. Most agar is extracted from species of *Gelidium* and *Gracilaria*, while most carrageenan is extracted from *Kappaphycus alvarezii* and *Eucheuma denticulatum*. The brown seaweed resources for alginates extraction are species of *Ascophyllum*, *Ecklonia*, *Laminaria* and *Macrocystis*(Dennis J. McHugh, 2003).

In addition, sulfated polysaccharides such as ulvan, carrageenan and fucoidan, have been recognized to possess a number of biological activities including anticoagulant, antiviral and antioxidant activates, which makes them potential therapeutic materials(Jiao *et al.*, 2011). However, as it is difficult to identify the relation between the chemical structure and bioactivities, the therapeutic applications of the sulfated polysaccharides have not been widely commercialized.

Besides, macroalgae have some other uses. For instance, macroalgae has a long history of use as a fertilizer and a soil conditioner by coastal people due to their suitable

content of nitrogen and potassium, and also the trace elements they contain. In some European countries such as France, Iceland and United Kingdom, animals that lived in coastal areas have been fed with seaweed for long time. Macroalgae also have the potential for use in wastewater treatment to reduce the nitrogen- and phosphorus-containing compounds and to remove the toxic metals from industry wastewater(Dennis J. McHugh, 2003).

1.3.5 Biofuels from macroalgae

Biofuels are addressed as partial solutions to the development problems facing our society, such as resource depletion, climate change and energy security(Daroch *et al.*, 2013). According to the difference of feedstocks, biofuels can be classified into:

- First-generation biofuels are mainly produced from food and oil crops like corn, rapeseed, sugarcane as well as animal fats by fermentation and transesterification. The most well-known and widely commercialized first generation biofuels are bioethanol and biodiesel. However, the controversy known as "food vs. fuel" issue emerged, and this promoted the development of second-generation biofuels.
- Second-generation biofuels are primarily produced from lignocellulosic biomass, which are either agriculture residues (e.g. wheat straw, corn stover, etc.) or non-edible plant biomass (e.g. wood, grass, etc.). Compared with first-generation biofuels, second-generation biofuels no longer compete with food sector, and worldwide distribution of lignocellulosic biomass guarantees the supply of feedstock. However, the technology and process to produce the second-generation biofuels are more complex than first-generation biofuels, which still need to be developed before commercialisation
- Third-generation biofuels are produced from algal biomass, including microalgae and macroalgae. Unlike terrestrial plants, algae do not require agriculture land for cultivation and many species grow in salt water or even in

wastewater(Pittman *et al.*, 2011). Algae grow faster and the potential biomass yield of algae per unit area is often higher than that of terrestrial plant(Williams & Laurens, 2010). Therefore, algae are considered as the one of the most promising feedstocks for biofuels production.

Here we focus on macroalgae. By using biochemical and/or thermochemical processing, macroalgae can be converted to a range of biofuels.

1.3.5.1 Biogas

Biogas can be produced from various macroalgae by anaerobic digestion (AD). Biogas mainly consists of methane (CH_4) and carbon dioxide (CO_2), with minor amounts of hydrogen sulfide (H_2S) and ammonia (NH_3)(Vergara-Fernandez *et al.*, 2008). Previous studies have shown that macroalgae are good raw materials to produce biogas through anaerobic digestion due to high conversion efficiencies, and methane yields are comparable to those using cattle manure as substrates with its best yield twice as high(Suutari *et al.*, 2015). Moreover, the digestate (residue after the AD process) contains both nitrogen- and phosphorus-rich compounds, which makes it a possible fertiliser(Milledge *et al.*, 2014). The chemical composition and species of macroalgae are important factors effecting the biogas production, while generally methane yield from brown algae are higher than those from green algae(Sutherland & Varela, 2014). It has been suggested that a co-digestion of macroalgae with N-rich substrates can increase biogas production(Hughes *et al.*, 2012). However, although seaweed AD has been proven to be closer to commercialisation than other fuels, the cost of raw materials must be reduced by at least 75% to make it economically competitive(Milledge *et al.*, 2014).

1.3.5.2 Syngas

Syngas is a combustible gas mixture of hydrogen (30-40%), carbon monoxide (20%-30%), methane (10-15%), ethylene (1%), nitrogen, carbon dioxide and water

vapour(Demirbas, 2001). It can be produced by gasification of organic matter. Conventional biomass gasification processes require dry feedstock, which is not suitable for wet macroalgae with high moisture content(Guan *et al.*, 2012). Drying process is needed for conventional macroalgae gasification, which will cause extra energy consumption. Supercritical water gasification (SCWG) is an alternative technology for wet feedstocks like algae. Several brown algae species were tested by SCWG(Schumacher *et al.*, 2011) and results show that all the species produced substantially higher hydrogen yields than lignocellulosic materials reported previously. Hydrogen yields were 11.8-16 g H_2 kg^{-1} algal biomass and the methane yields were 39-104 g CH_4 kg^{-1} biomass. While for lignocellulosic agricultural waste (e.g. corn stalk, sunflower stalk, corncob, etc.), hydrogen yields were 4.18-8.30 g H_2/kg biomass and methane yields were in the range of 30-70 g CH_4/kg biomass(Yanik *et al.*, 2007). The higher syngas yields from macroalgae may be due to the high concentration of inorganic salts, which act as catalysts promoting the gasification processes. Additions of NaOH and alumina-supported ruthenium (Ru/Al_2O_3) catalysts in the SCWG of macroalgae have been found to increase the H_2 and CH_4 by 2-3 times(Cherad *et al.*, 2013; Onwudili *et al.*, 2013). The syngas also can be converted into hydrocarbons and water through Fischer-Tropsch Synthesis (FTS), with aromatics and alcohols formed as secondary products(Milledge *et al.*, 2014).

1.3.5.3 Bioethanol

Bioethanol has been widely accepted as the most promising replacement of gasoline to act as a transport fuel(Li *et al.*, 2014). It can be produced by fermentation of sugars with appropriate microorganisms, mainly yeast and bacteria(Daroch *et al.*, 2013). Unlike terrestrial plants, the carbohydrate profiles of macroalgae are more complex, including various polysaccharides such as starch, cellulose, laminarin, mannitol, alginate, agar and some other sulfated polysaccharides. Therefore, the choice of microorganisms that can convert above polysaccharides is important (Table1.6). *Saccharomyces cerevisiae* is the most widely used yeast for bioethanol production from

glucose(Wei *et al.*, 2013). Bacterium *Zymobacter palmae* can convert mannitol to ethanol while two yeast strains *Kluyveromyces marxianus* and *Pacchysolen tannophilus* can ferment laminarin. Only yeast *Pichia angophorae* can use both mannitol and laminarin, and was reported to have the yield of 0.43 g ethanol g^{-1} substrate(Horn *et al.*, 2000a). Alginate, a major component in brown algae, cannot be fermented by any natural strains(Horn *et al.*, 2000a). In order to maximize the ethanol production from brown algae, an engineered strain of *Escherichia coli* was developed, which could convert all main sugars including laminarin, mannitol and alginate into ethanol, yielding 0.281 g g^{-1} dry seaweed, 80% of the maximum of theoretical conversion(Wargacki *et al.*, 2012).

Table1.6 Microorganisms that convert macroalgae into ethanol(Wei *et al.*, 2013; Yanagisawa *et al.*, 2013)

Microorganisms	**Major carbohydrate utilized**
Natural strains	
Saccharomyces cerevisiae	Glucose
Zymobacter palmae	Mannitol
Kluyveromyces marxianus	Laminarin, glucose
Pacchysolen tannophilus	Laminarin, glucose
Pichia angophorae	Mannitol, laminarin, glucose
Brettanomyces custersii	Galactose, glucose
Engineered strains	
Saccharomyces cerevisiae (engineered for improved galactose fermentation)	Galactose, or simultaneous cofermentation of galactose and cellobiose
Escherichia coli (engineered for alginate metabolism)	Glucose, mannitol, laminarin and alginate
Escherichia coli KO11	Glucose, mannitol

Bioethanol production from seaweed involves four major operations including pretreatment, hydrolysis, fermentation and distillation(Li *et al.*, 2014). Acid hydrothermal treatment is probably the most widely used pretreatment method to increase the digestibility of biomass (Ra & Kim, 2013). Hydrolysis of the seaweed

releases the fermentable sugars from polysaccharides, and is normally done by the use of acid or enzymes. Acid hydrolysis is a cheap and fast method for saccharification; however, inhibitors generated during the process can affect the microorganisms and need to be removed before fermentation(Meinita, Hong, *et al.*, 2012b). In comparison, enzymatic hydrolysis is a milder approach without inhibitor formation, but enzyme activity is generally specific to the type of polysaccharide while several types of polysaccharides exist in even one single species of macroalgae(Choi *et al.*, 2009). Distillation is an energy intensive process, and at least 4% of ethanol concentration is necessary for the reduction of energy consumption(J. S. Jang *et al.*, 2012), therefore, high ethanol yield from "total" utilisation of seaweed component is required. So far, all three green, brown and red algae have been investigated for ethanol production, and the highest concentrations of ethanol produced are shown in Table1.7.

Table1.7 The highest concentrations of ethanol produced from green, brown and red macroalgae(Yanagisawa *et al.*, 2013)

Macroalgae	Carbohydrates converted to ethanol	Ethanol concentration (g/L)	Ethanol yield (g ethanol g^{-1} seaweed)
***Ulva pertusa* (green)**	Glucans	27.5	0.092
***Laminaria japonica* (brown)**	Glucose, mannitol, alginate	37.8	0.291
***Gelidium elegans* (red)**	Agar (galactose), glucans	55	0.183

1.3.5.4 Biobutanol

Biobutanol has been suggested to be advantageous in practical use compare to ethanol due to its lower vapour pressure, higher energy density and non-corrosive nature(Potts *et al.*, 2012). Biobutanol is produced by a process known as the ABE fermentation, in which bacterium *Clostridium* converts a variety of sugars including both hexoses (C_6) and pentose (C_5) to acetone, butanol and ethanol(van der Wal *et al.*, 2013). As macroalgae is rich in carbohydrates, some species have been investigated as

potential feedstocks for biobutanol production. ABE fermentation of green algae *Ulva lactuca* yielded 0.35 g ABE g^{-1} sugar(van der Wal *et al.*, 2013). The same green algae species was also reported to yield 0.29 g butanol g^{-1} sugar(Potts *et al.*, 2012). The brown algae kelp *Saccharina* yielded 0.12 g butanol g^{-1} sugar and 0.16 g ABE g^{-1} sugar, but left alginate unused(Huesemann *et al.*, 2012). Although biobutanol production from macroalgae is still at the experimental level, a strong effort has been undertaken to make it economically feasible for large-scale commercial production(Suutari *et al.*, 2015).

1.3.5.5 Biodiesel

Biodiesel is produced by transesterification of lipids extracted from raw biomass. A lot of work has been done to produce biodiesel from microalgae, which typically has a high lipid content about 20%-50%, while some species can have over 70%(Murphy *et al.*, 2013). In comparison, the average lipid content of macroalgae is < 3.5%(Suutari *et al.*, 2015). Several macroalgae species such as *Chaetomorpha linum, Ulva lactuca* and *Enteromorpha compressa* have been tested for biodiesel production, however, the yields are low at 11% of the total dry biomass weight(Milledge *et al.*, 2014). Therefore, macroalgae may not be a good feedstock for biodiesel production.

1.3.5.6 Bio-oil

Bio-oil can be produced by pyrolysis or hydrothermal liquefaction of organic compounds. By varying the temperature and heating rate, pyrolysis can be optimised to favour the production of bio-oil, gas and solid char. Generally, fast pyrolysis is capable of producing higher quantities of liquid(Miao *et al.*, 2004). Compared to lignocellulosic materials, which are mainly composed of cellulose, hemicellulose and lignin, macroalgae is lignin free and rich in soluble carbohydrate. Therefore, pyrolysis of macroalgae can be achieved at lower temperatures(S. Wang *et al.*, 2007). However, overall yield of bio-oil derived from seaweed pyrolysis is lower than that from

lignocellulosic biomass due to the high concentration of ash content in the seaweed(S. Wang *et al.*, 2013). Bio-oil from pyrolysis cannot be used in conventional fuel engines directly before refining, as it is typically a complex mixture of highly oxygenated organic compounds, which are generally polar, viscous, corrosive and unstable(Bridgwater, 2012). In comparison, bio-oil from hydrothermal liquefaction is more stable, as it is lower in oxygen and moisture content(Neveux *et al.*, 2014). Another advantage that makes hydrothermal liquefaction more attractive for processing algal biomass is that there is no need to dry the algae after harvesting prior to the treatment(Minowa *et al.*, 1995).

Although bio-oil is a potential biofuel from macroalgae, there are problems, such as high nitrogen and sulfur content in the bio-oil that may lead to excessive formation of NO_X and SO_X compounds, still limit the commercialisation of bio-oil from macroalgae(Bae *et al.*, 2011; Elliott *et al.*, 2014).

1.3.6 Conclusion: benefits and challenges of algae

In recently years, algae have been intensively studied as one of the most promising and sustainable feedstocks for biorefinery process. Here, we summarise 6 reasons why algae can be of benefit:

1. **Macroalgae Do Not Compete With Agriculture.** Macroalgae grow in the sea; they do not compete with food production on either land or in fresh water.
2. **Macroalgae Grow Fast.** Algae grow fast, compared with the terrestrial biomass, which are harvested once or twice a year. Moreover, the average photosynthetic efficiency of aquatic biomass (6-8%) is much higher than terrestrial biomass (1.8-2.2%)(Li *et al.*, 2014).
3. **Macroalgae Have High Carbohydrate Content.** The carbohydrate content (e.g. starch, sugars) is abundant in the algae cell, which makes algae a good feedstock for biofuels.

4. **Macroalgae Can Consume CO_2.** Like any other plant, macroalgae can consume CO_2 from the atmosphere, and produce O_2. For high productivity, algae require more CO_2, resulting in the GHG reductions relative to fossil fuels.
5. **Macroalgae Can Be Used to Produce Many Products.** In addition to the biofuels, macroalgae can be also used to produce a variety of products: hydrocolloids, fertilizer, animal feed and medicines.
6. **Macroalgae industry can create job opportunities.** From research to engineering, from marketing to financial services, many macroalgae related jobs could be created(AllAboutalgae.com).

However, despite the advantages of macroalgae being used as a biorefinery feedstock, many challenges exist. Compared with the compositions of terrestrial biomass, which are mainly lignocellulosic materials, macroalgae contains unique carbohydrates. Therefore, the technologies developed to treat terrestrial biomass can't be directly applied to macroalgae, and more investigations are necessary. Furthermore, industrial scale production of biofuels (e.g. bioethanol and biodiesel) from crop biomass has been successfully established, while the macroalgae biorefinery is still in early stage.

1.4 Microwave technology

1.4.1 Introduction

Microwaves are electromagnetic waves with wavelengths ranging from as long as one meter to as short as one millimeter, or equivalently, with frequencies between 0.3 GHz and 300 GHz (Figure 1.4)(Pozar, 1993). For microwave heating purpose, there are two frequencies reserved by Federal Communications Commission (FCC) for use in industrial, scientific and medical systems, 915 MHz and 2.45 GHz(Thostenson & Chou, 1999). The domestic microwave oven and almost all lab MW reactors work on the frequency of 2.45 GHz.

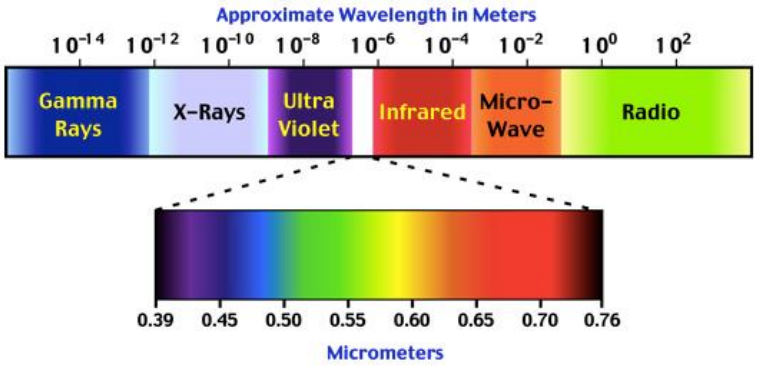


Figure 1.4 Electromagnetic spectrum with visible light highlighted(Pozar, 1993)

Besides heating purpose, microwave technology has a wide variety of applications, including communication, radar, radio astronomy, navigation etc. The earliest application of microwave technology in chemistry can be traced back to 1950s, then microwave heating in the laboratory began to gain wide acceptance following papers in 1986(Gedye *et al.*, 1986). In recent decades, microwave heating, which is considered as a green technology, has been more and more applied in chemistry because it offers a clean, cheap, and convenient method of heating which often results in higher yields and shorter reaction times(Thostenson & Chou, 1999).

1.4.2 Microwave heating

1.4.2.1 Heating mechanism

Generally, according to the interaction with the microwave field, there are three qualitative ways in which a material may be categorized (Figure 1.5): opaque (conductors, such as metals) — microwave are reflected and do not penetrate; absorbing (high dielectric loss materials, such as ethanol and water) — absorb microwave energy to a certain degree based on the value of the dielectric loss factor; and transparent (low dielectric loss materials, such as teflon, glass and quartz) — microwaves pass through with little attenuation (Sutton, 1989).

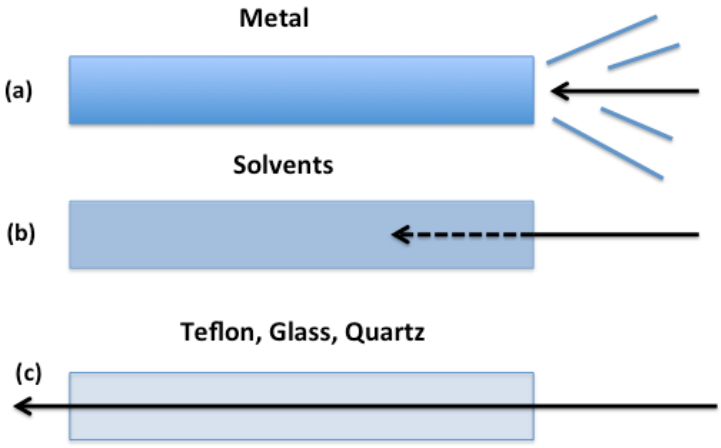


Figure 1.5 Interaction of microwaves with different materials. (a) Electrical conductors. (b) Absorbing materials (tanδ 0.05-1). (c) Insulators (tanδ < 0.01)(Kappe & Stadler, 2006)

Actually, microwave fields are a high frequency oscillating electric and magnetic fields. Anything that is put into this field will be affected by electrical or magnetic polarisation at this oscillation frequency. Two major heating mechanism exist: dipolar polarisation and ionic conduction(Clark & Sutton, 1996; Lidstrom *et al.*, 2001).

Dipolar polarisation

Under a high frequency electric field, the polar molecule will attempt to follow the field, however, the rapid reversal of electromagnetic waves makes it difficult to keep in phase with the field frequency. In this case, the random motion of molecules to attempt to follow the field results in molecules jostling, which is the heating we observe in the sample.

The ability of a material to be polarised is expressed in terms of the loss tangent:

$$\tan\delta = \varepsilon''/\varepsilon',$$

where ε'' is the dielectric loss factor, defining the capacity of a material to convert electromagnetic energy into heat, and ε' is the dielectric constant that represents the ability of a material be to polarized. Therefore, higher tanδ is favorable for microwave processing(Kappe, 2004).

Ionic conduction

Ionic conduction normally works on ion-containing solutions only. When there is electrical field applied, the charge carriers (ions) are moved through the material under the influence of the electric field, resulting in a polarisation. These induced currents will cause heating in the sample due to any electrical resistance. In an ion containing solution that is under microwave radiation, ionic conduction contributes much more than the dipolar polarisation(Lidstrom *et al.*, 2001).

Therefore, it seems that seaweed in water is a very good system for efficient microwave radiation. While water is a good microwave absorbent, high ash content will also contribute to the ionic conduction.

1.4.2.2 Comparison with conventional heating

In conventional heating manner, heat is transferred from an external heating source (e.g., an oil bath or heating mantle) into the material through conduction from the surfaces of the material towards the inner parts(Motasemi & Afzal, 2013). This is a comparatively slow method for energy transfer, and results in a temperature gradient within the materials, and also a higher temperature at the surface of the reaction vessel than that within the solution of reactants. Subsequently, some energy is used in heating the vessel rather than the solution. On the contrary, microwave energy is delivered directly into materials through molecular interaction with the electromagnetic field (in-core volumetric heating). Therefore, microwave irradiation heat up the whole volume simultaneously (bulk heating) rather than heat conduction. Since the reaction vessels used in microwave reactors are typically made out of (nearly) microwave-transparent materials, such as borosilicate glass, quartz, or teflon, an inverted and small temperature gradient results compared to conventional thermal heating (Figure 1.6)(Kappe, 2004).

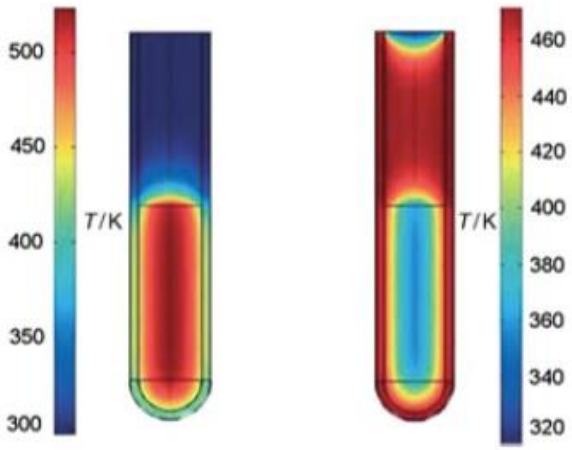


Figure 1.6 Inverted temperature gradients in microwave versus oil-bath heating: Difference in the temperature profiles (finite element modeling) after 1 min of microwave irradiation (left) and treatment in an oil-bath (right). Microwave irradiation raises the temperature of the whole volume simultaneously (bulk heating) whereas in the oil-heated tube, the reaction mixture in contact with the vessel wall is heated first.(Kappe, 2004)

1.4.2.3 Single and multimode microwaves

Common microwave reactors include single mode and multimode cavities. In a single mode microwave, the superposition of the incident and reflected waves gives a standing wave, which enables the sample to be placed in the position of maximum electric field for optimum conversion of the electromagnetic energy to heat (Figure 1.7a). However, single mode microwave can only work with small-scale samples and the maximum output power is limited to 300W, 400 W or 850W(Kappe & Stadler, 2006).

Multimode microwaves reactors are most widely used, comprising over 50% of industrial systems and almost all household microwaves. In a multimode system, a mode stirrer directs microwave irradiation into the cavity. The mode stirrer usually consists of a metal multi-blade fan and keeps rotating to distribute the microwave into the field continuously (Figure 1.7b). This results in regions of high and low electric fields, thus samples put into multimode microwave reactors need to be rotated to get

more uniform heating(Clark & Sutton, 1996). Compared with single mode microwave, it is difficult to heat up small individual samples (<3 mL) due to the low energy density in multimode microwaves(Kappe & Stadler, 2006) .

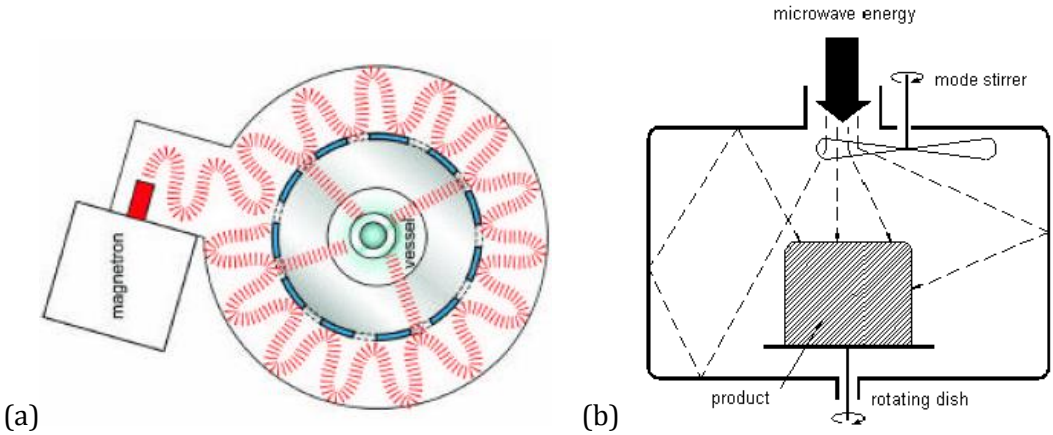


Figure 1.7 Schematic diagram of (a) single mode microwave (b) multimode microwave(Kuhnert, 2002)

1.4.3 Microwave treatment of biomass

1.4.3.1 Microwave assisted pyrolysis

Pyrolysis is the thermochemical decomposition of organic material in the absence of oxygen, producing solid (char), liquid (tar), gas fuels as well as valuable chemicals(Mokhtar *et al.*, 2012). Pyrolysis process was suggested to have five key elements: (1) biomass is heated, (2) volatiles evolve from the organics, and carbonization occurs, (3) outflow of hot volatiles, (4) volatiles condense to liquid (tar) with incondensable gas, and (5) autocatalytic secondary reactions (decomposition or repolymerisation) take place if long residence time is applied(Babu & Chaurasia, 2003).

Microwave assisted pyrolysis (MAP) has been growing in importance recently due to the advantages MAP offers compared with conventional pyrolysis (e.g. external heating by conduction, convection or radiation):

1. Controllability ---- uniform distribution of heat means it is easier to control the temperature, which means better control of the pyrolysis procedure and the final products. As can been seen in Figure 1.6, reactant is selectively heated by microwave heating and vessel is still cool, which means once microwave radiation stops, the reactant can be cooled down easily. While in conventional process, heating is conducted from vessel to reactant, therefore, when heating is turned off, heat will still be transferred from vessel to reactant due to the temperature gradient. More importantly, lower temperatures in MW cavity can help to avoid undesirable reactions in vapor and gas phase(Mushtaq *et al.*, 2014).

2. Time efficiency ---- dielectric heating is a rapid and efficient heating method, which can save lots of energy and time compared to the traditional conduction heating methods. Due to the special mechanism, microwave dielectric heating is very fast and efficient, however, microwave heating is restricted to microwave absorbing solvents and materials.

3. Energy efficiency ---- very fine feedstock grinding required by conventional pyrolysis is not necessary for microwave pyrolysis process, resulting in substantial energy savings(Mokhtar *et al.*, 2012). Moreover, microwave radiation selectively heat up reactant only rather than both reactant and reaction vessel, which also helps to save energy.

There are many factors affecting the behaviors of MAP, primarily including temperature, heating rate, residence time, microwave power, additives, and the original characteristics of feedstock(Motasemi & Afzal, 2013).

Many feedstocks have been investigated under microwave heating. Miura *et al.* investigated microwave pyrolysis of cylindrical wood blocks (diameter: 60–300 mm; weight: 80–12000 g) in a few minutes and demonstrated that temperature distribution, heat transfer and mass transfer are quite different from a conventional heating method(Miura *et al.*, 2004). While the conventional pyrolysis develops from the surface

to the centre, microwave assisted pyrolysis develops towards a direction from the centre to the surface, which can prevent the undesired secondary reactions that happens in conventional pyrolysis process when the prifmary volatiles produced near the centre must transfer through the high temperature surface region. Dominguez *et al.* found that microwave treatment of coffee hulls produces much higher H_2 and syngas (H_2+CO) contents than those obtained by conventional pyrolysis(Dominguez *et al.*, 2007). Du *et al.* investigated microwave-assisted pyrolysis of microalgae and indicated that compositions of bio-oil were mainly aliphatic hydrocarbons, aromatic hydrocarbons, phenols, long chain fatty acids and nitrogenated compounds, among which aliphatic hydrocarbons and aromatic hydrocarbons are highly desirable compounds as those in crude oil, gasoline and diesel(Du *et al.*, 2011).

There are many other feedstocks that have also been researched, such as rice straw, waste tea, corn stover, wheat straw etc.(Macquarrie *et al.*, 2012; Mokhtar *et al.*, 2012; Motasemi & Afzal, 2013). It is worth noting that Marin *et al.* investigated co-pyrolysis of wood biomass and synthetic polymers mixtures, which is a potential method for treatment (and upgrading) of biomass–plastic waste(Marin *et al.*, 2002).

1.4.3.2 Microwave assisted extraction

As one of the advanced extraction technologies, microwave assisted extraction (MAE) is gaining more attention due to its advantages, such as shorter extraction time, less use of solvent, moderately high recoveries and minimal extractive sample preparations(Garcia-Ayuso & de Castro, 1999, 2001). Microwave heating is a volumetrically distributed heat source, which is generated by ionic conduction of dissolved ions and dipole rotation of polar solvent. This rapid internal heating leads to the effective cell rupture, releasing the compounds into the solvent(Vazquez-Delfin *et al.*, 2014). Compared with conventional soxhlet extraction, which takes at least 1 h and sometimes 12-24 h and consumes a lot of solvent, microwave extraction takes around 30 min or less with less solvent(Routray & Orsat, 2012).

Solvent is one of the most important factors in an MAE process, as the solvent used must be able to absorb microwaves. The loss factors for some common solvents are shown in Table1.8. A solvent with a high tanδ is required to absorb microwave efficiently for rapid heating. Combining different solvent for selective extraction of different compounds can modify solvent properties(Routray & Orsat, 2012). Moreover, there have been intensive reports about solvent free MAE of essential oil from variety of biomass feedstocks(Lucchesi *et al.*, 2004; Ma *et al.*, 2012; X. L. Qi *et al.*, 2014).

Table1.8 Loss factors (tanδ) of different solvent; 2.45 GHz, 20 °C (Kappe, 2004)

Solvent	tanδ	Solvent	tanδ	Solvent	tanδ
Ethylene glycol	1.350	2-butanol	0.447	Chloroform	0.091
Ethanol	0.941	1,2-dichlorobenzene	0.280	Acetonitrile	0.062
DMSO	0.825	NMP	0.275	Ethyl acetate	0.059
2-propanol	0.799	Acetic acid	0.174	Acetone	0.054
Formic acid	0.722	DMF	0.161	Tetrahydrofuran	0.047
Methanol	0.659	1,2-dichloroethane	0.127	Dichloromethane	0.042
Nitrobenzene	0.589	Water	0.123	Toluene	0.040
1-butanol	0.571	Chlorobenzene	0.101	Hexane	0.020

MAE has many applications including the extraction of high-value compounds such as phytonutrients, nutraceutical and functional food ingredients, and pharma actives from biomass(Tripti Jain *et al.*, 2009). Green tea leaves have been extracted using microwave and the results showed that MAE was more effective than the conventional extraction methods (Soxhlet extraction and ultrasonic extraction) in terms of extraction time, extraction efficiency and the percentages of tea polyphenols or tea caffeine in extracts(Pan *et al.*, 2003). Bagherian *et al.*'s(Bagherian *et al.*, 2011) study showed that MAE could improve the yield and quantitative characteristics (degree of esterification and viscosity) of extracted pectin from grapefruits compared with ultrasonic extraction and water bath heating, and combination of ultrasonic (pretreatment) extraction and MAE gave better results than MAE.

1.4.3.3 Microwave assisted hydrothermal treatment

As one of the fastest and most efficient heating methods, microwave heating has been intensively applied for the hydrothermal treatment of biomass. It has been reported that microwave heating could promote the dilute acid(Yu *et al.*, 1996), dilute alkali(Zhu *et al.*, 2006) and solid acid(Wu *et al.*, 2010) catalysed hydrolysis of various lignocellulosic materials, usually resulting in high yield of reducing sugars in a short reaction time. Recently, Fan *et al.* demonstrated that microwave heating could successfully and efficiently convert crystalline cellulose into glucose with high yield and selectivity under catalyst free conditions(J. J. Fan *et al.*, 2013). Furthermore, Szabolcs *et al.* reported that polysaccharides of both plant and animal origin could be successfully converted to levulinic acid in the presence of sulfuric acid by using microwave heating. The yield of product was similar from microwave heating and conventional heating, while reaction time was significantly reduced by using microwave heating(Szabolcs *et al.*, 2013). And Yemis *et al.* demonstrated that microwave assisted acid-catalysed process provided highly efficient conversion of furfural from wheat straw, triticale straw and flax shives, giving yield of 48.4%, 45.7% and 72.1% (calculated from the pentose content available in the straw biomass), respectively(Yemis & Mazza, 2011). Microwave-assisted hydrothermal carbonization is an innovative approach to obtain carbonized lignocellulosic materials. It was reported that pine sawdust could be carbonised at 200 °C in acidic aqueous media by microwave heating(Guiotoku *et al.*, 2014).

1.4.3.4 Microwave assisted drying

Microwave assisted drying (MAD) is mainly used for drying food such as fruits, vegetables and meat, to preserve them for long periods. Currently, natural drying (drying in the shade) and hot air drying are still the most widely used methods due to their lower cost. However, natural drying has many disadvantages due to the inability to handle the large quantities and to achieve consistent quality standards. And major

drawbacks of hot air drying are low-energy efficiency and a lengthy drying time during the last stage of drying(Soysal, 2004).

There has been extensive research into microwave drying techniques, examining a broad spectrum of fruits and vegetables. Barba *et al.* compared MAD and hot air drying of banana samples, and results showed that MAD was faster, and dried samples were homogeneous in the water content and showed no radical profiles of water content, which is different from hot air dried samples. And also MAD process could save polyphenol total content, while it decreased during hot air drying. However, MAD decreased the reducing sugar content in the dried sample(Barba *et al.*, 2014). Yang *et al.* demonstrated that a combination of hot air drying followed by microwave-vacuum drying could shorten drying time and also greatly improved the retention of chlorophyll and ascorbic acid in the wild cabbage(Y. Y. Xu *et al.*, 2004). Furthermore, Fennell designed continuous microwave drying systems that can be more efficient and with higher throughputs than conventional air-blown systems to dry sweet sorghum bagasse biomass, suggesting that microwave assisted drying may be a viable future alternative for processing of biofuel feedstock materials(Fennell & Boldor, 2014).

1.4.4 Advantages and challenges of microwave processing

In summary, utilization of microwave as a heating source is gaining popularity due to its advantages like energy savings, reduced processing times and good performance in improving the properties of products(Singh *et al.*, 2015). Although microwave heating has been widely used in drying processes, the problems such as technical deficiencies and economic obstacle regarding microwave technology application in chemical industry need to be solved to make it a successful commercialization (Table 1.9).

Table1.9 Advantages and challenges of microwave processing (adjusted from (Clark & Sutton, 1996))

Advantages
● Rapid and selective heating ● Volumetric and uniform heating (due to deep energy penetration) ● Short processing times ● Increased yield and improved properties of products ● Synthesis of new materials
Challenges
● Unable to heat poorly microwave absorbing materials ● Controlling accelerated heating ● Equipment developing for industrial scale ● Reluctance to abandon proven technologies ● Economics (due to capital and training cost)

1.5 Aims and objectives

As biorefinery based on crop biomass is controversial due to its competing with world food supply, in recent years, algae biomass has been intensively investigated as a more sustainable feedstock for biorefinery process. However, compared with the mature technology and established industrial scale of crop biomass-based biorefinery, the algae-based biorefinery is in its infancy. This project developed the biorefinery concept using brown macroalgae *Ascophyllum nodosum* as an example.

Microwave heating is a fast and efficient method for hydrothermal treatment of biomass, and microwave heating has good performance on biomass extraction, hydrolysis and pyrolysis processes(J. J. Fan *et al.*, 2013; Macquarrie *et al.*, 2012; Rodriguez-Jasso *et al.*, 2011). Therefore, microwave technology was applied during the biorefinery process of *Ascophyllum nodosum.*

Specifically, the main objectives of this research are:

- Compositional analysis of the raw material *Ascophyllum nodosum.*
- Study the impact of different parameters on alginate extraction, and optimize the yield and properties of alginate obtained.
- Study the impact of different parameters on fucoidan extraction, and optimize the yield and properties of fucoidan obtained.
- Optimize the saccharification process of *Ascophyllum nodosum* for ethanol production.
- Develop a step-by-step process to produce alginate, fucoidan and fermentable sugars in a biorefinery way.

Chapter 2: Compositional Analysis of Brown Macroalgae *Ascophyllum nodosum*

Publication:

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2.1 Introduction to *Ascophyllum nodosum*

The brown macroalgae used in this work is *Ascophyllum nodosum*, in the order of Fucales (Figure 2.1). It is generally harvested in the wild and distributed around the coast of North Atlantic Ocean (Figure 2.2). *Ascophyllum nodosum* is one of the most economically important brown seaweeds(Hong *et al.*, 2007). It can be found over the coasts of Britain and Ireland. Around 32000 t of *Ascophyllum nodosum* is harvested per year for use in alginates, fertilisers and for the manufacture of seaweed meal for animal and human consumption(Hill & White, 2008). In addition, seaweed is well known to contain higher ash content than terrestrial biomass, which is a good aspect for using microwave extraction, as high ash content can contribute to ionic conduction mechanism and therefore to microwave activation.

Figure 2.1 Picture of *Ascophyllum nodosum*

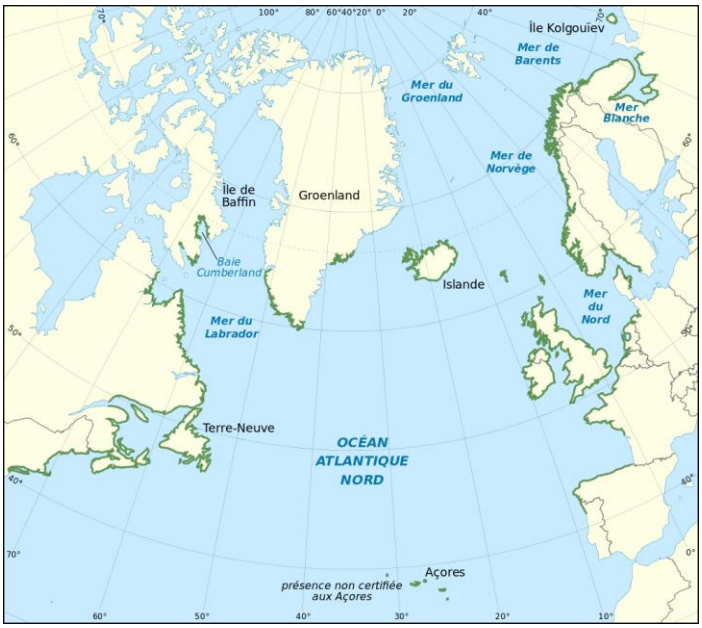


Figure 2.2 Distribution of *Ascophyllum nodosum* (in green area) (WikimediaCommons, 2011)

2.2 Chemical compositional analysis of *Ascophyllum nodosum*

The fresh seaweed with high water content was dried by microwave heating (described in Chapter 7) and stored at room temperature. Then the crude seaweed sample was analysed to determine moisture, protein, ash, lipid and phenolic content as described in Chapter 9. Carbohydrate content was determined by acid hydrolysis, as there is no official method for carbohydrate determination. The seaweed powder was initially treated by 2 M trifluoroacetic acid (TFA) for 2 h at 121 ^{o}C, then residual seaweed was treated by 72% sulfuric acid for 4 h at room temperature, followed by dilute acid (diluted down to 3.2% sulfuric acid) for 4 h at 120 ^{o}C. The sugar content was measured by phenol-H_2SO_4 method described in section 9.3.3.

Table 2.1 Composition of brown seaweed *Ascophyllum nodosum*

Component	Composition % (w/w)
Carbohydrate	44.66±2.1
Protein	5.24±0.22
Lipid	2.99±0.07
Ash	18.61±0.89
Moisture	13.48±0.32
Phenolic	1.4±0.21
Others	13.62

Table 2.1 shows the compositions of *Ascophyllum nodosum* used in this study. The carbohydrate content of this seaweed was 44.66% (w/w), which is within the range (40-60%) reported for brown seaweed(Borines *et al.*, 2013; J. S. Jang *et al.*, 2012; H. Kim *et al.*, 2013). However, it is lower than the carbohydrate content (50-70%) of most red seaweed reported(Chirapart *et al.*, 2014; Li *et al.*, 2014; F. C. Wu *et al.*, 2014). Wu *et al.*(F. C. Wu *et al.*, 2014) and Jang *et al.*(J. S. Jang *et al.*, 2012)reported even high content of carbohydrate 76.67% in *Gracilaria* sp and 74.4% in *Gelidum amansii*, respectively. The ashes in *Ascophyllum nodosum* was 18.61%, which is lower than the reported value 22.5% of same seaweed species(Rioux *et al.*, 2007), but is similar with *Fucus vesiculosus* that has ash content of 18.32%(Rodriguez-Jasso *et al.*, 2011). The differences in the chemical compositions of seaweed have been demonstrated to depend on species, maturity and various environmental factors such as water temperature, light and salinity(John *et al.*, 2011; Munier *et al.*, 2013). The other components were protein 5.24%, Lipid 2.99%, moisture 13.48%, phenolic 1.4% and others 13.62%, respectively.

2.3 Elemental analysis of *Ascophyllum nodosum*

Elemental analysis of *Ascophyllum nodosum* is shown in Table 2.2. As can be seen, C%, H% and N% of raw material is 36.26%, 4.86% and 0.84% respectively.

Table 2.2 Elemental analysis of *Ascophyllum nodosum*

CHN analysis (wt%)		Elemental analysis by ICP* (wt%)							
C	36.26	**Al**	**As**	**Ca**	**Cu**	**Fe**	**K**	**Mg**	**Mn**
H	4.86	0.015	0.006	0.520	0.002	0.077	0.386	0.619	0.009
N	0.84	**Na**	**P**	**Rb**	**S**	**Si**	**Sn**	**Sr**	**Zn**
		3.721	0.148	0.001	3.334	0.027	0.001	0.025	0.009

* There are some other elements such Ba, Cr, La, Ni, etc, which exist as very low concentration < 10ppm.

In comparison (Table 2.3), macroalgae are lower in C, H and O than terrestrial plants and higher in N and S(Ross *et al.*, 2008). It is well know that ash content of macroalgae is much higher than terrestrial plants due to the metal ion rich environment they live in. The elemental analysis by ICP indicates that major minerals present in this seaweed are Na (37210 ppm), S (33340 ppm), Mg (6187 ppm), Ca (5200 ppm), K (3865 ppm), P (1479 ppm).

Table 2.3 Comparison on elemental content of macroalgae and terrestrial plants (Ross *et al.*, 2008)

Materials	Ash content	C%	H%	N%	S%	O%
Oat straw	9.4	42.75	5.22	1.06	<0.2	38.71
Miscanthus	10.9	46.32	5.58	0.56	<0.2	41.79
Willow coppice	5.9	52.69	5.92	0.48	<0.2	41.90
Fucus vesiculosus	22.82	32.88	4.77	2.53	2.44	35.63
Ascophyllum nodosum (this work)	18.61	36.26	4.86	0.84	3.33	36.10*

*Estimated by difference [O=100-(C+H+N+S+Ash)]

Chapter 3: Microwave Assisted Extraction of Sulfated Polysaccharides (Fucoidan) from *Ascophyllum nodosum*

Publication:

Yuan, Y., & Macquarrie, D. (2015). Microwave assisted extraction of sulfated polysaccharides (fucoidan) from *Ascophyllum nodosum* and its antioxidant activity. *Carbohydrate Polymers,* 129, 101-107.

Conference presentation:

RRB-10 – "Important Chemical Products from Seaweed Biorefinery by Microwave Assisted Technology", June 2014, Valladolid, Spain.

3.1 Introduction

Fucoidan is a fucose rich polysaccharide in brown seaweed, which has been shown to have diverse biological activities including anticoagulant, antithrombotic, anti-inflammatory, antitumoral, contraceptive, antiviral and antioxidant(Feldman *et al.*, 1999; Jhamandas *et al.*, 2005; Nishino & Nagumo, 1992; Patankar *et al.*, 1993; Ponce *et al.*, 2003). The typical structure of fucoidan is shown in Figure 3.1. Fucoidan is traditionally extracted by using large volumes of aqueous or acidic solutions at temperatures from room temperature to 100 ^{o}C for several hours (Ale *et al.*, 2011; Hu *et al.*, 2010). Recently, microwave heating has been reported to present a potentially faster, more efficient and selective method for the thermal treatment of biomass(J. Fan *et al.*, 2013; Macquarrie *et al.*, 2012). Studies showed that microwave assisted extraction (MAE) was a promising method for extracting active ingredients from a variety of natural resources including seaweed(Bagherian *et al.*, 2011; Lucchesi *et al.*, 2004; Pan *et al.*, 2003; Shu *et al.*, 2003; Sousa *et al.*, 2010; Vazquez-Delfin *et al.*, 2014). However, little information is available on MAE of fucoidan, only one report by Rodriguez-Jasso *et al.*(Rodriguez-Jasso *et al.*, 2011) demonstrated that MAE was an effective method to recover fucoidan in a high yield (18.22%) from *F. vesiculosus,* but the key properties such as molecular weight and biological activities were not studied.

In this chapter, fucoidan was extracted from *Ascophyllum nodosum* by MAE technology. The influence of temperature and extraction time on fucoidan yield was studied. The molecular weight, chemical composition, and antioxidant activity of recovered fucoidan were also investigated.

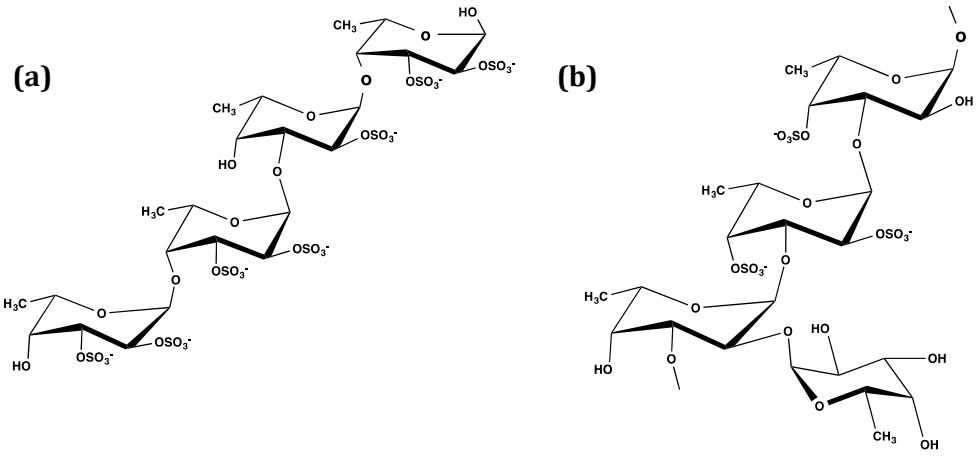


Figure 3.1 Typical structure of fucoidan from (a) *Ascophyllum nodosum*; (b) *Laminaria saccharina*: consisting primarily of α-(1→3)- and (1→4)-linked-L-fucose residues, that may be organized in stretches of (1→3)-α-fucan or of alternating α-(1→3)- and α-(1→4)-bonded L-fucose residues. The L-fucose residues may be substituted with sulfate (SO_3^-) on the oxygen of the C-2 or C-4 (rarely on C-3) position(Ale *et al.*, 2011).

3.2 Effect of time and temperature on extraction process

The yields of fucoidan after various extractions are shown in Table 3.1. The highest fucoidan yield (16.08%) was obtained after 120 ^oC for 15 min extraction. Temperature played an important role on the fucoidan yields. For 150 ^oC, yield decreased with extraction time while for 90 ^oC, yield kept increasing to 30 min extraction time. The yield of fucoidan was 20.98% from conventional method (3*3h at 70 ^oC), which was higher than MAE method. However, considering the extraction time, 15 min extraction of MAE is much shorter than 9-hour extraction. Moreover, the volume of solvent used for the MAE method (15 mL) was 3 times less than conventional method (3*15 mL).

Table 3.1 Fucoidan yields, alginate removed and residue left during the extraction processes

	Fucoidan yields (%)			Alginate removed (%)			Residue left (%)		
Extraction condition	**30 min**	**15 min**	**5 min**	**30 min**	**15 min**	**5 min**	**30 min**	**15 min**	**5 min**
150 °C	6.48±0.97	6.98±0.99	9.08+0.20	Trace	Trace	Trace	28.09±0.11	30.19±0.76	31.47±0.24
120 °C	14.02±0.28	16.08±0.24	14.49±0.96	0.60±0.07	0.64±0.08	0.60±0.04	36.21±0.12	38.32±0.98	41.29±0.78
90 °C	14.55±0.20	14.09±0.78	11.97±0.76	2.07±0.09	1.84±0.03	1.75±0.10	46.86±1.22	50.04±0.67	52.79±0.55
Conventional method	20.08±0.44			4.29±0.13			37.37±0.76		

The fucoidan yield of this work is much higher than the value ranging from 1 % - 8 %, reported for the same seaweed species *Ascophyllum nodosum* by other researchers(Foley *et al.*, 2011; Nakayasu *et al.*, 2009; Rioux *et al.*, 2007). This high yield may be due to the harvest time (October) of our seaweed materials, as fucoidan levels are highest towards the end of the main photosynthetic period(Rioux *et al.*, 2009). Fucoidan extracted from *Fucus vesiculosus* were reported to yield 18.22% crude fucoidan(Rodriguez-Jasso *et al.*, 2011), while sporophyll parts of *Undaria pinnatifida* produced remarkably high yields of fucoidan, about 57.3-69.9%(Mak *et al.*, 2013).

Little information is available about mass balance of the whole fucoidan extraction process. In this work, alginate was removed during the extraction process and the residues, which could be separated from fucoidan, were also studied. It can be seen that more alginate was removed at lower temperatures, which illustrates that high temperature may hydrolyse the alginate into uronic acid blocks; this is in accordance with the results of uronic acid content in supernatant from different extraction conditions (Figure 3.2). Chhatbar *et al.*'s research also demonstrated that alginate could be hydrolysed by microwave irradiation in the presence of low concentration acid(Chhatbar *et al.*, 2009). In terms of the residue left, the higher the temperature and the longer the extraction time were, the less the residue left. The yield of residue left from conventional method was comparable with those from MW 120 °C extraction

processes, around 38%.

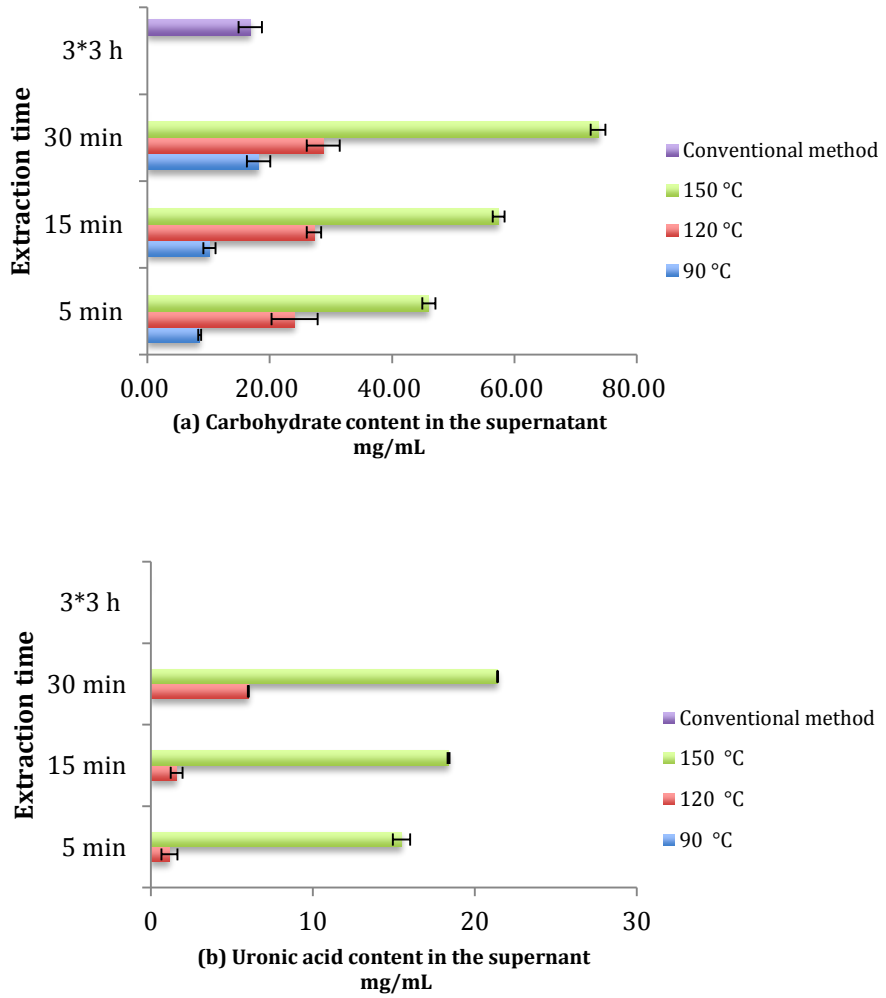


Figure 3.2 Carbohydrate content (a) and uronic acid content (b) in the supernatant after fucoidan precipitation

As the utilization of possible by-product can further optimize the economic value of the extraction process, the supernatant after fucoidan precipitation was concentrated to 5 ml and further analysed for carbohydrate content. Figure 3.2 shows that the supernatant has significantly high carbohydrate content for 120 °C and 150 °C, of which about one third is uronic acid. No uronic acid was detected for MAE at 90 °C and conventional method (70 °C), which indicates that high temperature can hydrolyse alginate into uronic acid. The carbohydrate content for 150 °C is 40-75 mg/ml, which might be used as sugar feedstock for the fermentation process(Hwang *et al.*, 2011).

3.3 Characterisation of extracted fucoidan

3.3.1 Chemical compositional analysis of extracted fucoidan

The chemical compositional analysis of extracted fucoidan was carried out by decomposition into monomers and is shown in Table 3.2. The monosaccharide composition indicates that glucuronic acid is the major component of fucoidan extracted at the highest temperature (150 °C) while fucose is the major component of fucoidan from 90 °C. This is in agreement with Ponce *et al.*(Ponce *et al.*, 2003), who extracted fucoidan from *Adenocystis utricularis* and demonstrated that higher temperatures would lead to a lower proportion of fucose and a higher proportion of glucuronic acid. Similar behavior was observed by Rodriguez *et al.* who also used microwave assisted extraction technology for fucoidan extraction from *F. vesiculosus*, and the results showed that fucose content decreased from 100% to 27% with extraction pressure decreasing from 120 psi to 30 psi(Rodriguez-Jasso *et al.*, 2011). Hence, the temperature and possibly pressure used for extraction has a strong influence on the fucoidan compositions. Similar to previous reported data, fucoidan from this work also contains other sugars like rhamnose, galactose, glucose, xylose and mannose (Ale *et al.*, 2012; Foley *et al.*, 2011; Mak *et al.*, 2013; J. Wang *et al.*, 2008).

Table 3.2 Chemical composition of extracted fucoidan

	Extraction temperature (°C)	Extraction time (min)	Sulphate (%)	Mw (kDa)	M_w/M_n	Monosaccharide composition (mol %)						
						Fuc	*Rha*	*Gal*	*Glc*	*Xyl*	*Man*	*GluA*
1	150	30	7.82	1.34	1.06	8.12	0.00	5.38	5.38	3.44	18.50	62.00
2		15	9.25	1.41	1.06	9.06	0.00	6.49	6.49	2.37	17.77	61.79
3		5	6.10	1.96	1.30	9.95	0.00	12.23	12.23	3.80	3.19	46.31
4	120	30	16.87	6.30	2.40	8.96	0.68	6.67	6.67	16.75	17.60	41.28
5		15	14.71	9.04	2.65	11.32	0.60	6.39	6.39	18.37	17.04	37.88
6		5	15.74	18.61	2.39	12.99	0.60	5.41	5.41	18.72	15.89	38.22
7	90	30	28.60	27.96	2.29	30.14	1.75	5.01	5.01	18.24	11.52	30.50
8		15	27.12	34.42	2.55	41.25	0.00	5.95	5.95	15.01	11.26	24.55
9		5	27.83	37.54	2.54	42.25	0.00	5.69	5.69	17.42	10.41	22.21
10	Conventional method	3*3 h	29.33	40.23	2.66	42.49	0.00	1.12	5.54	16.67	10.51	23.67

Sulfate content of extracted fucoidan was within the range of those reported, 16%-30%(Chizhov *et al.*, 1999; Cumashi *et al.*, 2007; Duarte *et al.*, 2001). Especially for the fucoidan extracted from 90 °C, it has a sulfate content around 28%, which is higher than the reported data of fucoidan from *Ascophyllum nodosum* (19% - 22%) (Foley *et al.*, 2011; Nakayasu *et al.*, 2009; Rioux *et al.*, 2007). This is an advantageous aspect since it has been reported that the increase of sulfate degree can increase the anti-HIV activity(Schaeffer & Krylov, 2000) and anticoagulant activity(Haroun-Bouhedja *et al.*, 2000). The fucoidan extracted from *Laminaria japonica* was reported to have sulfate content as high as 41.99% after fractionation of crude fucoidan that contained 30.14% sulfate content(J. Wang *et al.*, 2010). Sulfate content also plays an important role in antioxidant activities. It was suggested that sulfate group could activate the hydrogen atom of the anomeric carbon, thus contributing to the hydrogen-donating ability of the polysaccharides(J. Wang *et al.*, 2010). Fucoidan from *Undaria pinnitafida* was found to exhibit stronger antioxidant activities compared to its de-sulfated polysaccharides(Hu *et al.*, 2010). And furthermore, sulfated polysaccharide from red seaweed *Corollina officinalis* had more excellent antioxidant abilities than de-sulfated polysaccharides(Yang *et al.*, 2011). Results of this work also show that sulfate content of fucoidan decreases with increasing extraction temperature, and this trend was also reported by Ponce *et al.* (2003) and Ale *et al.* (2012). It is worth mentioning that fucoidan extracted from MW 90 °C within 30 min has very similar composition profile with the one extracted by conventional method for 3*3 h.

3.3.2 Molecular weight determination by gel permeation chromatography analysis

The molecular weight of extracted fucoidan was determined by gel permeation chromatography. Gel permeation chromatography (GPC) is a type of liquid chromatography “in which polymer molecules in solution are sorted according to their size in a column packed with a porous material”(Vankreve.Me & Vandenho.N, 1973).

GPC analysis can present the molar mass distribution, which describes the relationship between the number of moles of each polymer species (N_i) and the molar mass (M_i) of that species. The distribution can be characterized by the number average molar mass (M_n), mass average molar mass (M_w) as well as the polydispersity index PI. These parameters can be calculated by the equations below:

$$M_n = \frac{\sum_i N_i M_i}{\sum_i N_i}$$

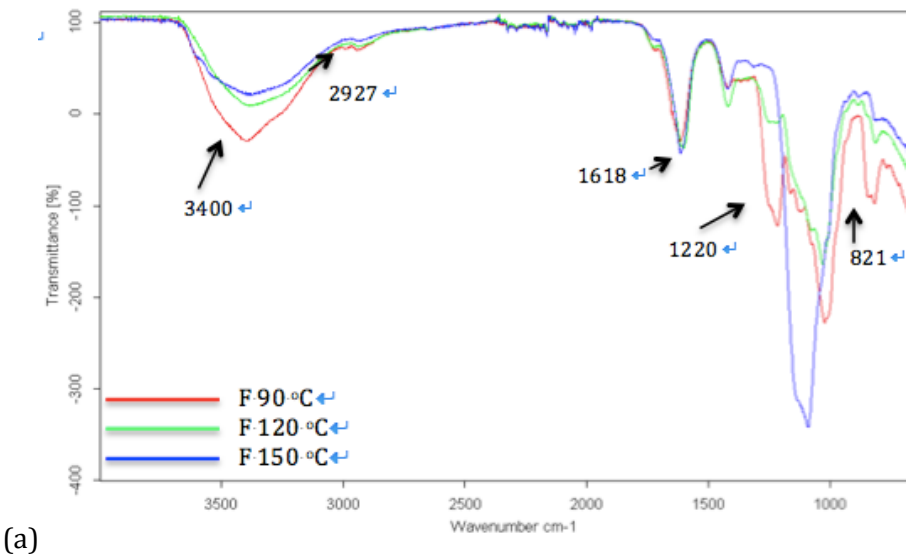
$$M_w = \frac{\sum_i N_i M_i^2}{\sum_i N_i M_i}$$

$$PI = \frac{M_w}{M_n}$$

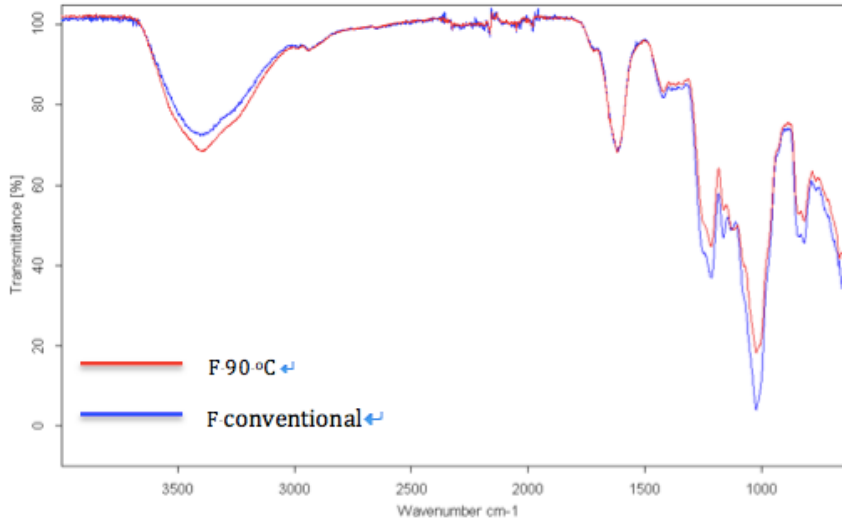
The molecular weight profiles determined by GPC suggest that fucoidan extracted in this work has molecular weight ranging from 1.4 kDa to 40 kDa, and the polydispersity was between 1.06-2.66 (Table 3.2). The molecular weight of fucoidan in this work was lower compared with reported data ranging from 40 – 1300 kDa for *Ascophyllum nodosum*(Foley *et al.*, 2011; Rioux *et al.*, 2007). The variation in M_w from the same species can be due to the different fucoidan extraction procedures(Zvyagintseva *et al.*, 2003). Foley *et al.* extracted fucoidan at 70 °C with water and two fucoidan fractions with molecular weight 47 kDa and 420 kDa were obtained. The polydispersity of fucoidan was around 4.6, which means a broader distribution of different size molecules than our work. Rioux *et al.* used 0.01 M HCl at 70 °C to extract fucoidan and molecular weight was 1323 kDa; the polydispersity of fucoidan was unknown. In this work, 0.1 M HCl was used for the extraction process and high acidic solution may hydrolyse the ether bonds on the polymeric chain(Gustavo Herna´ndez-Carmona, 1999), resulting in the low molecular weight fucoidan.

3.3.3 FT-IR analysis of extracted fucoidan

Extracted fucoidans were further analysed by infrared (Figure 3.3). The bands around 3400 and 2927 cm^{-1} were due to O-H stretching and C-H stretching vibration, respectively. The absorption at around 1618 cm^{-1} was assigned to carbonyl groups of uronic acid. The signal around 1220-1230 cm^{-1} was attributed to the asymmetric stretching of S=O and suggesting the presence of sulfate ester. The band around 1000-1100 cm^{-1} was assigned to C-O vibrations of polysaccharides(J. Wang *et al.*, 2010). It can be observed that there was some shift of C-O absorption (around 1000-1100 cm^{-1}) of fucoidan from 150 ^{o}C, this might be because the fucoidan from 150 ^{o}C has short chain with high polarity, which may lead to the shift of C-O band to higher frequency; or this shift may be due to the high glucuronic acid content in the fucoidan, which can make the molecular polar. It could also be due to the loss of sulfate, which should have an impact on the C-O bond to which it is attached. However, it is not clearly known. It is reported that absorption at 840 cm^{-1} was due to sulfate groups at the axial C-4 position and sulfate groups at equatorial C-2 and C-3 positions gave band around 820 cm^{-1}(Duarte *et al.*, 2001; Foley *et al.*, 2011; Marais & Joseleau, 2001; Rodriguez-Jasso *et al.*, 2011). It is also observed in the infrared spectrum that fucoidan extracted from 90 ^{o}C has stronger absorption around 1220 and 820 cm^{-1} than those from higher temperature, which indicates that sulfate content degree of fucoidan decreases with extraction temperature. This result was well agreed with the sulfate degree determined by $BaCl_2$-gelatin turbidimetric method. Figure 3.3b shows that fucoidan from MW 90 ^{o}C has very similar IR spectrum with that extracted from conventional method.



(a)



(b)

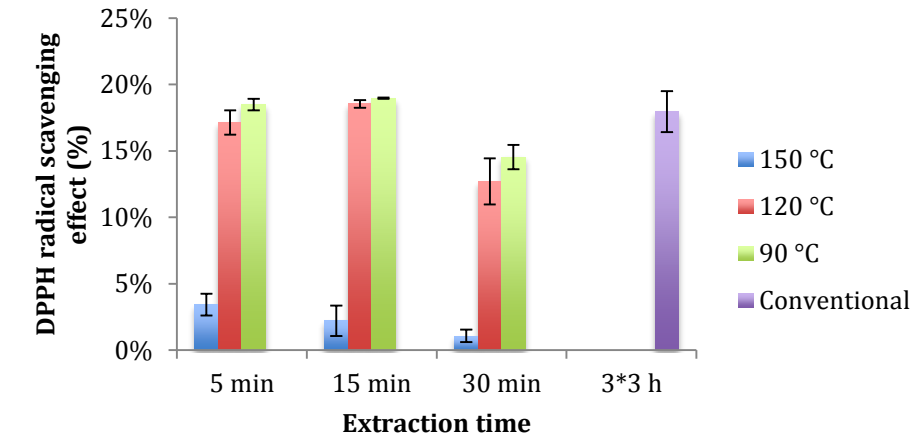
Figure 3.3 Infrared analysis of fucoidans from *Ascophyllum nodosum* scanned between 4000 and 400 cm^{-1}. (a) Fucoidans from different temperature (b) Fucoidans from MW 90 °C and conventional method.

3.4 Antioxidant activities of extracted fucoidan

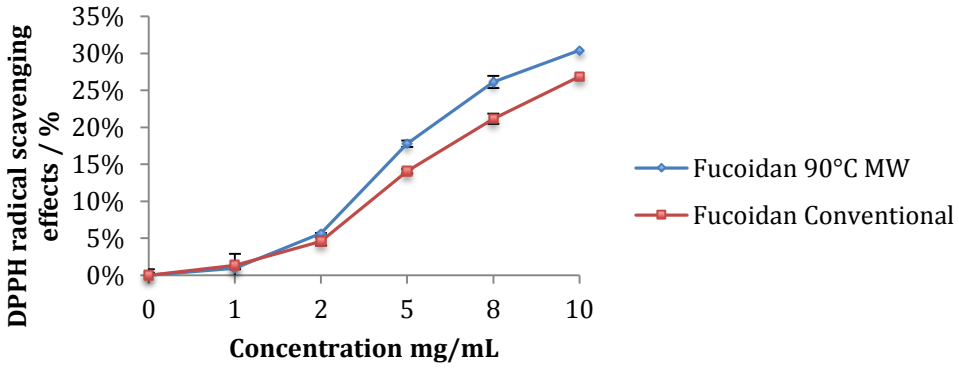
3.4.1 Scavenging activity on DPPH radical

DPPH is a stable free radical that has been widely used to determine the free radical-scavenging activities of antioxidants(Hu *et al.*, 2010). DPPH free radical shows a characteristic absorption at 517 nm (purple), and when it is reduced by antioxidants to

the non-radical form DPPH-H, the purple colour fades rapidly(Yang *et al.*, 2011). In this work, antioxidant activity of fucoidan was investigated by this DPPH scavenging assay (Figure 3.4). The results show that fucoidan extracted from 150 °C has much lower scavenging effects than those from lower temperature, and fucoidan extracted from 90 °C and 120 °C for 15 min have similar scavenging effects compared with those extracted by conventional method, about 18%. This value is similar with the reported literatures in which fucoidan was extracted from *Sargassum vulgare*(Dore *et al.*, 2013) and *Sargassum pallidum*(Ye *et al.*, 2008), with inhibition percentages of 22% (at 3 mg/mL) and 19.1% (at 3.8 mg/mL) respectively. However, compared with ascorbic acid, which is a well-known antioxidant reagent and has an inhibition of 95.65% at the same concentration, the scavenging effects of fucoidans on DPPH radicals were all relatively lower. Only Fitton *et al.* reported a fucoidan that had a stronger antioxidant activity than ascorbic acid, but details of species of seaweed used and extraction method were unknown(Fitton *et al.*, 2007). The scavenging ability of samples from MW 90 °C and conventional method was also tested at different concentrations between 1-10 mg/mL, and it is concentration related for both fucoidan samples (Figure 3.4b). At a concentration of 10 mg/mL, fucoidan extracted from MW 90 °C has a slightly higher scavenging ability (30.44%) than that extracted by conventional method (26.87%).



(a)



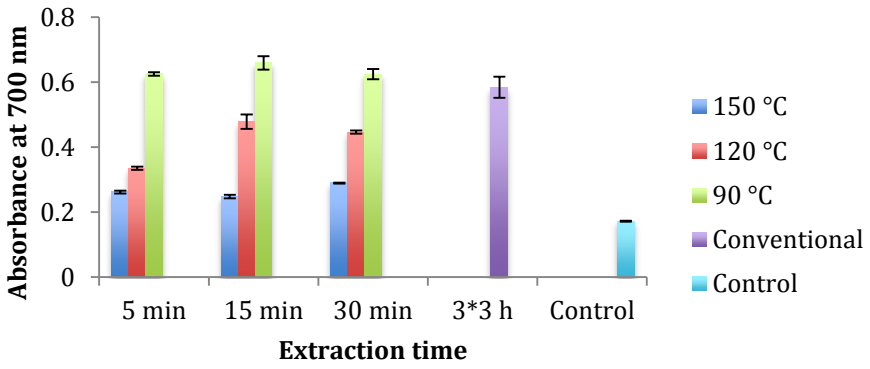
(b)

Figure 3.4 Scavenging effects of extracted fucoidan on DPPH free radicals. (a) Fucoidan extracted from different conditions, sample concentration is 3 mg/ml (b) Different concentrations of fucoidan from MW 90 °C and conventional method. Values are means from three independent tests.

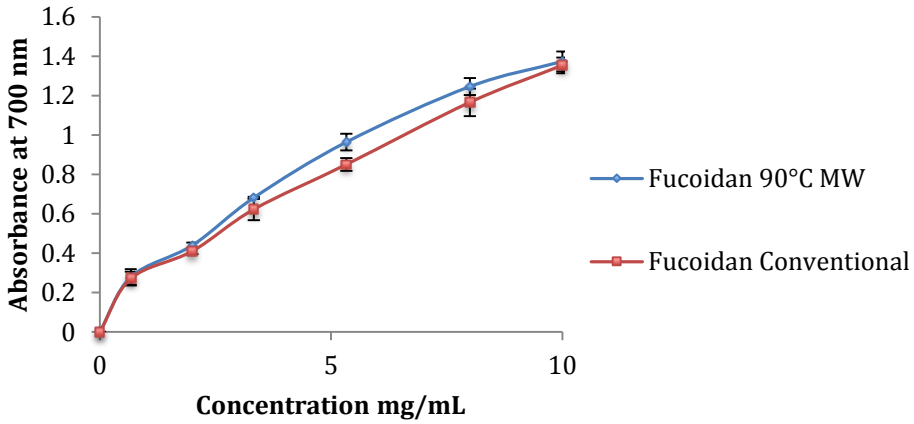
3.4.2 Reducing power assay

The antioxidant activity of substances has been reported to have a positive correlation with the reducing power(Abu *et al.*, 2013). In this assay, the yellow colour of test solution would change into green and blue colours when the reductant in the test sample reduces Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}). As shown in Figure 3.5, the reducing power of fucoidan increases with the decrease of extraction temperature. This result might suggest the relation of reducing power to sulfate content and the molecular weight, which is the higher the sulfate content and

molecular weight, the higher the reducing power of fucoidan. This is in agreement with results of Yang *et al.*(Yang *et al.*, 2011) and Hu *et al.*(Hu *et al.*, 2010) who demonstrated that the antioxidant activities have certain relationship with the degree of sulfate. And Ponce *et al.*(Ponce *et al.*, 2003) also stated that both high molecular weight and sulfate content are necessary for bioactivity. However, analysis of polysaccharides prepared from *Ulva pertusa* showed that lower molecular weight fraction had stronger reducing power due to higher number of reducing and non-reducing ends existing(H. M. Qi *et al.*, 2005). This is in contrast with our results, as fucoidan from 150 °C had low chain length with more reducing ends, but it inhibited low antioxidant activity, therefore, it can be concluded from this work that it is the sulfate degree rather than the reducing end contributing to the antioxidant activity. The reducing power of fucoidans extracted from MW 90 °C and conventional method was also tested at different concentrations between 1-10 mg/mL, and it is concentrated related for both fucoidan samples (Figure 3.5b). At a concentration of 10 mg/mL, fucoidan extracted from MW 90 °C has a similar reducing power (1.37) than that extracted by conventional method (1.35).



(a)



(b)

Figure 3.5 Reducing power assay of extracted fucoidan. (a) Fucoidan extracted from different conditions, sample concentration is 3 mg/ml (b) Different concentrations of fucoidan from MW 90 °C and conventional method. Values are means from three independent tests.

3.5 Conclusion and future work

The results obtained in the present study demonstrate that fucoidans could be successfully extracted from *Ascophyllum nodosum* by microwave assisted extraction technology in a much faster process compared with conventional extraction method. The characterisation of fucoidans shows that fucoidan recovered from MW 90 °C has similar composition and molecular weight with that extracted by conventional method. Moreover, antioxidant tests show that fucoidan recovered from MW 90 °C has similar DPPH scavenging effects and even higher reducing power than that extracted by conventional method. These results support the utility of microwave assisted

extraction technology for an efficient extraction method for fucoidan extraction.

However, the relation between fucoidan structure and antioxidant activities is not clearly known, thus further work will be necessary to unravel the precise mechanism. Other bioactivities such as anticoagulant, antithrombotic, antiinflammatory, antitumoral etc. maybe tested for the extracted fucoidan.

Chapter 4： Microwave Assisted Extraction of Alginate from *Ascophyllum nodosum*

Conference presentation:

RRB-10 – “Important Chemical Products from Seaweed Biorefinery by Microwave Assisted Technology” June, 2014, Valladolid, Spain.

4.1 Introduction

Alginic acid is a family of linear copolymers of (1 → 4) –β-d-mannuronic acid (M) and (1 → 4) a-l-guluronic acid (G) units. Figure 4.1 shows the structure of its sodium form. The chemical composition and sequence of M and G units depend on the biological source, growth, and stationary conditions.(Chee *et al.*, 2011) Alginates are widely used in the pharmaceutical, cosmetic, food and biotechnology industries(Hernandez-Carmona *et al.*, 1999) to produce products such as paper coatings, adhesives, dyes, gels and explosives. In addition, naturally occurring biopolymers extracted from algae have been known to exhibit excellent adsorption ability for metal ions,(Pathak *et al.*, 2008) thus it can be used in environmental technology such as the recovery of valuable metals and the removal of toxic metals.(Chen *et al.*, 1993) (Deans & Dixon, 1992) (L. K. Jang *et al.*, 1991)

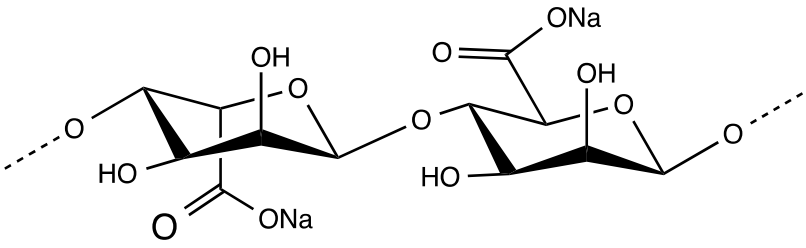


Figure 4.1 The chemical structure of alginic acid sodium salt

A lot of work has been done to investigate the reaction conditions that could impact the extraction process, such as acid pretreatment, extraction temperature, extraction time, concentration of alkaline solution, methods of separation and purification as well as different seaweed species, etc.(Chee *et al.*, 2011) (Nishide *et al.*, 1987) (Gustavo Herna´ndez-Carmona, 1999) (Hernandez-Carmona *et al.*, 1999) (D. J. McHugh *et al.*, 2001) (Hernandez-Carmona *et al.*, 2002) (Gomez *et al.*, 2009). The alginate industry extraction protocol is divided into five steps: acidification, alkaline extraction, solid/liquid separation, precipitation, and drying(Vauchel *et al.*, 2008). Briefly, acid pre-treatment is to eliminate polyvalent cations, making the alginate more readily

soluble in an alkaline solution. It is worth mentioning that acid will destroy acetal bond, causing lower molecular weight, thus this pretreatment step could be used to control the properties of final product. The purpose of the extraction step is to convert the alginate to the soluble form of sodium alginate and remove it from the algae. After separation, alginate could be precipitated out from sodium alginate solution by several different methods.(Gomez *et al.*, 2009) Finally, the product is dried. As described above, extraction of alginate by conventional heating has been investigated thoroughly; however, little information is available about MAE of alginate. In this chapter, *Ascophyllum nodosum* will be used to extract sodium alginate by microwave heating.

4.2 Open vessel system extraction

4.2.1 Influence of difference particle size on extraction processes

In this section, three different particle sizes (<1 mm, 1-2 mm, 2-2.8 mm) were used to investigate the influence of different particle size on the yield and molecular weight distribution of extracted alginate, pre-extraction fractions were analysed as well.

4.2.1.1 Analysis of pretreatment fraction from different particle size seaweed

Seaweed was pretreated by dilute HCl (0.1 M) as described in section 9.2.2.1. The pretreated liquid of three different particle size seaweed was concentrated to 5 ml by rotary evaporation for determination of carbohydrate and uronic acid content and then dried in an oven at 50 ^{o}C for 48 h to get dry weight.

Table 4.1 shows the mass balance of the pretreatment process. It can be seen that seaweed with smaller particle size had more weight loss during the pretreatment step. The weight loss for <1 mm, 1-2 mm and 2-2.8 mm are 27.38%, 20.84% and 14.66%, receptively. There is around 10% of mass difference, which is due to the sand that was left on the filter paper during the filtration.

Table 4.1 Effect of particle size on pretreatment step

Particle size	Soluble component (wt.%)	Seaweed residue (wt.%)	Mass difference (wt.%)
<1 mm	27.38%	62.60%	10.02%
1-2 mm	20.84%	70.65%	8.51%
2-2.8 mm	14.66%	76.64%	8.70%

Figure 4.2 shows the carbohydrate content and uronic content in the 5 ml concentrated pre-treated fraction. The loss of carbohydrate content in this fraction is more likely due to the extraction of water-soluble short polysaccharides and monosugars. Obviously, the chain length of polysaccharides could be affected when seaweed was cut into small pieces and also smaller particle size has larger surface area, therefore, some monosaccharides and oligosaccharides are easily extracted during pretreatment step. It is observed that pretreatment fraction from <1 mm seaweed has highest carbohydrate (20.36 mg/mL) and uronic content (14.35 mg/mL) while fraction from 2-2.8 mm seaweed has lowest content. This indicates that smaller particle size seaweed will have higher carbohydrate content loss during the pretreatment step.

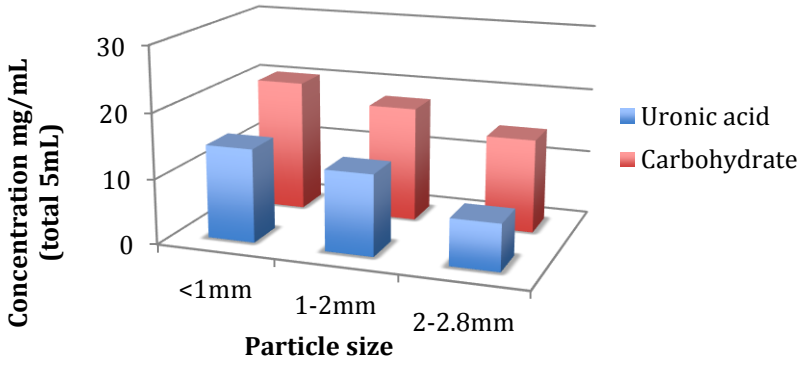


Figure 4.2 Effect of particle size on carbohydrate and uronic acid content of pretreatment fractions

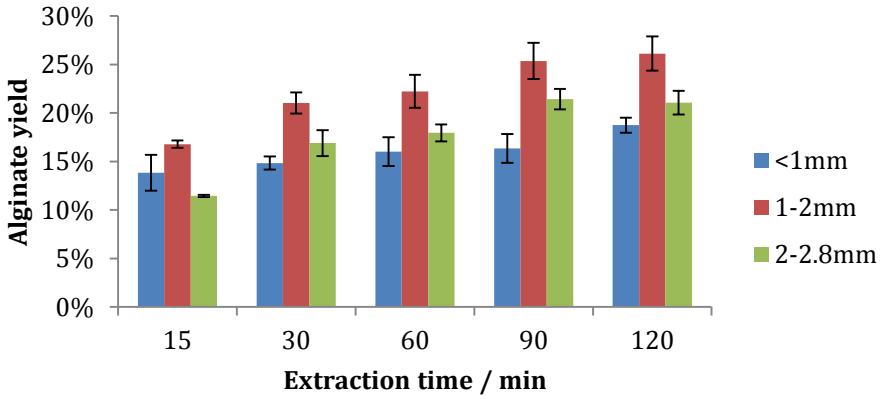
The liquid pretreatment fractions were dried for further analysis. The seaweed pretreatment dry material and raw seaweed was analysed by ICP (Table 4.2). In comparison, large amount of inorganics were exchanged out by HCl, especially K, Na and S. Besides the elements shown in the table, some other elements also appeared in small amount below 100 ppm, such as Ba, Cu, Mn, Rb, etc.

Table 4.2 Elemental analyses of dry pretreatment fractions and raw seaweed

Pretreated fractions	Element (ppm)										
	Al	**As**	**Ca**	**Fe**	**K**	**Mg**	**Na**	**P**	**S**	**Si**	**Zn**
< 1mm	221.4	120.8	5643.8	456.6	10325.4	8876.5	112546	2087.8	65788	657.7	123.7
1-2 mm	201.3	130.2	4394.9	552.2	9488.9	7336.7	102563	1848.1	51885	535.7	102.9
2-2.8 mm	198.4	67.87	4234.5	456.7	7685.4	6989.6	78658	1546.7	4354.7	356.7	67.6
Raw seaweed	147.1	61.2	5200.9	769.2	3864.7	6187.1	37210	1478.9	33340	270.9	86.2

4.2.1.2 Influence of particle size on alginate yield

Seaweed with different particle size (< 1 mm, 1-2 mm, 2-2.8 mm) was used for extraction process under 60 ^{o}C for 15-120 min. According to Figure 4.3, highest yield was achieved by 1-2 mm seaweed, followed by particle size 2-2.8 mm and < 1 mm. For all three cases, alginate yield was still increasing in the 120 min extraction. In terms of the residue yield, <1mm seaweed also yielded least, followed by 1-2 mm and 2-2.8 mm. The low yields of alginate and residue from <1 mm seaweed maybe because of the high carbohydrate content loss during the pretreatment step.



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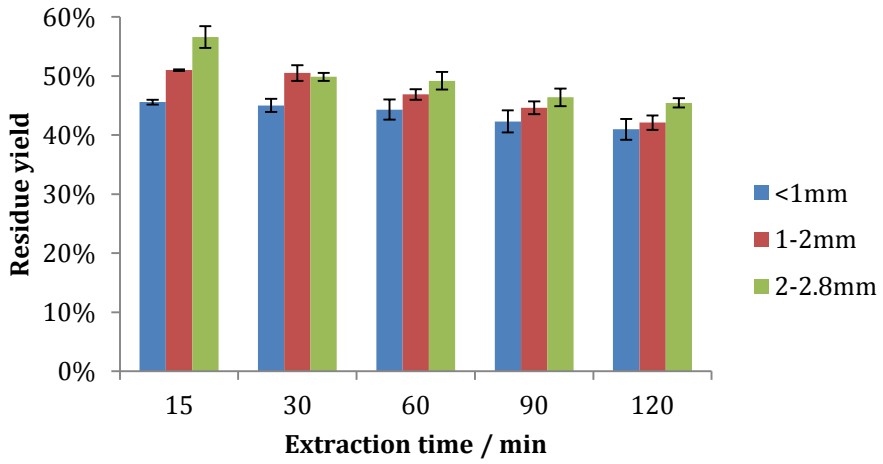


(b)

Figure 4.3 (a) Effect of seaweed particle size on alginate yield (b) Effect of particle size on residue yield

4.2.1.3 Molecular weight determination of alginate

The molecular weight of alginate was analysed by GPC as descried in section 9.3.9, and the weight average molecular weight (Mw) of alginate is shown in Figure 4.4. It can be seen that the highest molecular weight alginate was obtained in 60 min for all particle sizes, and longer extraction time would lead to the decrease of molecular weight. As shown in Figure 4.4, the molecular weight patterns for 1-2 mm and 2-2.8 mm are quite similar. The molecular weight increased dramatically from 15 min extraction (120-140 kDa) to 60 min extraction (200-210 kDa), and then decreased for 120 min extraction (170-180 kDa). In comparison, the molecular weight of alginate from <1 mm didn't have a obvious change during the whole extraction process. This indicates that 1 mm

particle size is probably a critical point for the high molecular weight alginate, and <1 mm seaweed particles only contain short chain alginate. Therefore, seaweed <1mm is not a good option to get high molecular weight alginates. The polydispersity index (PI) of alginate from three different particle sizes was similar, between 2.9-3.9 (Table 4.3).

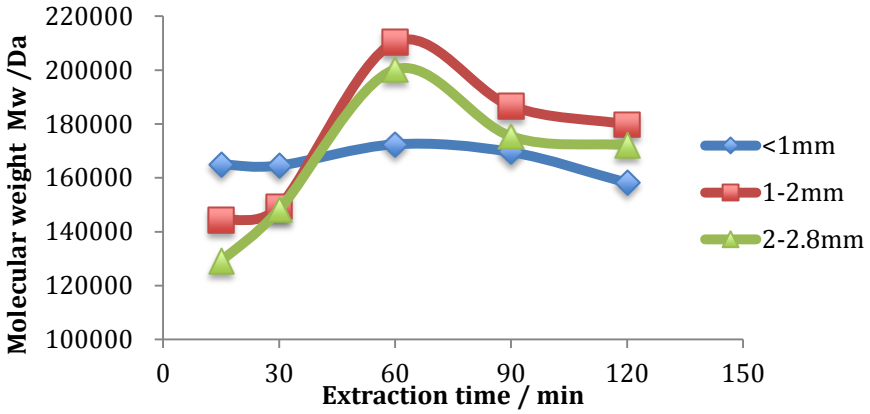


Figure 4.4 Effect of seaweed particle size on molecular weight of alginate

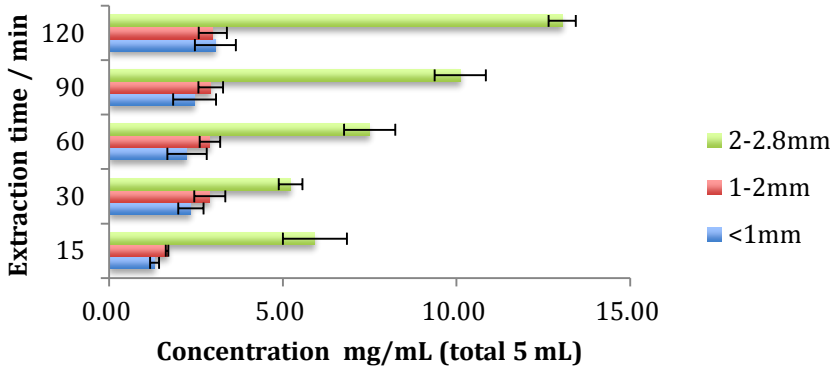
Table 4.3 The polydispersity index of alginate from different particle size seaweed

	15 min	30 min	60 min	90 min	120 min
<1 mm	3.42	3.45	3.57	3.47	3.13
1-2 mm	3.20	3.11	2.91	3.30	3.35
2-2.8 mm	3.35	3.42	3.73	2.98	3.30

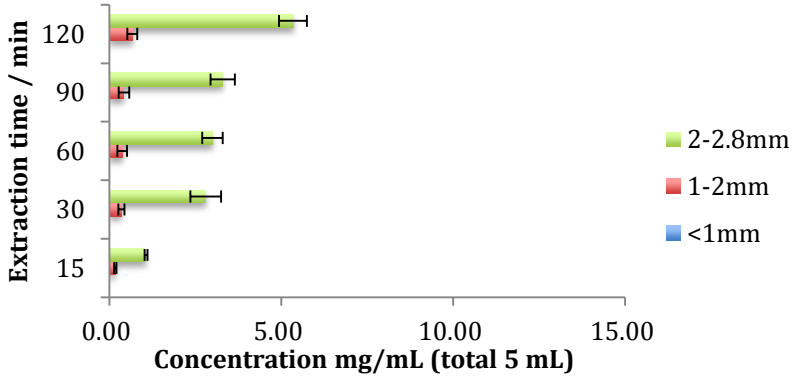
4.2.1.4 Analysis of supernatant after alginate precipitation

Figure 4.5 shows total carbohydrate and uronic acid content in the supernatant after alginates precipitation. 2-2.8 mm supernatant has higher carbohydrate and uronic acid than the other two. It is supposed that, during the pretreatment step, the

monosaccharides and oligosaccharides in smaller particle size seaweed, which has higher surface area, were easily extracted out, while after microwave extraction process, the structure of 2-2.8mm seaweed was more opened, so the monosaccharides and oligosaccharides were released during the extraction process.



(a)



(b)

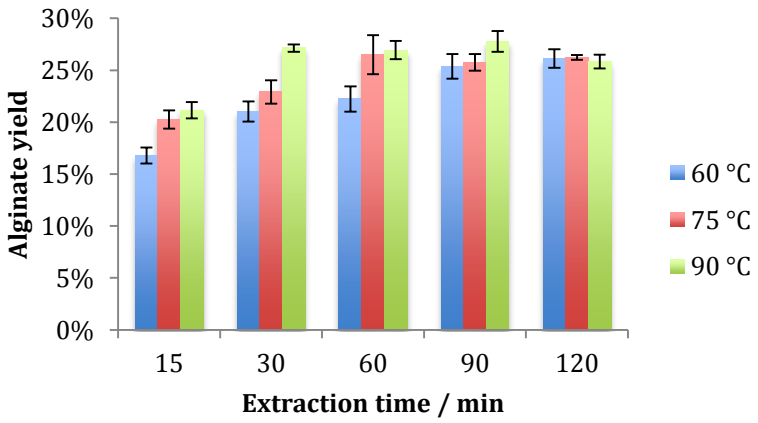
Figure 4.5 (a) Carbohydrate content in the supernatant after alginates precipitation (b) Uronic acid content in the supernatant after alginates precipitation

4.2.2 Influence of temperature on extraction processes

As seaweed with particle size 1-2 mm had highest alginate yield (section 4.2.1.2), it was used for this section. Alginate is typically extracted by aqueous alkali at temperature ranging from room temperature to 100 °C(Chee *et al.*, 2011; Fenoradosoa *et al.*, 2010; Gomez *et al.*, 2009). In this section, extraction processes were done at different temperatures (60 °C, 75 °C, 90 °C) by microwave heating for 15-120 min.

4.2.2.1 Influence of temperature on alginate yield

Figure 4.6 shows that the higher the temperature was, the less time was needed to get a high yield. For 60 °C, 75 °C, 90 °C, highest yields were obtained in 2 h, 1 h, 0.5 h respectively. Hernandez-Carmona *et al.* studied the effect of temperature (70, 80, 90 °C) and time on alginate extraction from *Macrocystis pyrifera* at pilot plant scale (10 kg seaweed in 166 L alkali solution), and demonstrated that alginate yield increased with temperature and time, maximum yield was obtained around 3.5 h treatment ranging from 19.4% at 70 °C to 21.9% at 90 °C(Hernandez-Carmona *et al.*, 1999). In terms of residue left, the higher the temperature was, and the longer the extraction time was, the less the residue left. It is worth mentioning that very low power (<10 W) was used to keep the temperature. In the case of 60 °C, 75 °C and 90 °C, power needed for keeping the temperature were 1-3 W, 2-5 W and 5-8 W, respectively.



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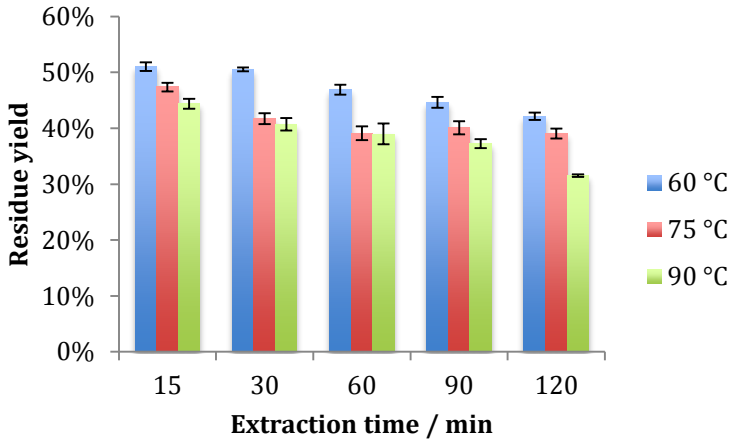


(b)

Figure 4.6 (a) Effect of temperature on alginate yield (b) Effect of temperature on residue yield

4.2.2.2 Molecular weight determination of alginate

The effect of temperature (60 °C, 75 °C, 90 °C) on molecular weight of alginate is shown in Figure 4.7. The highest molecular weight for all temperatures was obtained within 1 hour. It also can be observed that molecular weight increases with temperature within 1 hour extraction time, which indicates that high temperature helps to open the structure of seaweed in less time, thus releasing large molecules. However, long extraction time for all temperatures will lead to a decrease of molecular weight, probably due to chain scission. The molecular weight (Mw) in this work is in line with Rioux *et al.*(Rioux *et al.*, 2007) and Fourest *et al.*'s(Fourest & Volesky, 1997) research who obtained molecular weight of 177.3 kDa and 132 kDa for the same seaweed species *Ascophyllum nodosum*. The polydispersity index of alginate from different temperature falls in range 2.8-3.6 (Table 4.4).

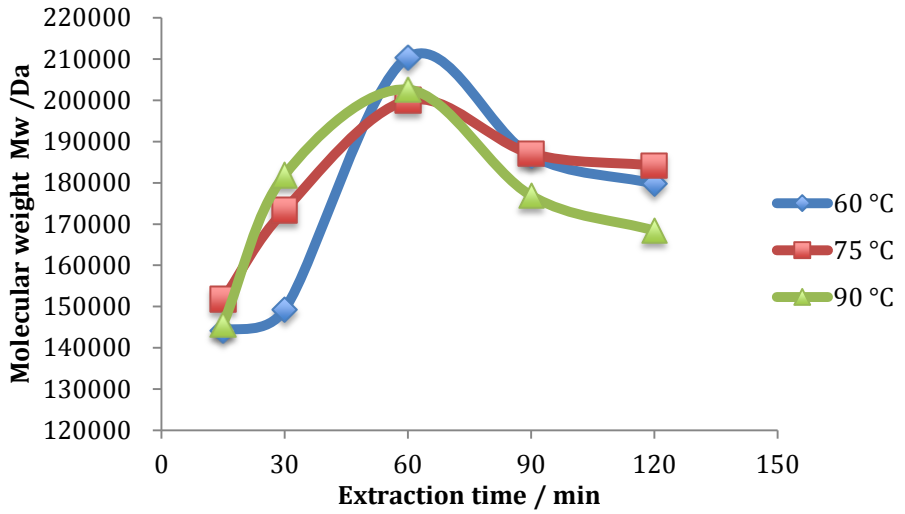


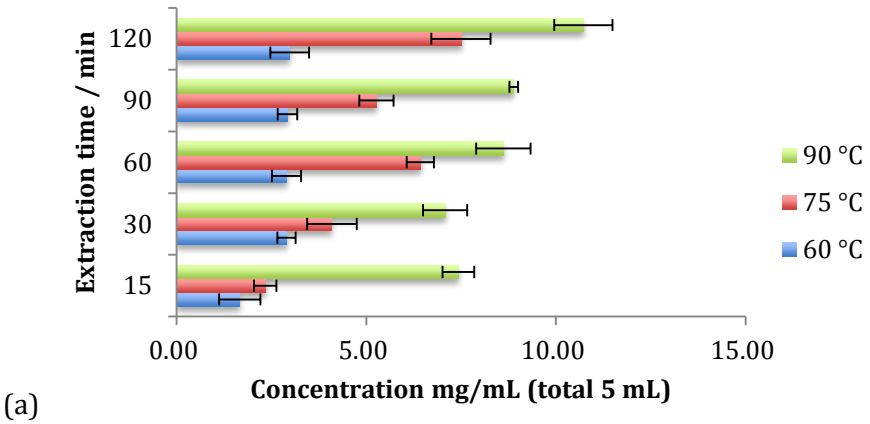
Figure 4.7 Effect of different temperature on molecular weight of alginate

Table 4.4 Polydispersity index of alginate extracted from different temperature

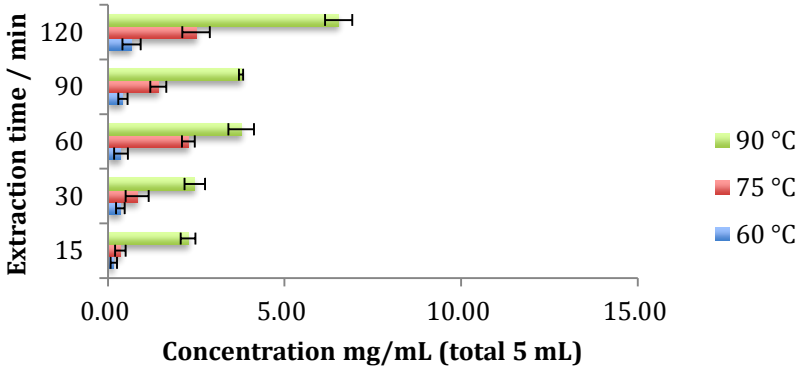
	15 min	30 min	60 min	90 min	120 min
60 °C	3.20	3.11	2.91	3.30	3.35
75 °C	3.52	3.59	2.96	3.10	2.99
90 °C	3.29	3.22	3.06	2.86	2.99

4.2.2.3 Analysis of supernatant after alginate precipitation

The supernatant after alginate precipitation was concentrated to 5 mL and analysed for carbohydrate and uronic acid content (Figure 4.8). It can be seen that the content of carbohydrate and uronic acid in the supernatant is increasing with the extraction time and temperature. It may be because that high temperature and long extraction time will help to open the structure of seaweed cells as well as to break the acetal bond of polysaccharide. As a consequence, more monosaccharides and oligosaccharides were released into the solution.



(a)



(b)

Figure 4.8 (a) Effect of temperature on carbohydrate content in the supernatant after alginates precipitation (b) Effect of temperature on uronic acid content in the supernatant after alginates precipitation

4.3 Closed vessel system extraction

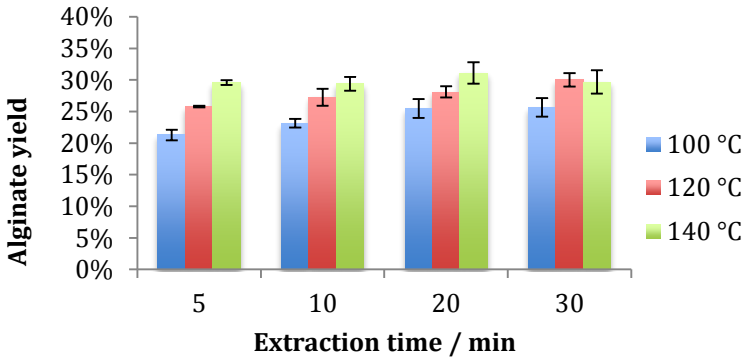
To investigate whether higher temperature and microwave heating can optimize the yield and properties of the alginate in short extraction time, extraction processes were done at different temperature (100 °C, 120 °C, 140 °C) in closed vessel system. Seaweed with particle size of 1-2 mm was used for this section.

4.3.1 Influence of temperature on alginate yield

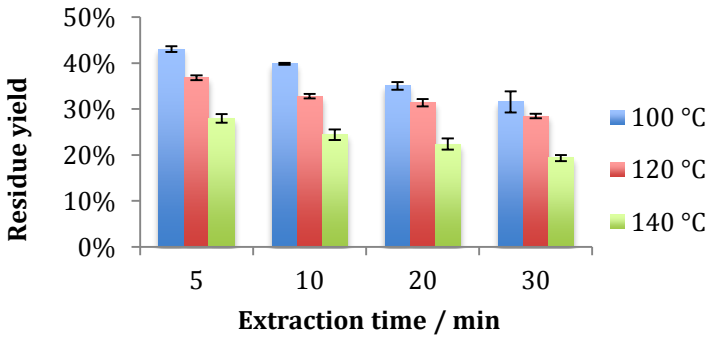
Similar with low temperature (<100 °C) extraction process, Figure 4.9 shows that for 100 °C and 120 °C, alginate yield increased with temperature and extraction time within 30 min. However, when 140 °C was applied, extraction yield increased up to 20

min and then started to decrease, which indicates that under such a high temperature, alginates begin to degrade. It is worth mentioning that in closed system, pressure was generated during the heating process (Figure 4.10), and this pressure may also help to open the seaweed structure for alginate release as well as to break the alginate polysaccharide chain. Rodriguez-Jasso *et al.*'s research (Rodriguez-Jasso *et al.*, 2011) on microwave-assisted extraction of fucoidan demonstrated that fucoidan yield was improved when pressure was increased, however, high pressure with longer reaction time had significant negative effect for fucoidan yield. This is in agreement with our results.

Compared with low temperature extraction process, the yield of alginate was slightly higher and could be obtained in much shorter time when high temperature was applied. In terms of residue, while residue left from open vessel (60-90 °C) was between 31-51%, residue left from closed vessel was about 20%-43%.



(a)



(b)

Figure 4.9 (a) Effect of temperature on alginate yield (b) Effect of temperature on residue yield

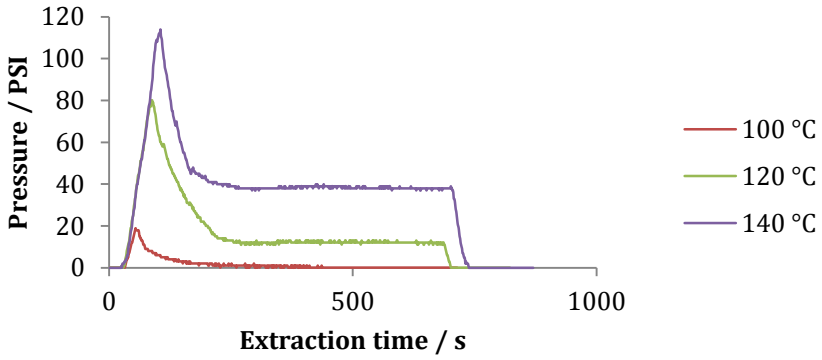


Figure 4.10 Pressure profile of extraction process under different temperature for 10 min

4.3.2 Molecular weight determination of alginate

According to GPC results (Figure 4.11), alginate molecular weight was increasing to 30 min extraction time under 100 °C, remaining stable after 20 min extraction under 120 °C, however, the molecular weight decreased with the extraction time at 140 °C. Furthermore, the polydispersity index of alginate extracted at 140 °C for 20 - 30 min was much higher than those from lower temperature (Table 4.5). This indicates that high temperature together with high pressure can break down the polysaccharides chain, therefore leading to the wide molecular weight distribution. The molecular weight of alginate extracted from 100 °C and 120 °C ranged between 200-220 kDa, which is very similar with those extracted from 60-90 °C. Therefore, alginate could be extracted efficiently at high temperature (100 -120 °C) in short time.

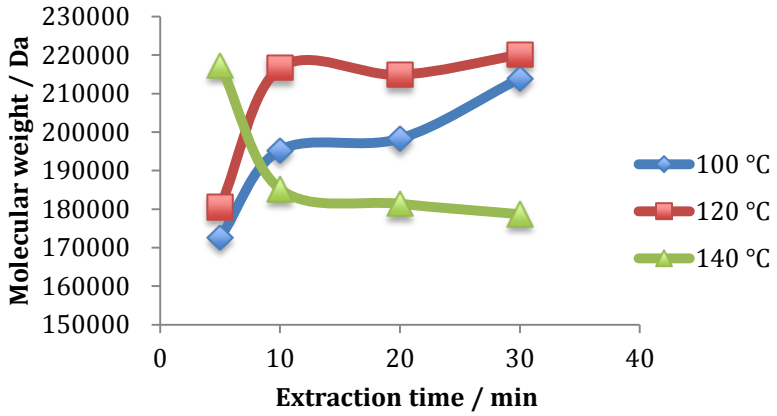


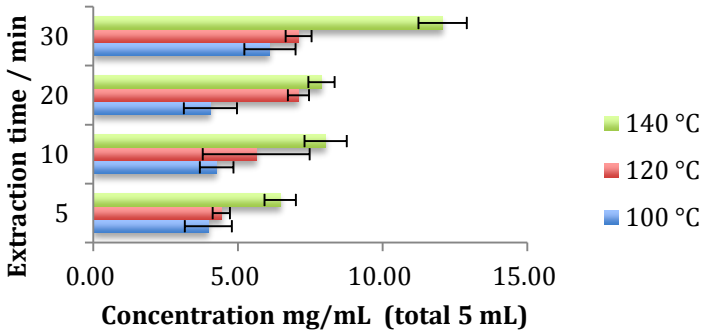
Figure 4.11 Effect of temperature and time on molecular weight of alginate

Table 4.5 Polydispersity index of alginate extracted from different temperature (100, 120, 140 °C)

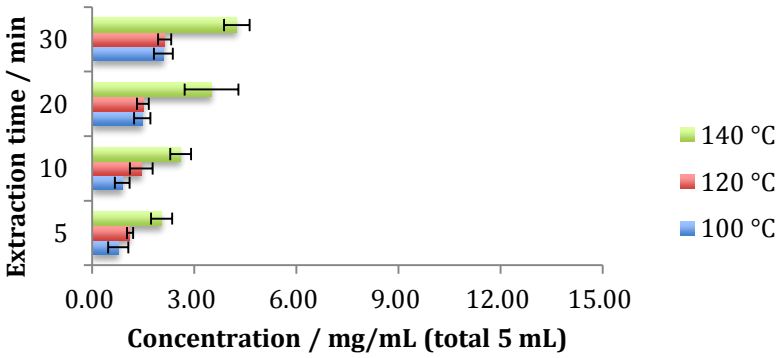
	5min	10 min	20 min	30 min
100 °C	2.88	3.14	2.82	3.23
120 °C	3.10	3.34	2.90	3.64
140 °C	3.48	3.07	5.15	6.54

4.3.3 Analysis of supernatant after alginate precipitation

Analysis of supernatant after alginate precipitation is shown in Figure 4.12. Similar to low temperature extraction, the carbohydrate and uronic acid content of supernatant after alginate precipitation increased with both temperature and extraction time.



(a)



(b)

Figure 4.12 (a) Effect of temperature on carbohydrate content in supernatant after alginate precipitation (b) Effect of temperature on uronic acid content in supernatant after alginate precipitation

It is unexpected that the value of carbohydrate and uronic acid content in high temperature supernatant is within the same range as low temperature supernatant, around 5-10 mg/ml (total 5 mL), which suggests that the temperature range 100-140 ^{o}C can't further hydrolyse the alginate to monomers, although smaller molecular weight alginate was obtained at 140 ^{o}C.

4.4 Chemical compositional analysis of alginate

NMR and FT-IR were measured for the chemical compositional analysis of extracted alginate.

4.4.1 NMR analysis

^{1}H NMR spectroscopy is suitable for characterizing both the composition and the distribution sequence of the two uronate residues in alginate samples.(Heyraud *et al.*, 1996) 4.9 (area A), 4.5 (area B), and 4.3 (area C) ppm were assigned to H1-G, H1-M + H5-GM, and H5-GG, respectively (Figure 4.13). Following Grasdalen *et al.*'s method,(Grasdalen *et al.*, 1979) the relative areas of above peaks contain information on the uronic acid composition and the fraction of nearest neighbours along the copolymer chain. Quantitatively, the mole fraction of G and doublet frequency F_{GG} are related to the areas (A) by the following relationships:

$$F_G = A_A / (A_B + A_C); \quad F_{GG} = A_C / (A_B + A_C)$$

The mole fraction of M is then derived from the normalization condition:

$$F_G + F_M = 1$$

The relations between the mole fractions and the doublet frequencies are given by

$$F_{GG} + F_{GM} = F_G; \quad F_{MM} + F_{MG} = F_M$$

For long chains (DPn > 20), corrections for the reducing-end residues may be neglected, so that $F_{MG} = F_{GM}$. Hence, numerical values for the uronic acid composition and for the doublet frequencies may be calculated. In this work, the degree of polymerization (DPn) of alginate is about 1000.

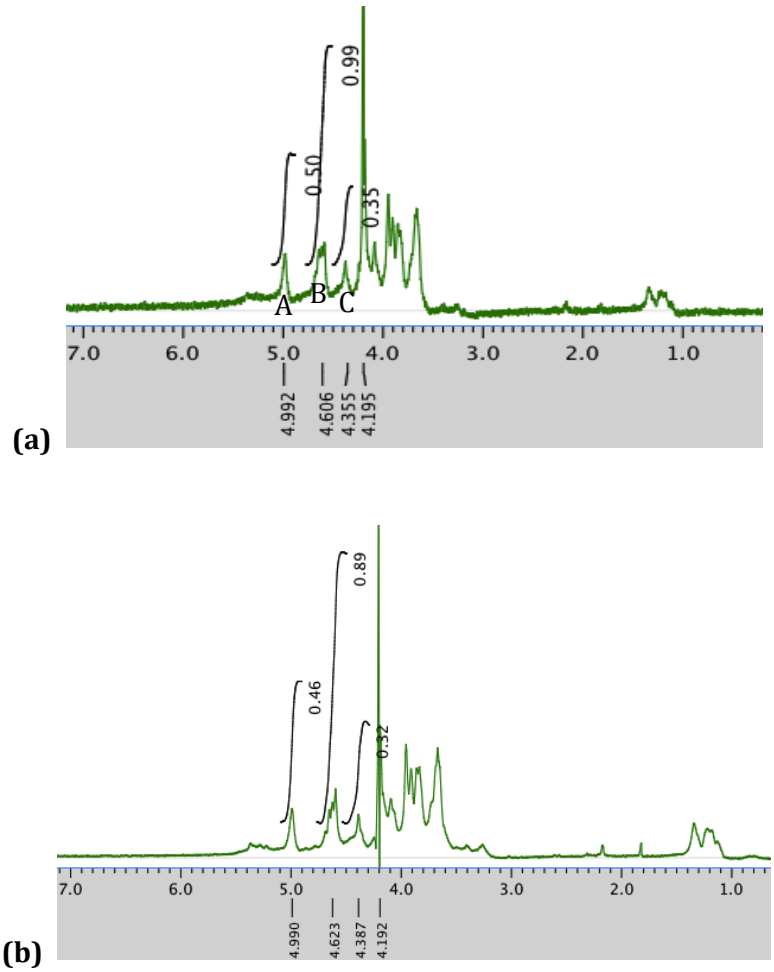


Figure 4.13 ^{1}H NMR spectrum of alginate (a) extraction condition: 75 °C 60 min; (b) extraction condition 140 °C 10 min

The importance of monomers and block proportion is essential to the understanding of the gelling process and characteristics of alginate gels. A high proportion of GG block leads to a rigid and brittle gel where MM block will induce a soft and elastic gel and MG blocks will give flexibility(Draget *et al.*, 2000). Table 4.6 shows the chemical composition of alginate extracted from 75 °C and 140 °C, and M, G block composition is quite similar. Table 4.6 also presents two literature results in which alginate was also extracted from *Ascophylum nodosum.* The difference with this work may be due to the harvest period, location and the part of seaweed that is used(Rioux *et al.*, 2007).

From ^{1}H NMR spectrum (Figure 4.13), it can also be seen that there is a peak around 1-1.5 ppm for both alginate samples. This peak is probably a methyl group (-CH_3) from

other polysaccharide or fatty acid and need to be further confirmed. It is worth mentioning that many researches using ^{1}H NMR analysis of alginate (Gomez *et al.*, 2009; Grasdalen, 1983; Larsen *et al.*, 2003) just show the spectrum between 3-5 ppm and little information is given about whether or not there are some other compounds co-extracted in the product and how these impurities will affect the properties of final product.

Table 4.6 Comparison of chemical composition of alginate from this work and literatures

Extraction condition	F_M	F_G	F_{MM}	F_{GM}	F_{MG}	F_{GG}
75 ^{o}C 60 min	0.63	0.37	0.52	0.11	0.11	0.26
140 ^{o}C 10 min	0.62	0.38	0.50	0.12	0.12	0.26
(Haug *et al.*, 1974)	0.61	0.39	0.35	0.26	0.26	0.13
(Rioux *et al.*, 2007)	0.46	0.54	0.28	0.18	0.18	0.36

4.4.2 FT-IR analysis

The FT-IR spectrum of sodium alginate extracted and commercial alginate is presented in Figure 4.14. In the 3600–1600 cm^{-1} region three bands appear: a broad band centred at 3361 cm^{-1} assigned to hydrogen bonded O–H stretching vibrations, the weak signal at 2933 cm^{-1} due to C–H stretching vibrations, and the asymmetric stretching of carboxylate O–C–O vibration at 1598 cm^{-1}. The band at 1409 cm^{-1} may be due to C–OH deformation vibration with contribution of O–C–O symmetric stretching vibration of carboxylate group(Mathlouthi & Koenig, 1986). The weak bands at 1294, 1125 and 1082 cm^{-1} may be assigned to C–C–H and O–C–H deformation, C–O stretching, and C–O and C–C stretching vibrations of pyranose rings, respectively; the band at 1027 cm^{-1} may be also due to C–O stretching vibrations. The fingerprint, or anomeric, region (950–750 cm^{-1}) is the most discussed in carbohydrates(Mathlouthi & Koenig, 1986) (Tulchinsky *et al.*, 1976). The spectrum shows a band at 949 cm^{-1}, which was assigned to the C–O stretching vibration of uronic acid residues, and one at 871 cm^{-1} assigned to

the C1–H deformation vibration of b-mannuronic acid residues. The band at 812 cm^{-1} seems to be characteristic of mannuronic acid residues.(Leal *et al.*, 2008)

It can be seen that there is some difference between alginate product and commercial one around peak 1235 cm^{-1}, which is usually a position of S=O stretching(Rodriguez-Jasso *et al.*, 2011). Combine the peak around 1-1.5 ppm in ^{1}H spectrum and the peak 1235 cm^{-1} in FT-IR spectrum, it is highly possible that the product may contain sulfated polysaccharides such as fucoidan(Foley *et al.*, 2011), which can also be precipitated out with ethanol(Hahn *et al.*, 2012). Moreover, alginate from 140 ^{o}C contains more possible fucoidan, as peak intensity of 140 ^{o}C for both ^{1}H spectrum (1-1.5 ppm) and FT-IR spectrum (1235 cm^{-1}) is higher than those of 75 ^{o}C samples.

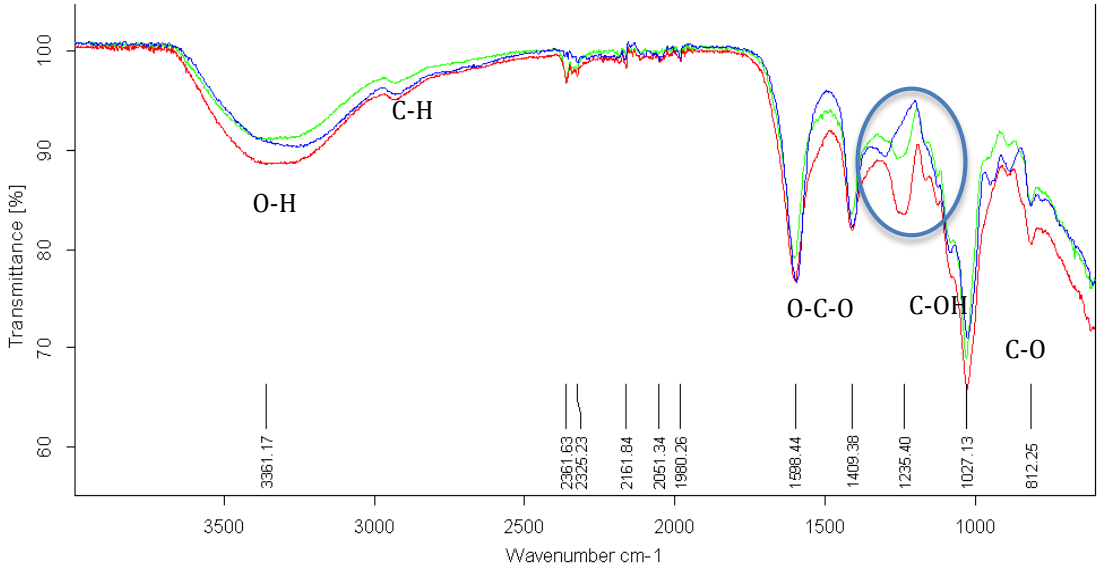


Figure 4.14 FT-IR spectrum of alginate: green line represents alginate from this work (extraction condition: MW 60 °C 60 min); red line represents alginate from this work (extraction condition: MW 140 °C 10 min); blue line represents commercial alginate purchased from Sigma-Aldrich)

4.5 Influence of different purification conditions on final properties of alginate

As discussed above, alginate may contain some other compounds; therefore, different purification conditions were studied to obtain high quality alginate. In addition to the separation step that used ethanol to precipitate sodium alginate directly, which has been described in section 9.2.2.1, another three different processes of purification were studied. They have been named HCl, DCM and $CaCl_2$ routes respectively. Among them, HCl and $CaCl_2$ routes were based on the methods reported by Gomez *et al.*(Gomez *et al.*, 2009). The reason why DCM route was studied here is that according to ^1H NMR spectrum (Figure 4.13) of ethanol route, a peak appears round 1-1.5 ppm, which might be a kind of fatty acid or methyl group ($-CH_3$) of fucoidan.(Chizhov *et al.*, 1999). As DCM is well known to dissolve fatty acids, so DCM washing of sodium alginate solution after extraction process was tried. A schematic representation of the steps developed in the processes is shown in Figure 4.15.

(I). Ethanol route

Described in section 9.2.2.1 as separation step: aqueous solution of sodium alginate obtained in the extraction step was directly precipitated, under stirring, by addition of ethanol until reaching a proportion 1:1 in volume. Thus, the insoluble polymer was separated and then washed with ethanol. Finally, the biopolymer was dried at 50 ^oC until constant mass and then ground to powder using mortar and pestle.

(II). DCM route

The aqueous solution of sodium alginate was added with DCM (1/1 volume ratio) followed by shaking in a separating funnel. After that, the aqueous phase and ethanol were mixed in a proportion 1:1 by volume to precipitate the sodium alginate. Then the same procedure of washing and drying described in Section (I) was applied.

(III). HCl route

In this route of purification, the solution of extracted sodium alginate was added with 1 mol/L HCl to pH between 1-1.5. Next, the precipitate of alginic acid was separated by centrifugation and consequently mixed with 20 mL of 0.1 mol/L Na_2CO_3. Then, this mixture was left under stirring at room temperature for 10 min in order to obtain the soluble sodium alginate. Later, the polymer was precipitated from this solution by slow addition of ethanol (1/1 volume ratio), employing the same procedure of washing and drying described in Section (I).

(IV). $CaCl_2$ route

The aqueous solution of sodium alginate was precipitated by the addition of 1mol/L $CaCl_2$ (20 mL). The precipitate of calcium alginate was washed with distilled water. Then, 1 mol/L HCl was added to the precipitate until pH between 1-1.5 in the supernatant, maintaining this mixture under agitation at room temperature for 10mins. The insoluble material obtained (alginic acid) was separated from the supernatant by centrifugation. Next, the alginic acid was treated as in Section (III).

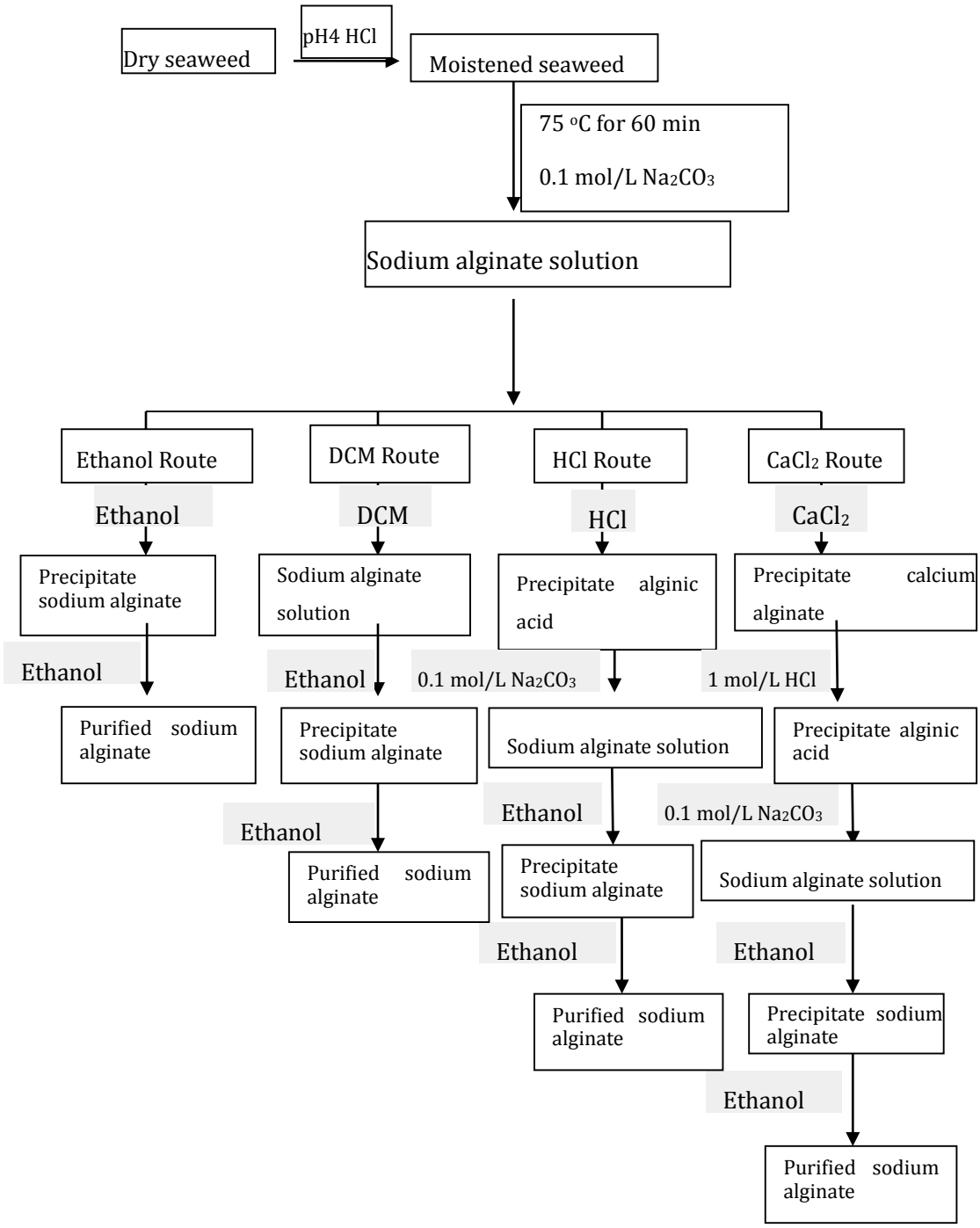


Figure 4.15 Scheme corresponding to the four routes of alginate purification

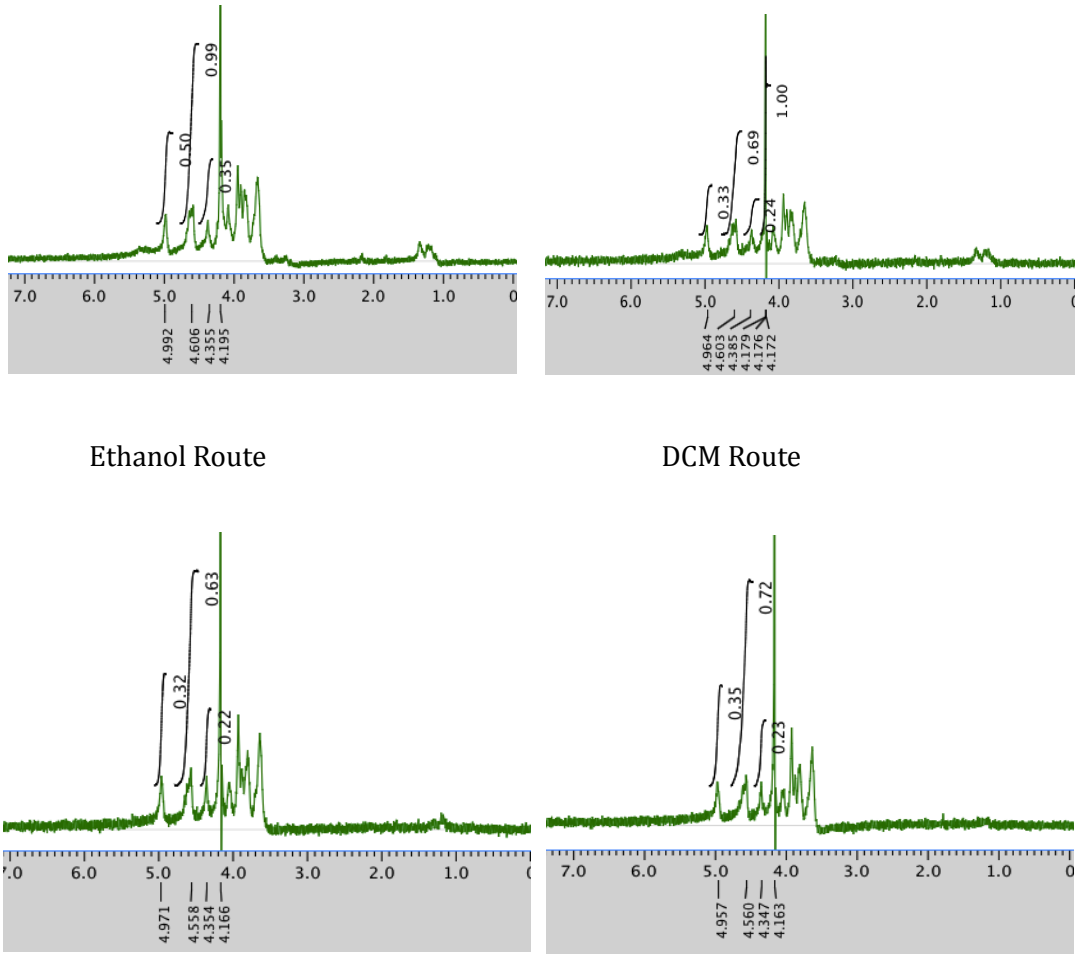
A comparison of four different purification methods is shown in Table 4.7. As can be seen, ethanol route gave highest yield as well as highest molecular weight while had shortest purification steps. For ethanol, HCl and $CaCl_2$ route, the effect on alginate yield and molecular weight here is similar with Gomez *et al.*(Gomez *et al.*, 2009), who

demonstrated that ethanol route displayed the highest yield and best rheological properties with the lowest number of steps compared with HCl and $CaCl_2$ route. The decrease of Mw from HCl and $CaCl_2$ routes may be due to the chain scission caused by HCl.

Table 4.7 Comparison of four different purification methods

	Ethanol route	**DCM rote**	**HCl route**	**$CaCl_2$ route**
Yield	26.49%	24.48%	13.53%	15.09%
Mw	200 kDa	192.1 kDa	157.3 kDa	152.3 kDa
Polydispersity	2.96	3.15	2.83	2.88
Number of steps	2	3	4	5

The ^1H NMR spectrum of alginate from four routes is shown in Figure 4.16. For DCM route, it still shows the peak around 1-1.5 ppm, so there might be fucoidan in the product instead of fatty acid. Also from Figure 4.16, it can be seen that there is no peak around 1-1.5 ppm for HCl and $CaCl_2$ route, which indicates that acid precipitation can help to avoid co-precipitation of sulfated polysaccharides with alginate. According to Table 4.8, there is no significant dispersion of M/G ratio for the four routes.



Ethanol Route

DCM Route

HCl Route

$CaCl_2$ Route

Figure 4.16 ^{1}H NMR spectrum of sodium alginate from four different purification methods

Table 4.8 Chemical compositions of alginates from four different purification methods

	F_M	F_G	F_{MM}	F_{GM}	F_{MG}	F_{GG}
Ethanol Route	0.63	0.37	0.52	0.11	0.11	0.26
DCM Route	0.64	0.36	0.54	0.10	0.10	0.26
HCl Route	0.62	0.38	0.51	0.12	0.12	0.25
$CaCl_2$ Route	0.63	0.37	0.50	0.13	0.13	0.24

Figure 4.17 shows the colour of products from four purification methods. The product from $CaCl_2$ had lightest colour and that from DCM had darkest colour.

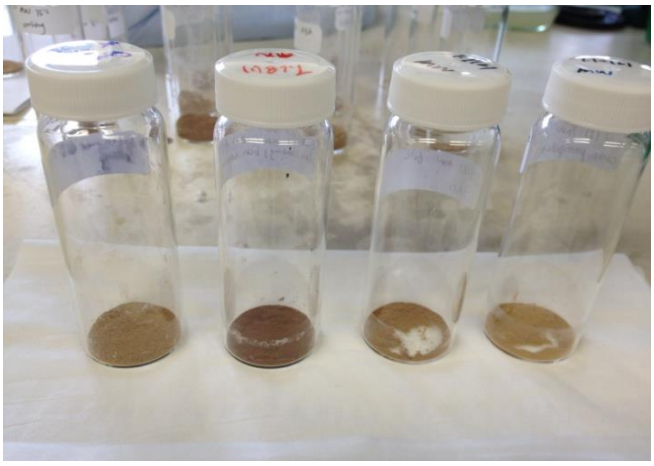


Figure 4.17 Picture of samples from four purification methods (From left to right: ethanol route, DCM route, HCl route and $CaCl_2$ route)

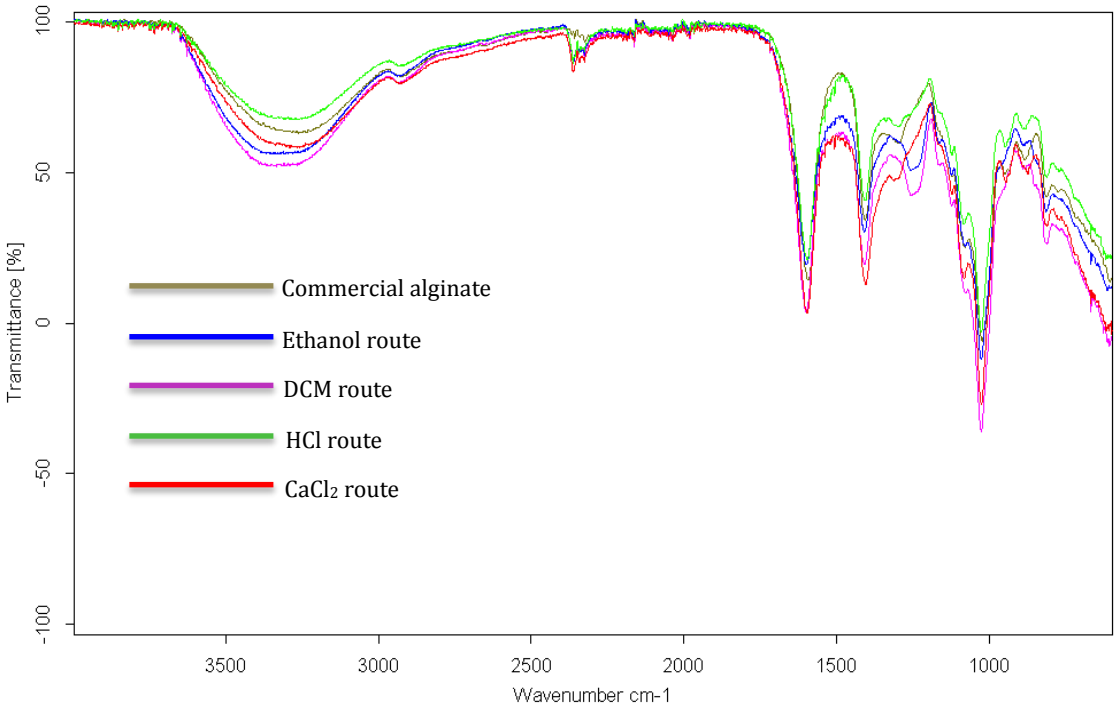


Figure 4.18 FT-IR spectrum of alginate sodium from four purification methods

FT-IR spectrum of sodium alginate from four methods is shown in Figure 4.18. Apparently, HCl and $CaCl_2$ routes have exactly same peaks with commercial one; the peak around 1250 cm^{-1} of ethanol and DCM route is no longer in HCl and $CaCl_2$ route. This indicates that the HCl and $CaCl_2$ purification methods could remove the co-extracted fucoidan efficiently, resulting in higher purity alginate.

Table 4.9 ICP analyses of alginates from four purification methods

	Ca (ppm)	Na (ppm)	*S (ppm)*	P (ppm)	Mg (ppm)	K (ppm)	Si (ppm)	Al (ppm)	Fe (ppm)
Ethanol route	3687.82	119250	***21832***	368.57	5669.15	1680.99	505.16	69.86	269.66
DCM route	3167.16	111837	***21861***	594.14	6555.89	1589.89	695.84	284.01	533.86
HCl route	5258.32	134598	***9754***	1154.45	1619.91	537.48	942.41	596.10	953.08
$CaCl_2$ route	5729.85	137735	***5836***	756.23	469.55	336.80	763.86	395.86	604.26

Products from four purification methods were also analysed by ICP (Table 4.9). As product is sodium form alginate, it is reasonable that sodium has much higher concentration than other metal elements such as Ca, Mg, K, etc. Moreover, HCl route and $CaCl_2$ route could help to decrease the concentration of S, which is in accordance with FT-IR results. It can also be seen that for HCl and $CaCl_2$ routes, the concentrations of Mg and K decreased significantly compared with direct ethanol precipitation, which suggests that the use of HCl and $CaCl_2$ can help to exchange more metal ions attached in polysaccharides.

4.6 Conclusion and future work

Microwave assisted extraction technology can help to extract alginate from seaweed successfully. Different reaction systems and parameters such as time, temperature and seaweed particle size have influences on both yields and molecular weight, so different conditions can be used to control the properties of final product.

Four different purification methods were investigated. While ethanol route gave highest alginate yield, molecular weight as well as fewest purification steps, HCl and $CaCl_2$ route could help remove impurities, thus resulted in higher purity alginate.

Supernatant after alginate precipitation was also analysed. It contains relatively low amount of sugars and uronic acids. Further investigation may be done to identify whether the carbohydrate in the supernatant can be used in fermentation for ethanol production.

As the industrial application of alginate depends mainly on its ability to thicken aqueous solutions and forms gels, the rheological properties of alginate should be studied.

Chapter 5: Microwave Assisted Acid Hydrolysis of *Ascophyllum nodosum* for Bioethanol Production

Publication:

Yuan, Y., & Macquarrie, D. (2015). Microwave assisted acid hydrolysis of brown seaweed *Ascophyllum nodosum* for bioethanol production and characterization of alga residue. *ACS Sustainable Chemistry & Engineering,* 3(7), 1359-1365.

5.1 Introduction

The interest of using seaweed as potential biomass for ethanol production has been increasing recently as it offers several advantages such as high biomass productivity, high carbohydrate content as well as lignin free structure. Various seaweed species such as red seaweed *Palmaria palmata* (Mutripah *et al.*, 2014), *Gelidiella acerosa* (Babujanarthanam & Kavitha, 2014), *Eucheuma cottonii* (Tan *et al.*, 2013), *Gracilaria sp.* (Kumar *et al.*, 2013; F. C. Wu *et al.*, 2014), brown seaweed *Saccharina japonica* (J. Y. Lee *et al.*, 2013; Ra & Kim, 2013), *Undaria pinnatifida* (H. Kim *et al.*, 2013), green seaweed *Ulva intestinalis* and *Rhizoclonium riparium* (Chirapart *et al.*, 2014) have been investigated.

While most saccharification processes were achieved by enzymatic hydrolysis, only a few reports were about bioethanol production directly using the hydrolysate from dilute acid hydrothermal treatment without further detoxification(Chirapart *et al.*, 2014; Mutripah *et al.*, 2014), as it is more likely that the by-product generated during the acid hydrolysis process may inhibit cell growth and ethanol production from the hydrolysate(Herrera *et al.*, 2004; Meinita, Hong, *et al.*, 2012b). However, it is still worth investigating due to its low cost and high reaction rate(Mutripah *et al.*, 2014).

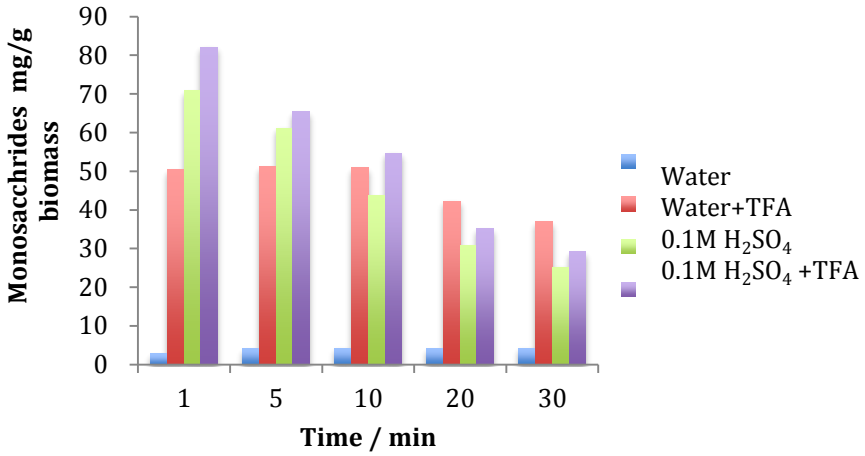
Recently, it has been reported that microwave heating presents a potentially faster, more efficient and selective method for the thermal treatment of biomass(Borges *et al.*, 2014; Budarin *et al.*, 2011; Macquarrie *et al.*, 2012; C. F. Wu *et al.*, 2014). This chapter combines microwave heating and sulfuric acid to perform a fast hydrolysis of *Ascophyllum nodosum.* Variables such as temperature, time, acid concentration and biomass loading were optimized to obtain highest monosaccharide yield. Then the hydrolysate was further explored for bioethanol production without detoxification. Moreover, the biomass residue after hydrolysis was also analysed and its potential to be used as a biochar fuel was also evaluated.

5.2 Microwave assisted hydrolysis of *Ascophyllum nodosum*

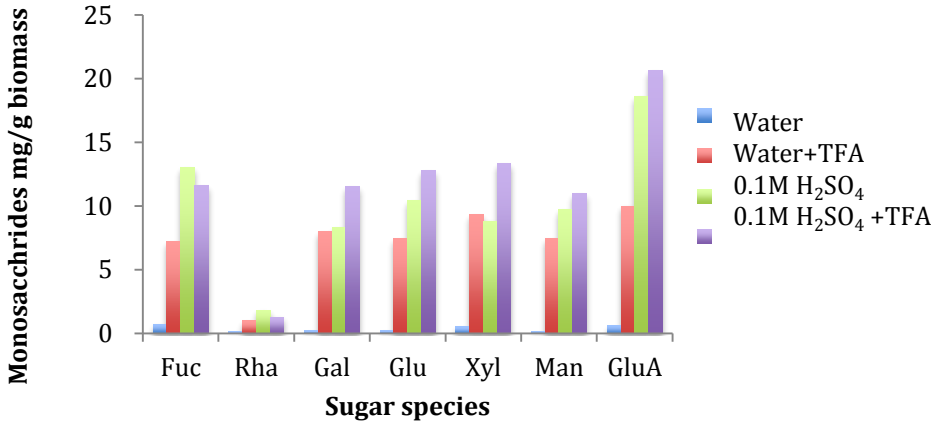
5.2.1 Preliminary work

To investigate if hydrolysis could happen under acid free condition, seaweed was treated with just water at 180 °C for 1-30 min. The results show that monosaccharides yields were extremely low for H_2O treatment, less than 10 mg monosaccharides g^{-1} seaweed. Then the liquid was dried and further treated with 2 M TFA at 121 °C for 2 h for monosaccharides release(Arnous & Meyer, 2008). As can be seen from Figure 5.1a, significant increase of monosaccharides yields was observed after TFA treatments, which indicates that water treatment could extract oligo/polysaccharides from seaweed instead of monosaccharides, and further acid treatment is necessary for the hydrolysis of these oligo/polysaccharides into sugars. Therefore, sulfuric acid (0.1 M) was tested with same condition as water treatment. Similar amount of saccharide are released as with water, but the majority are also hydrolysed into monosaccharide in the acidic conditions. To further identify the efficiency of acid hydrolysis, liquid after sulfuric acid treatment was also dried and hydrolysed by TFA. As shown in Figure 5.1a, there is slight increase of sugar yields after further TFA treatment compared with sulfuric acid only treatment. Therefore, sulfuric acid is efficient for hydrolysis of seaweed and was used for the following work.

Meanwhile, according to the results, 180 °C appeared to be too high a temperature for the hydrolysis, as total monosaccharide yield decreased with treating time, and it is very likely that sugars were degraded to secondary by-products such as HMF and furfural (Meinita, Hong, *et al.*, 2012b). Thus, lower temperatures were tried in the later work.



(a)



(b)

Figure 5.1 (a) Total monosaccharides yield from different treatment (b) Individual monosaccharide yield from different treatment (1 min holding time)

Figure 5.1b shows the composition of the monosaccharides released for 1 min holding time. 9 monosaccharides were detected by HPEAC analysis, including fucose, rhamnose, arabinose, galactose, glucose, mannose, galacturonic acid and glucuronic acid. Arabinose and galacturonic acid were not detected in this work, and other 7 sugars have similar trends as total monosaccharide yields with water and/or acid treatments (Figure 5.1b).

5.2.2 Effect of the reaction temperature

The effect of reaction temperature is shown in Figure 5.2. It can be seen that the

temperature had a significant effect on the hydrolysis process and the optimal temperature for high monosaccharide yield was 150 °C, where 70 – 85 mg/g seaweed of monosaccharides can be produced. Figure 5.2 also shows that fucose and mannose could be obtained efficiently at 120 °C, however, other sugars like galactose, glucose, xylose and glucuronic acid required a higher temperature. The monosaccharide yield decreased at 180 °C, especially with longer holding time. This is probably because the secondary reactions of monosaccharides to other chemicals such as HMF or levulinic acid(Jeong & Park, 2010) were more prevalent at this elevated temperature. Therefore, 150 °C was chosen as the optimum hydrolysis temperature for this study.

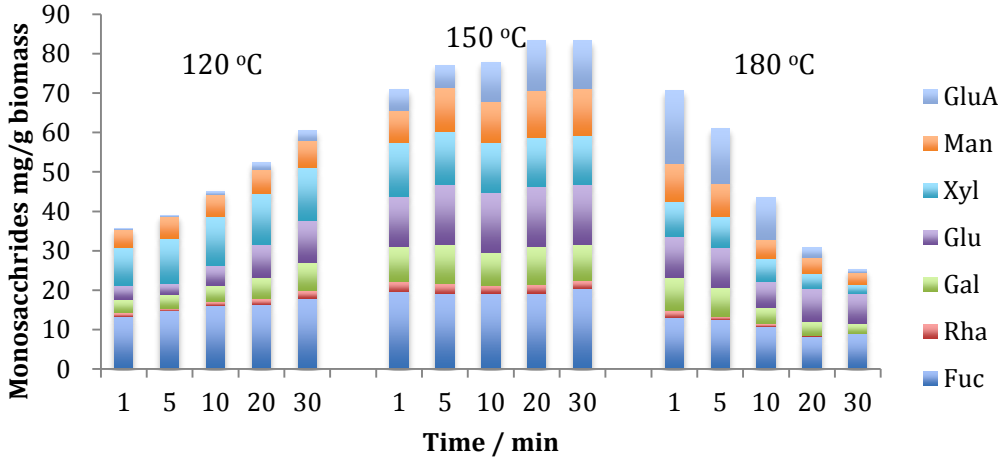


Figure 5.2 Effects of temperature on the monosaccharides yield (Reaction condition: 0.1 M H_2SO_4, biomass loading 3.13%)

As hydrolysis process was conducted at temperature from 120 °C to 180 °C, which is within the range of hydrothermal carbonization temperature(Kruse *et al.*, 2013), seaweed residue was also characterized as potential biochar for fuel. Table 5.1 shows the effect of temperature on biochar properties. It can be seen that compared with raw seaweed, carbon content was dramatically increased and it also increased with process temperature. As a result, the higher heating values of biochar were much higher than the feedstock (13.73 MJ/kg), ranging between 19.36-23.26 MJ/kg, about a 40-70% increase in energy density. This increase is comparable or even higher than the

reported data. For example, Liu *et al.* reported 34-66% and 32-55% energy density increase of coconut fiber and Eucalyptus leaves, at 200-375 °C(Liu *et al.*, 2013), and Xu *et al.* reported increase 10 - 44% of macroalgae at 180-200 °C(Q. Xu *et al.*, 2013).

Table 5.1 Effect of temperature on biochar properties

Sample	% C	% H	% N	HHV[a](MJ/kg)	ED[b]	Mass yield %	EY[c] %
Raw seaweed	36.26	4.86	0.84	13.73	/		/
120 °C	48.66	5.35	1.75	19.36	1.41	40.13	56.58
150 °C	51.08	5.11	1.31	19.72	1.44	34.53	49.72
180 °C	58.82	5.46	0.81	23.26	1.69	26.62	44.99

Reaction condition: 0.1 M H_2SO_4, 30 min, biomass loading 3.13%

[a] Higher heating value

[b] ED (energy densification) = $HHV_{sample}/HHV_{seaweed}$

[c] EY (energy yield)= HHV biochar * Biochar yield/HHV seaweed

To determine the efficiency of the hydrothermal carbonization process, energy densification and energy yield were also studied. As defined by Yan *et al.*, the energy densification was determined by energy content of biochar divided by the energy content of raw feedstock, while the energy yield was defined as the char mass yield multiplied by the energy densification ratio(Yan *et al.*, 2009). As shown in Table 5.1, energy densification increased with temperature, from a low value of 1.41 at 120 °C to a high value of 1.69 at 180 °C. This value is comparable with hydrocarbons derived from food waste (1.82), mixed municipal waste stream (1.73), and anaerobic digestion waste (1.5), which were treated at 250 °C for 20 h(Berge *et al.*, 2011). However, although better solid fuels can be obtained at higher temperature, the overall energy yield was decreasing with temperature due to the decreasing mass yield.

5.2.3 Effect of the acid concentration

To investigate the effect of acid concentration on the release of monosaccharides, *Ascophyllum nodosum* was hydrolysed under various sulfuric acid concentrations (0.01-0.4 M) at 150 °C. According to Figure 5.3, the yield of monosaccharide increased

dramatically with acid concentration. In addition, the monosaccharide yield increased with holding time when acid concentration was lower than 0.2 M, however, it began to decrease with longer holding time when acid concentration was 0.2-0.4 M. This indicated that high acid concentration with longer reaction time would result in degradation of sugars and formation of by-product, which has also been reported by other researchers(Harun & Danquah, 2011; Nguyen *et al.*, 2014). The optimal acid concentration for higher monosaccharide yield was 0.4 M, 5 min, 136 mg/g seaweed of monosaccharides. 0.4 M H_2SO_4 was also reported in the literature as the optimal concentration for hydrolyzing seaweed biomass(Borines *et al.*, 2013; Chirapart *et al.*, 2014; Mutripah *et al.*, 2014). So 0.4 M H_2SO_4 was chosen as the optimum hydrolysis concentration for this study.

As shown in Table 5.2, carbon content of biochar increased with acid concentration and the higher heating values were ranging of 19.17-23.30 MJ/kg. The higher heating values of biochar are strongly dependent on the original feedstock. The biochar derived from food waste(Berge *et al.*, 2011), wheat straw(Thomsen *et al.*, 2008), sewage sludge(He *et al.*, 2013) and pine sawdust(Guiotoku *et al.*, 2014) were reported to have HHVs of 29.1, 19.0, 14.74 and 25.42 MJ/kg, respectively. Energy densification also increases with acid concentration, from a low value of 1.40 of H_2O to a high value of 1.70 with 0.4 M H_2SO_4. Mass yield recovered decreased with acid concentration, however, the efficient carbonization with acid still makes high energy yields.

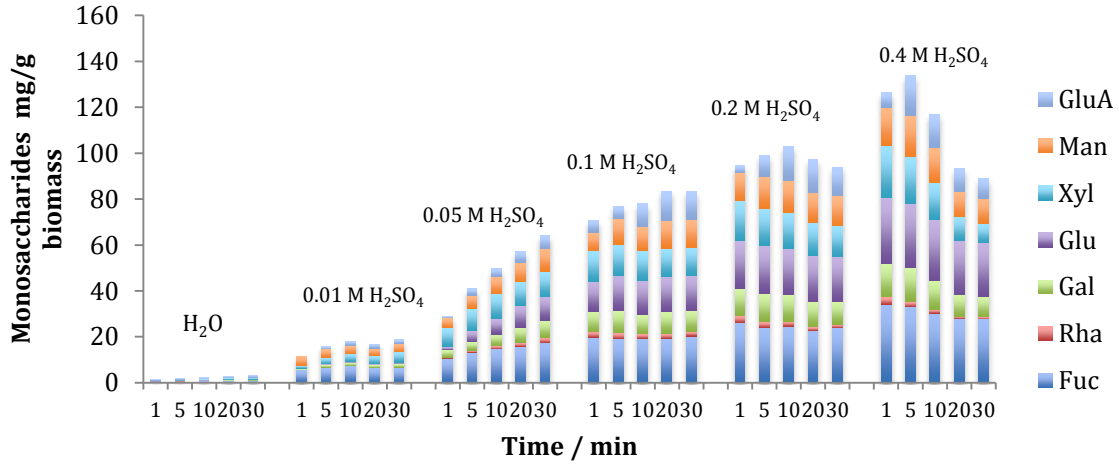


Figure 5.3 Effects of acid concentration on the monosaccharides yield (Reaction condition: MW 150 °C, biomass loading 3.13%.)

Table 5.2 Effect of acid concentration on biochar properties

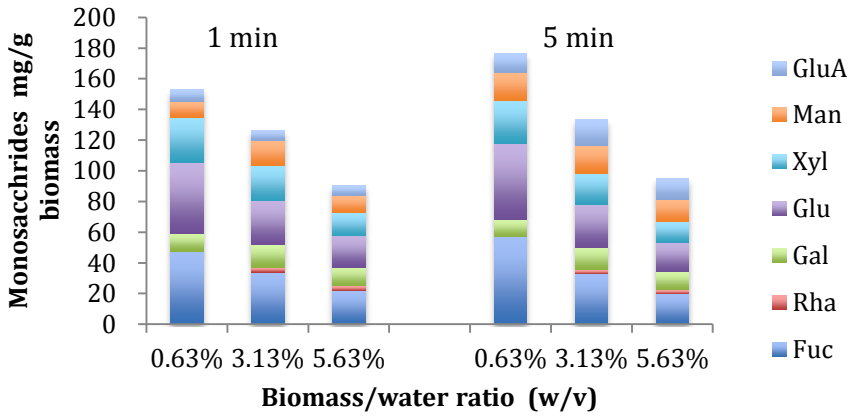
Sample	% C	% H	% N	HHV (MJ/kg)	ED	Mass yield %	EY %
Raw seaweed	36.26	4.86	0.84	13.73	/		/
H_2O	47.77	5.64	1.86	19.17	1.40	39.07	54.70
0.1 M H_2SO_4	51.08	5.11	1.31	19.72	1.44	34.53	49.72
0.2 M H_2SO_4	56.69	5.39	0.82	23.01	1.67	33.56	56.24
0.4 M H_2SO_4	57.24	5.32	0.82	23.30	1.70	32.16	54.67

Reaction condition: MW 150 °C, 30 min, biomass loading 3.13%.

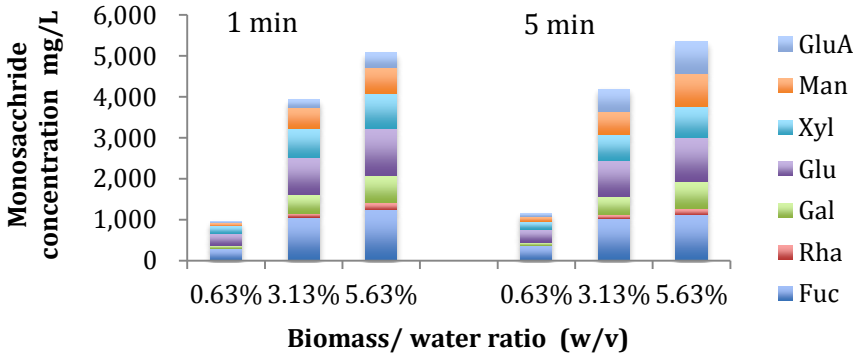
5.2.4 Effect of the biomass loading

Figure 5.4a shows the effect of different biomass concentrations on the hydrolysis process. As expected, lower biomass concentration resulted in higher monosaccharide yield, 176.18 mg/g seaweed of monosaccharide. This is probably because, in the low biomass concentration system, seaweed particles were better distributed, and better contact with water makes the hydrolysis more efficient. However, when under these conditions the actual monosaccharide concentration is considered (Figure 5.4b), 0.63% biomass ratio was quite low, which means more energy will be consumed during

concentration process afterwards. Therefore, 3.13% was chosen as the best biomass concentration considering both monosaccharide yield and concentration. Figure 5.4 also shows that although monosaccharide yield and concentration of 5 min holding time was slightly higher than 1 min holding time, the increase was mainly from glucuronic acid, which is not available for ethanol fermentation with currently available methodology(S. M. Lee & Lee, 2012). Thus, 1 min holding time was chosen for the hydrolysis process, which is much shorter than literature using conventional heating for hours(Babujanarthanam & Kavitha, 2014; Chirapart *et al.*, 2014; J. S. Jang *et al.*, 2009; Meinita, Hong, *et al.*, 2012a; Meinita, Kang, *et al.*, 2012; Mutripah *et al.*, 2014; Nguyen *et al.*, 2014).



(a)



(b)

Figure 5.4 Effects of biomass concentration on the monosaccharides yield (a) Monosaccharides yield (b) Monosaccharides concentration of liquid (Reaction condition: MW 150 °C, 0.4 M H_2SO_4)

Table 5.3 shows the effect of biomass ratio on biochar properties. It reveals that carbon content of biochar decreased with increasing biomass concentration and the higher heating values were ranging between 24.21-22.52 MJ/kg. The energy densification also decreased with biomass concentration, from a high value of 1.76 at 0.63% to a low value of 1.64 at 5.63%. Neither mass yield nor energy yield recovered varies significantly within the range studied.

Table 5.3 Effect of biomass loading on biochar properties

Sample	% C	% H	% N	HHV (MJ/kg)	ED	Mass yield %	EY %
Raw seaweed	36.26	4.86	0.84	13.73	/	/	/
0.63%	53.89	5.67	1.01	24.21	1.76	33.66	59.24
3.13%	54.05	5.70	1.60	22.93	1.67	33.23	55.49
5.63%	52.71	5.36	1.21	22.52	1.64	35.21	57.74

Reaction condition: MW 150 °C, 0.4 M H_2SO_4, 1 min

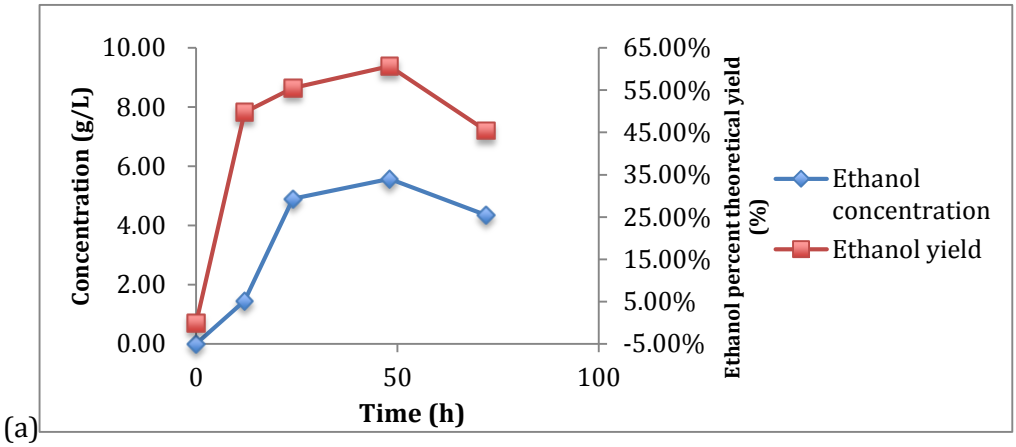
From these results, it could be concluded that the optimum condition for hydrolysing *Ascophyllum nodosum* is 0.4 M H_2SO_4, 3.13% (w/v) of biomass concentration, reaction temperature at 150 °C for 1 min, resulting in 127 mg/g monosaccharides of seaweed. The seaweed residue obtained under the above reaction condition has a higher heating value of 22.93 MJ/kg, the energy densification is 1.67 and energy yield recovered is 55.49%.

5.3 Fermentation

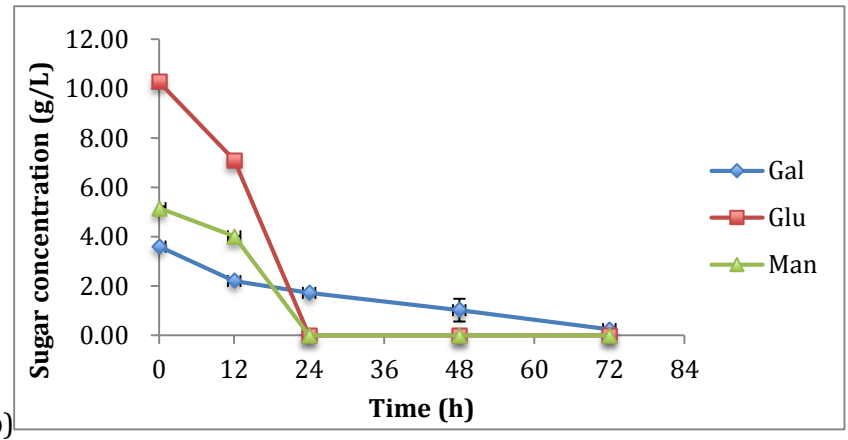
5.3.1 Ethanol fermentation

The hydrolysate solution obtained was freeze dried and then dissolved at a concentration of 100 g/L for fermentation. Figure 5.5a shows that ethanol concentration increased significantly in 24 h and continued to increase until 48 h. The decline in ethanol production after 48 h fermentation might be attributed to consumption of accumulated ethanol by the organism(R. Gupta *et al.*, 2009). The maximum ethanol concentration was 5.57 g/L, 60.7% theoretical yield, and 20.8 mg/g

seaweed. This is higher than those that also used acid hydrolysate for fermentation (Table 5.4). Chirapart *et al.* obtained an ethanol yield of 4.5 mg/g sugar (0.9% theoretical yield) of *Gracilaria tenuistipitata*, Mutripah *et al.* reported to have 15.7mg/g seaweed of ethanol from *Palmaria palmata* while Meinita *et al.* obtained 41% theoretical ethanol yield from *Kappaphycus alvarezii.* This suggests that fast microwave heating could decrease the formation of by-products, which severely inhibit the fermentation process. Brown seaweed has not been previously studied for ethanol production using hydrolysate from acid treatment directly.



(a)



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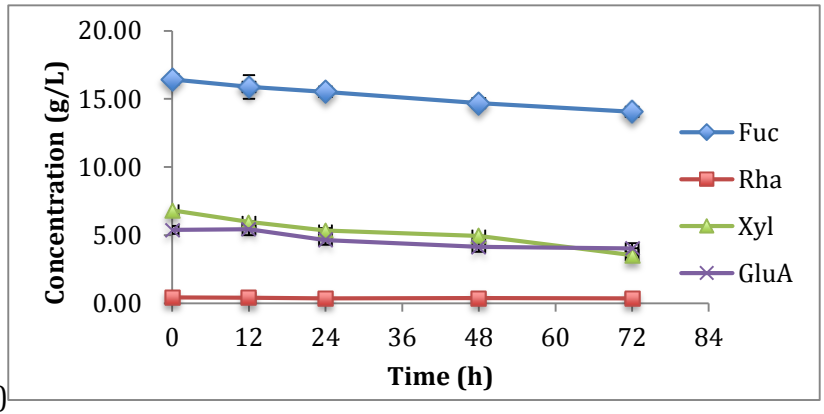


(c)

Figure 5.5 Ethanol production (a) and changes in sugar content (b) mostly consumed sugar (c) less consumed sugar

Table 5.4 Comparison of saccharification and ethanol production from various seaweed species

Seaweed species	Hydrolysis method	Sugar released	Fermentation conditions	Ethanol yields	References
Gracilaria tenuistipitata **(Red seaweed)**	4% biomass loading (w/v) in 0.1 M HCl at 95 °C for 15 h	NA	S.cerevisiae TISTR (10%, v/v) 30 °C, 120 rpm, 18 h	0.004g/g reducing sugar (0.9%)	(Chirapart *et al.*, 2014)
Kappaphycus alvarezii **(Red seaweed)**	10% biomass loading (w/v) in 0.2% (w/v) H_2SO_4, 130 °C, 15 min	305 mg/g (including 256 mg galactose)	Commercial brewer's yeast 30 °C, 120 rpm, 72 h	0.21g/g galactose (41%)	(Meinita, Kang *et al.*, 2012)
Palmaria palmate **(Red seaweed)**	10% biomass loading (w/v) in 0.4 M H_2SO_4 at 125 °C for 25 min	218.4 mg/g (including 27 mg glucose, 127.6 mg galactose)	Commercial brewer's yeast (1.5 g/L) 30 °C, 130 rpm, 72 h	17.3 mg/g seaweed	(Mutripah *et al.*, 2014)
Ascophyllum nodosum **(Brown seaweed)**	3.13% biomass loading (w/v) in 0.4 M H_2SO_4 at 150 °C for 1 min	127 mg/g (including 15 mg galactose, 29 mg glucose, 23 mg mannose)	*S.cerevisiae* ATCC (10%, v/v) 37 °C, 130 rpm, 72 h	20.9 mg/g seaweed 0.31g/g glucose + mannose + galactose (60.7%)	This work

Figure 5.5 b and c reveal that glucose, galactose and mannose were the three major sugars that were consumed, while the concentration of fucose, xylose, rhamnose and glucuronic acid just had slight decreases. It has been reported widely that glucose and galactose can be consumed by *S.cerevisiae* (Mutripah *et al.*, 2014; Tan *et al.*, 2013; F. C. Wu *et al.*, 2014), and according to this work, S.cerevisiae also can consume mannose. *S.cerevisiae* cannot ferment xylose directly, however, recent researches demonstrated that metabolic engineering of *S.cerevisiae* could result in strains capable of efficiently producing ethanol from xylose(Casey *et al.*, 2013; Matsushika *et al.*, 2009). Compared with the high content of fermentable sugars (e.g. glucose, galactose) in red seaweed, the carbohydrate composition in brown seaweed is more complex. Little information is available on fermenting fucose, rhamnose and glucuronic acid to ethanol. Recently, Wargacki *et al.* discovered an engineered microbial platform that can metabolize alginate polysaccharides(Wargacki *et al.*, 2012). And Hwang *et al.* investigated the possibility of fermenting the fucose, rhamnose and glucuronic acid into lactic acid by *Lactobacillus* strains(Hwang *et al.*, 2011). It is reported that at least 4% of ethanol concentration is necessary for the reduction of energy consumption during the distillation step(J. S. Jang *et al.*, 2012; Zacchi & Axelsson, 1989). However, the ethanol concentration in this study was about 0.7% (v/v, 7.06 ml/L), therefore, more investigation needs to be done to optimize the ethanol production.

5.3.2 Analysis of possible inhibitors

As the fermentation inhibitors might be generated during hydrolysis process, furfural (FF), hydroxymethyfurfural (HMF) and phenolic concentration of the initial fermentation medium were analysed (Table 5.5). FF is degraded from pentose sugars while HMF is a degradation compound from hexose sugars, and their toxicity depend on the concentration in the fermentation medium (Mussatto & Roberto, 2004). As shown in Table 5.5, there was no FF detected and the concentration of HMF was only 0.01 g/L, which indicated that fast microwave heating could minimize the degradation

of sugars. Phenolic compounds can also inhibit fermentation process, as they partition into biological membranes and cause loss of integrity, thereby affecting their ability to serve as selective barriers and enzyme matrices(Palmqvist & Hahn-Hagerdal, 2000). Although seaweed is well known to contain no or little lignin, according to the compositional analysis of our seaweed sample, there is 1.4% phenolic content present. Therefore, the phenolic content in the fermentation medium was estimated by the Folin-Ciocalteau (FCR) method (using gallic acid as reference) to be about 1.8 g/L. However, the specific phenolic molecule is unknown. Among the phenolics, 4-hydroxybenzoic acid was reported to have no significant effect on either growth or ethanol productivity with 2 g/L concentration(Palmqvist *et al.*, 1999), however, 4-hydroxycinnamic acid and ferulic acid severely inhibited ethanol productivity at low concentrations in *S. cerevisiae*(Klinke *et al.*, 2004). Therefore, to minimize the inhibition from phenolic, a pre-extraction of phenolic might be useful.

Table 5.5 The by-product compounds contained in initial fermentation medium.

By product	Concentration (g/L)
Phenolic	1.82
HMF	0.01
Furfural	Not detected

Salt is another problem for bioethanol production from seaweed as ash content in seaweed is much higher than terrestrial plants. High alkali metal concentration was reported to inhibit ethanol production by fermentation(Suutari *et al.*, 2015). Casey *et al.* tested 6 pairs of anions (chloride and sulfate) and cations (sodium, potassium and ammonium) from concentration 0.1 M to 0.5 M, all 6 pairs resulted in reduced cell growth rate, sugar consumption rate, and ethanol production rate(Casey *et al.*, 2013). While magnesium is important for many metabolic and physiological functions in yeast

and bacteria(Klinke *et al.*, 2004), calcium could cause an 80% decline in cell mass production at 0.23 mol/L concentration during ethanol fermentation by *S. cerevisiae* (Maiorella *et al.*, 1984). Heavy metal ions such as iron, chromium, nickel and copper could slightly reduce the microbial activity in quantities below 4, 5, 100 and 150 mg/L respectively, however, a 60% reduction was observed by nickel ions at concentration of 100 mg/L(Mussatto & Roberto, 2004). Table 5.6 shows the major minerals concentration in the fermentation medium. As can be seen, salts concentration is relatively high, especially the Na, Mg and K. Heavy metals such as Fe, Cr, Ni and Cu were also detected. The high concentration of salts is very likely to inhibit the fermentation process, thus salts removal from hydrolysates may be helpful.

Table 5.6 Elemental analysis of initial fermentation medium by ICP

Minerals	**Na**	**Mg**	**K**	**Ca**	**Zn**	**Mn**	**Al**
Concentration (ppm)	8813.27	2483.87	4552.26	247.26	15.11	19.53	10.78
Minerals	**Cr**	**Fe**	**Ni**	**Cu**	**Si**	**P**	**S**
Concentration (ppm)	3.71	57.53	1.75	0.43	41.06	206.75	2162.16

5.4 Conclusion and future work

In conclusion, brown seaweed *Ascophyllum nodosum* was successfully used as a potential feedstock for bioethanol production. Microwave assisted hydrothermal treatment provided a fast and efficient saccharification with minimal by-product that ensured the fermentability. A total of 127 mg/g monosaccharides of seaweed were released in 1 min holding time and 20.8 mg/g ethanol of seaweed was obtained. The ethanol concentration and conversion efficiency were 5.57 g/L and 60.7% (based on glucose, galactose and mannose) respectively, which was comparatively higher than those also used hydrolytes from hydrothermal treatment. In addition, more than 50% weight of alga residue was recovered after hydrolysis, and the energy densification

ranged from 1.4 to 1.7, with HHVs from about 19 - 24 MJ/kg, which can also be potentially used as solid fuel.

To achieve better ethanol productivity, more work needs to be done to identify and remove the possible inhibitors. Inhibitor-tolerant yeasts may be chosen for the fermentation. Moreover, more analysis could be done to test the properties of alga residue to be used as solid fuels.

Chapter 6: Microwave Assisted Hydrothermal Treatment of *Ascophyllum nodosum* — A Biorefinery Approach

Publication:

Yuan, Y., & Macquarrie, D. (2015). Microwave assisted step-by-step process for the production of fucoidan, alginate sodium, sugars and biochar from *Ascophyllum nodosum* through a biorefinery concept. *Bioresource Technology,* 198, 819-827.

6.1 Introduction

The biorefinery is an important concept for the development of alternative routes to a range of interesting and important materials from renewable resources. It ensures that the resources are used fully and that all parts of them are valorized. In this chapter, a step-by-step process was designed to obtain a variety of products from *Ascophyllum nodosum* consecutively. The products obtained include fucoidan, alginates, sugars and biochar, which have been produced individually by assistance of microwave technology successfully in previous chapters. The efficiency of the process was also evaluated by comparisons with fucoidan extraction only process, alginate extraction only process and hydrolysis for sugars only process in previous chapters.

6.2 Design of the biorefinery process

Based on the methods of fucoidan extraction, alginate extraction and hydrolysis process in previous chapters, a step-by-step microwave assisted thermal treatment of *Ascophyllum nodosum* as a biorefinery was designed and summarized in Figure 6.1. This process was carried out using Discover and Explorer SP, CEM Corporation.

Pre-extraction and fucoidan extraction steps were same as section 9.2.1.1. *Ascophyllum nodosum* (30 g) was extracted with 80% ethanol (300 ml) under mechanical stirring at room temperature for 18 h and then 70 ^{o}C for 4 h to remove pigments, proteins, mannitol and some salts. The residue was recovered by centrifugation and dried. The resulting seaweed (25.6 g) then was split into 30 parts for the following process. 0.85 g *Ascophyllum nodosum* was suspended in 15 ml 0.1 M HCl solution and put in a 35 ml microwave tube. Then the microwave tube was placed into the CEM Discover reactor for microwave irradiation at 90 ^{o}C for 15 min. After irradiation, the suspension was centrifuged to separate the residual alga, which was washed prior to alginate extraction. At this stage, the supernatant contains predominately fucoidan, but also traces of alginate. Therefore, 2% (w/v) $CaCl_2$ solution was added to the liquid fraction

and the mixture was maintained overnight at 4 °C for alginate removal. The solid fraction obtained by treatment with $CaCl_2$ was separated by centrifugation. Double volume of absolute ethanol was added to the resultant filtrate and the ethanol-precipitated fucoidan was recovered by centrifugation, then washed and dried at 40 °C. The supernatant after fucoidan precipitation was concentrated to 5 ml and neutralized for HPAEC analysis.

Residual seaweed after fucoidan extraction was suspended in 15 mL 0.1 M Na_2CO_3 solution and put into the 35 mL reactor for microwave irradiation at 100 °C for 10 min. After irradiation, the suspension was centrifuged to separate the residual alga, which was washed prior to further hydrolysis. The aqueous solution of sodium alginate obtained in the extraction step was directly precipitated, under stirring, by addition of ethanol until reaching a proportion 1:1 in volume. The ethanol-precipitated alginate was recovered by centrifugation, then washed and dried at 40 °C. The supernatant after alginate precipitation was concentrated to 5 ml and neutralized for HPAEC analysis.

According to the results from Chapter 5, residual seaweed after alginate extraction was treated under the optimal hydrolysis condition. Sample was subjected to 15 mL 0.4 M H_2SO_4 solution and put into microwave reactor for irradiation at 150 °C for 1 min. After irradiation, the suspensions were centrifuged to separate the residual alga, which was washed with distilled water and dried at 80 °C until constant weight. The liquid was neutralized for HPAEC analysis. The alga residue was characterized by elemental analysis and calorific value test.

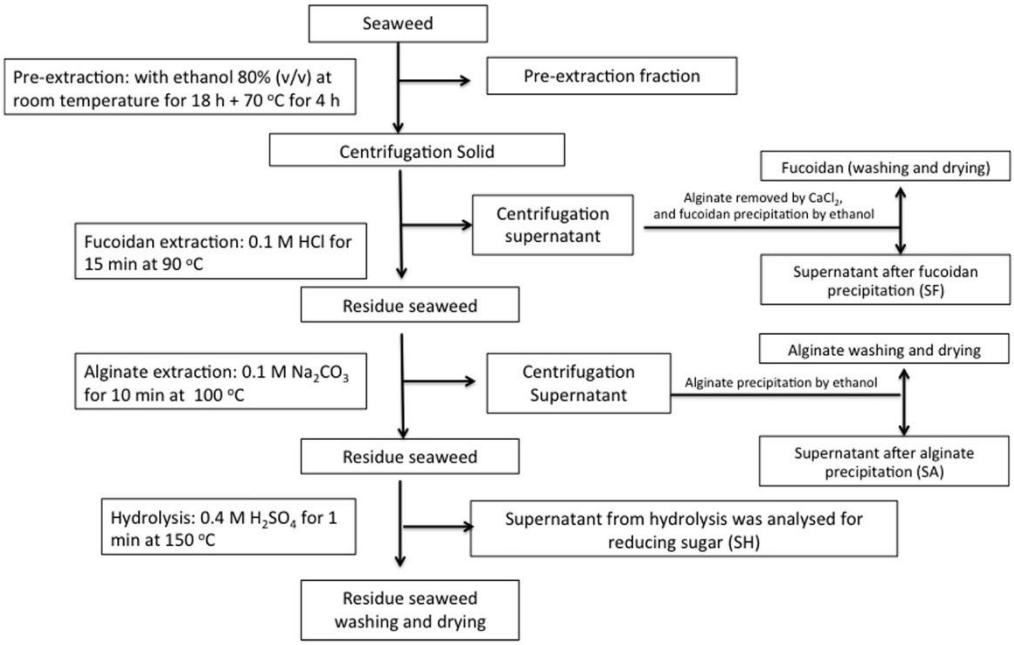


Figure 6.1 Schematic diagram of biorefinery process

To make a comparison, seaweed sample was treated for fucoidan extraction only, alginate extraction only and hydrolysis only, respectively. The microwave irradiation and separation method for each process were same as above.

6.3 Yield for each fraction

The yield for each fraction obtained from the biorefinery process is shown in Table 6.1. The yields of pre-extraction fraction, fucoidan, alginate and algal residue were 14.67%, 14.09%, 18.24% and 21.44%, respectively. To make a comparison, seaweed sample was also treated for fucoidan extraction only, alginate extraction only and hydrolysis only under the same microwave irradiation and separation method as for biorefinery process. As fucoidan was extracted as first product in the biorefinery process, the yield of fucoidan for fucoidan only extraction process was same as it is in biorefinery process, 14.09%. The alginate yield of alginate only process was 23.13%, which is about 5% higher than that from biorefinery process (18.24%), and this indicated that although there was some loss of alginate during the first fucoidan extraction step, the majority

of alginate could still be recovered as second product. The reason that alginate could not be extracted as first product is that alginate is principal materials of cell wall, the extraction of alginate will destroy the structure of the cell, resulting in both alginate and fucoidan being soluble in solution. As discussed in section 3.2, dilute acid could extract fucoidan without dissolving the majority of alginate at same time, although there was a little alginate precipitated out by $CaCl_2$ in fucoidan rich solution. In terms of algal residue, it is reasonable that residues left after the three single processes were higher than the biorefinery process.

Table 6.1 Extraction yield for each fraction

Method	Pre-extraction	Fucoidan	Alginate	Algal residue
Biorefinery process	14.67%	14.09%	18.24%	21.44%
Fucoidan only		14.09%	/	50.04%
Alginate only		/	23.13%	39.82%
Hydrolysis only		/	/	33.23%

6.4 Characterization of each fraction

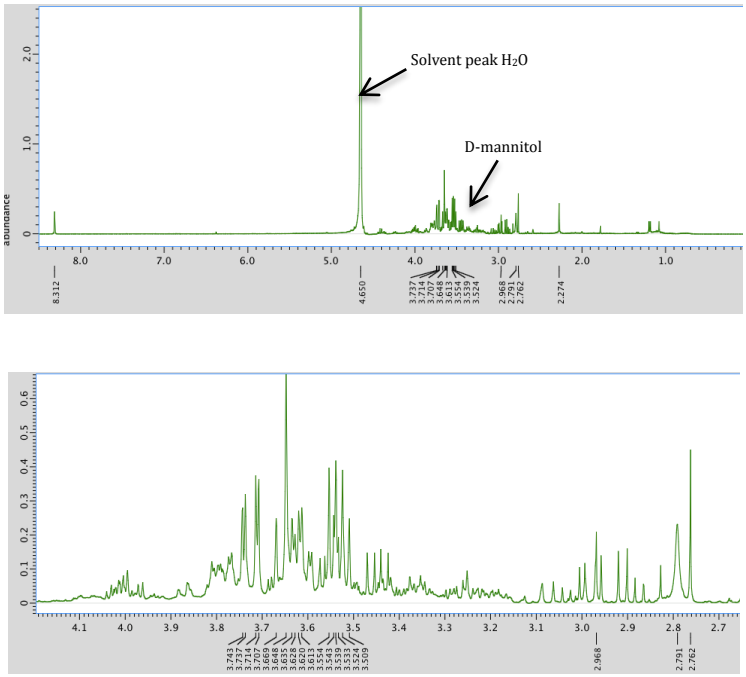
6.4.1 Characterization of pre-extracted fraction

Seaweed samples were pretreated by 80% ethanol before fucoidan extraction to remove pigments, proteins,(Rioux *et al.*, 2007) mannitol and some salts(Foley *et al.*, 2011). The pre-extracted fraction was freeze-dried and the chemical composition was determined and shown in Table 6.2. High ash content (42.07%) was observed, which means part of salts can be removed from seaweed sample during pretreatment step. Previous work indicates that the main carbohydrate content in this fraction is mannitol, which is soluble in ethanol(Foley *et al.*, 2012). The 1H NMR analysis of pre-extraction fraction suggests that in addition to mannitol as major carbohydrate content, there are some possible lipids in the pre-extraction fraction (Figure 6.2). The phenolic content was determined to be 8.4% by the Folin-Ciocalteau (FCR) method (using gallic acid as

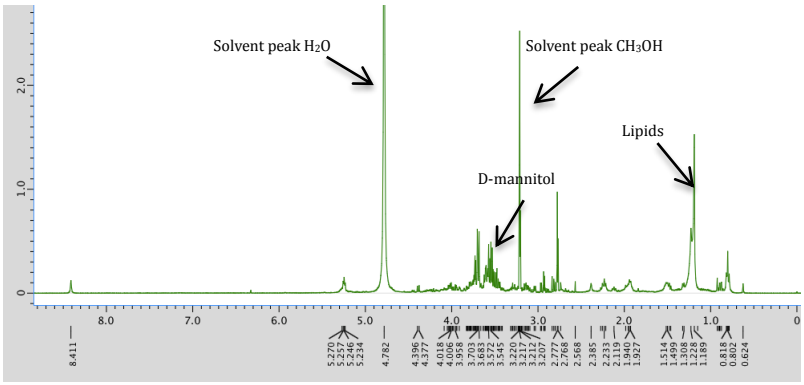
standard), as it is well known that phenols can be used as antioxidant reagent(Zhang *et al.*, 2014), the antioxidant activity of pre-extracted fraction was also investigated, and this will be discussed later together with fucoidan, which was demonstrated to exhibit antioxidant activity in section 3.4. The other compositions were moisture 1.8%, protein 2.51%, trace sulfate, and other components 26.07%, respectively.

Table 6.2 Chemical composition of pre-extracted fraction

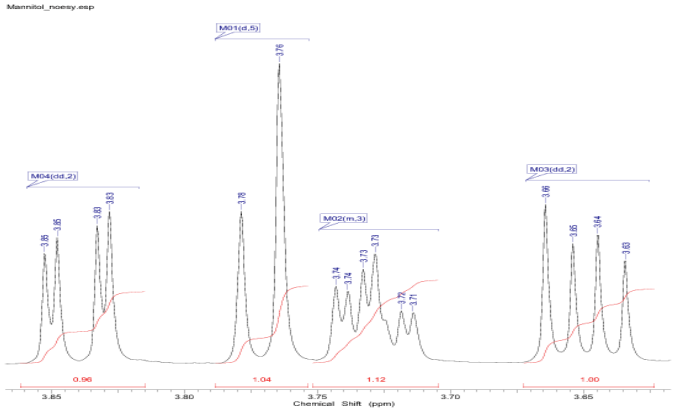
	Moisture (%)	Carbohydrate (%)	Protein (%)	Phenolic (%)	Ash (%)	Sulfate (%)	Other (%)
Pretreatment fraction	1.8	19.15	2.51	8.4%	42.07	Trace	26.07



(a) ^{1}H NMR spectrum of ethanol pre-extraction fraction (sample in D_2O)



(b) ^{1}H NMR spectrum of ethanol pre-extraction fraction (sample in d-methanol), showing the presence of lipids.



(c) Standard D-mannitol ^{1}H NMR in D_2O. (Data from http://www.ymdb.ca/compounds/YMDB00830)

Figure 6.2 ^{1}H NMR analysis of ethanol pre-extraction fraction

6.4.2 Characterization of fucoidan

The chemical composition of extracted fucoidan has been discussed in detail in Chapter 3. The comparison of extracted fucoidan with literature is summarized in Table 6.3. The sulfate content of this fucoidan was 27.12%, which is within the 15%-30% range of those reported. After hydrolysis of the fucoidan, the monosaccharide analysis by HPAEC showed that it is composed of mainly fucose (41.25%) and glucuronic acid

(24.55%), and smaller percentage of galactose (5.95%), glucose (5.95%), xylose (15.01%) and mannose (11.26%). The chemical composition of fucoidans strongly depends on species, growing conditions, extraction procedures etc.(Rodriguez-Jasso *et al.*, 2011). The molecular weight of fucoidan extracted in this work is 34.42 kDa, which is lower compared with reported data for the same seaweed species *Ascophyllum nodosum*. As discussed in Chapter 3, this might be due to the different fucoidan extraction procedures, and the acidic extraction solution (0.1 M) used in this work may hydrolyse partly the polymeric chain(Gustavo Herna´ndez-Carmona, 1999).

Table 6.3 Chemical compositions of extracted fucoidan and comparison with references

Seaweed species	Sulfate (%)	Mw (kDa)	Monosaccharide composition							Reference
			Fuc	Rha	Gal	Glc	Xyl	Man	GluA	
Ascophyllum nodosum	27.12	34.42	41.25	nd	5.95	5.95	15.01	11.26	24.55	This work
Ascophyllum nodosum	19.0	420 and 47	52.10	nd	6.10	21.30	16.50	nd	nd	(Foley *et al.*, 2012)
Ascophyllum nodosum	19.4	nr	62.89	nr	10.69	3.77	10.06	1.89	10.69	(Nakayasu *et al.*, 2009)
Fucus vesiculosus	22.6	nr	62.5	nr	6.88	1.88	5.63	2.5	20.63	(Nakayasu *et al.*, 2009)
Laminaria japonica	33.01	nr	62.08	nd	24.33	1.93	nd	6.06	1.93[a]	(J. Wang *et al.*, 2008)
Undaria pinnatifida	15.02	171	39.24	nr	26.48	0.95	28.85	5.04	1.23[a]	(Mak *et al.*, 2013)

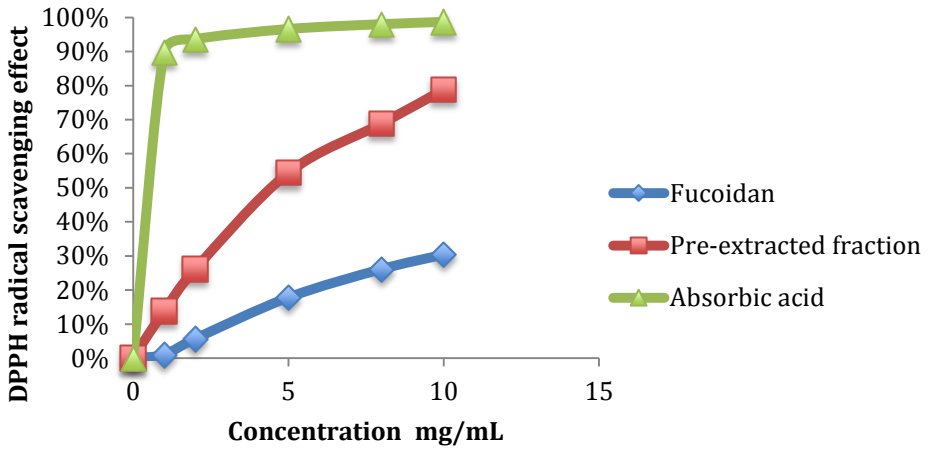
nd, not detected; nr, not reported

[a] reported as uronic acid in the references

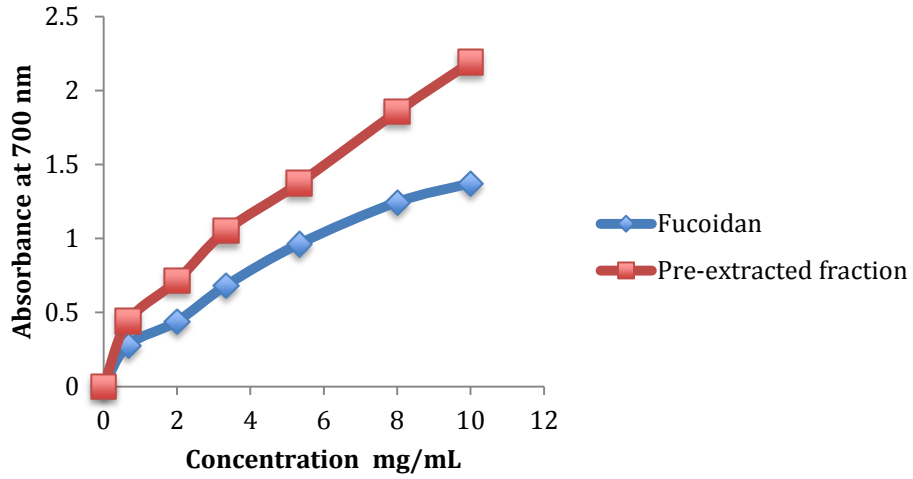
As pre-extracted fraction contains phenolic, and also fucoidan has been previously shown to have antioxidant activity, both pre-extracted fraction and fucoidan were tested for antioxidant activities. Figure 6.3 shows that pre-extracted fraction exhibits strong antioxidant activities. At 10 mg/mL, the DPPH free radical scavenging effect and reducing power absorbance were 79% and 2.19, respectively. Ye *et al.* tested antioxidant activities of ethanol extract from brown seaweed *Sargassum pallidum*, the polyphenol content standardized against chlorogenic acid was 5.34% in the extract(Ye *et al.*, 2009). The DPPH scavenging effect was 11.55% at 2 mg/mL, while for our work, it is 26% at the same concentration. The reducing power absorbance for Ye *et al.*'s extract was 0.267 at 0.2 mg/mL, while it is 0.67 at 1 mg/mL in our work. This indicates that our system leads to enhanced antioxidant behavior.

Although fucoidan has lower DPPH free radical scavenging of 25% and reducing power absorbance of 1.37 at concentration 10 mg/mL in comparison with pre-extracted fraction, the values are comparable with reported literature of fucoidan as discussed in Chapter 3.

However, the scavenging effects of both pre-extraction fraction and fucoidan were relatively lower than that of ascorbic acid, which is a well-known antioxidant and has a DPPH free radical scavenging of 90% at 1 mg/mL (Figure 6.3). In addition, the scavenging effects and reducing power for both samples increase with concentration.



(a)



(b)

Figure 6.3 (a) Scavenging effects of extracted fucoidan and pretreatment fraction on DPPH free radicals. (b) Reducing power assay of extracted fucoidan and pre-extracted fraction

6.4.3 Characterization of alginate

The method of characterization of alginate by ^{1}H NMR has been discussed in detail in Chapter 4. Figure 6.4 presents the ^{1}H NMR of alginate from alginate process and biorefinery process. Table 6.4 compares the chemical composition of alginate from biorefinery process, alginate only process and literature that used *Ascophyllum nodosum* and some other species for the extraction. It can be seen that alginates from alginate only process and biorefinery process have similar M/G ratios around 1.5. It is

reasonable that different species of seaweed have different chemical composition, however, even for the same species, the chemical composition is not quite similar. The difference may be due to the harvest period, location and the part of seaweed that is used(Rioux *et al.*, 2007).

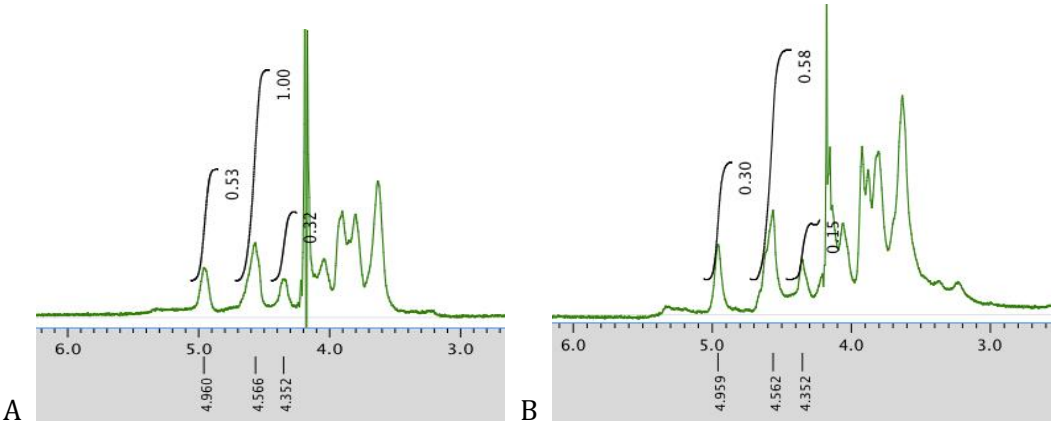


Figure 6.4 ^{1}H NMR spectrum of alginate from (A) biorefinery way (B) alginate only extraction

The molecular weight of alginate from this work and literatures is also shown in Table 6.4. The result shows that the molecular weight of alginate from alginate only process was 195.3 kDa, while that from biorefinery process was only 75.13 kDa. The reasons for this reduction are possibly due to the acid treatment and extra heating during the first fucoidan extraction, which might cause partial depolymerisation of the alginate. This is supported by Chharbar *et al.* 's research, which demonstrated that alginate could be partially hydrolysed by low concentration acids (0.25 M H_2SO_4) under microwave irradiation(Chhatbar *et al.*, 2009). Furthermore, polysaccharide chain of alginate could also be broken down during the alkaline extraction at 100 ^{o}C. The polydispersity index (PI) of alginate from alginate only process and biorefinery process are 3.142 and 4.123, respectively. There is no reported data for PI of alginate in the literature.

Table 6.4 Chemical composition of extracted alginate and comparison with references

References	Species	Mw / kDa	Chemical compositions						
			M/G	F_M	F_G	F_{MM}	F_{GM}	F_{MG}	F_{GG}
This work: Biorefinery way	*Ascophyllum nodosum*	44.91	1.5	0.60	0.40	0.44	0.16	0.16	0.24
This work: Alginate only	*Ascophyllum nodosum*	143.8	1.44	0.59	0.41	0.39	0.20	0.20	0.21
(Haug *et al.*, 1974)	*Ascophyllum nodosum*	nr	1.56	0.61	0.39	0.35	0.26	0.26	0.13
(Rioux *et al.*, 2007)	*Ascophyllum nodosum*	177.3	0.85	0.46	0.54	0.28	0.18	0.18	0.36
(Rioux *et al.*, 2007)	*Fucus vesiculosus*	154.9	1.44	0.59	0.41	0.39	0.19	0.19	0.22
(Rioux *et al.*, 2007)	*Saccharina longicruris*	106.6	0.69	0.41	0.59	0.07	0.34	0.34	0.25
(Fenoradosoa *et al.*, 2010)	*Sargassum turbinarioides Grunow*	552.8	0.94	0.48	0.52	0.36	0.25	0.25	0.39
(Larsen *et al.*, 2003)	*Cystoseira trinode*	nr	0.59	0.37	0.63	0.24	0.13	0.13	0.50

6.4.4 Sugar analysis for each liquid fraction

Supernatant after fucoidan precipitation (SF), supernatant after alginate precipitation (SA), supernatant from hydrolysis (SH) processes were concentrated and analysed for monosaccharides composition (Table 6.5). The monosaccharides content for each fraction were 39.64 mg/g biomass, 24.07 mg/g biomass and 44.97 mg/g biomass, respectively. This made a recovery of 108.68 mg/g monosaccharides in total. In comparison, *Ascophyllum nodosum* was hydrolysed directly for monosaccharides. It can be observed that 126.24 mg/g monosaccharides could be obtained, and this was slightly higher than total monosaccharides recovered from three fractions in the biorefinery process. Therefore, a similar amount of sugars could be obtained via the biorefinery process compared with hydrolysis only process, despite there being 2 more products (fucoidan and alginate) obtained. The sugars recovered can be further used for fermentation processes. The result of Chapter 5 has demonstrated that ethanol could be successfully produced from hydrolysate obtained by hydrolysis of seaweed samples without further detoxification. It is well reported that glucose, galactose, mannose and xylose are fermentable for production of ethanol and lipids(Li *et al.*, 2014; Liang *et al.*, 2012), however, the fermentation of more typical brown seaweed sugar such as fucose and uronic acid are still at early stage investigation. Wargacki *et al.* discovered an engineered microbial platform that can metabolize alginate polysaccharides(Wargacki *et al.*, 2012), while Newman *et al.* discovered a synthetic yeast platform, which can grow on mannitol and alginate monomers(Enquist-Newman *et al.*, 2014). Moreover, Hwang *et al.* identified the patterns of seaweed sugars fermentation into lactic acid by *Lactobacillus* strains(Hwang *et al.*, 2011).

Table 6.5 Sugar analysis for each liquid fraction

Sugar	SF / mg	SA / mg	SH / mg	Total /mg	Hydrolysis only / mg
Fucose	13.18	6.38	5.21	24.77	33.96
Rhamnose	0.15	0.11	0.33	0.59	3.33
Galactose	2.03	1.98	4.26	8.27	14.52
Glucose	13.91	2.91	17.11	33.93	28.77
Xylose	6.65	3.26	5.40	15.31	22.84
Mannose	0.92	1.23	5.78	7.93	16.48
Glucuronic acid	2.80	8.20	6.88	17.88	6.34
Total sugar	39.64	24.07	44.97	108.68	126.24

6.4.5 Characterisation of alga residue

Elemental analysis and calorific value were determined for alga residue from the biorefinery process and from the three single processes. As shown in Table 6.6, carbon content of all alga residue samples increased compared with raw material, especially for algal residue from the biorefinery process and the hydrolysis only process, where significant increases of carbon content were observed. In this way, we may say that hydrothermal carbonization happened. The energy densification (ED) and energy yield (EY) of alga residue were evaluated by the method suggested by Yan *et al.*(Yan *et al.*, 2009) to study the efficiency of the hydrothermal treatment. It can be seen that residue from fucoidan extraction only and alginate extraction only had lower ED (1.12 and 1.21), and this might be due to the lower temperature applied for the extraction processes, as hydrothermal processing temperature strongly affects the carbon content in biochar(Q. Xu *et al.*, 2013). In comparison, alga residue from the biorefinery process and hydrolysis only process had much higher ED (1.55, 1.67), which is also higher than the reported value of 1.45 for macroalgae *Sargassum horneri*(Q. Xu *et al.*, 2013). As expected, the HHVs of residue from fucoidan extraction only and alginate extraction only processes were 15.42 and 16.65 MJ/kg, which was lower than those from biorefinery process (21.23 MJ/kg) and hydrolysis only processes (1.67). In terms of the energy yield, as mass yield of alga residue from biorefinery process was low compared

with other three single processes, the energy yield was also relatively low, 33.23%.

Table 6.6 Characterization of residue

	% C	% H	% N	HHV[a] (MJ/kg)	ED[b]	Mass yield %	EY[c] %
Raw seaweed	36.26	4.86	0.84	13.73	/	/	/
Biorefinery	51.05	5.29	1.87	21.23	1.55	21.44	33.23
Fucoidan extraction only	39.91	5.50	1.68	15.42	1.12	50.04	56.04
Alginate extraction only	41.47	5.55	1.34	16.65	1.21	39.82	48.18
Hydrolysis only	54.05	5.7	1.6	22.93	1.67	33.23	55.49

[a] Higher heating value

[b] ED (energy densification) = $HHV_{sample}/HHV_{seaweed.}$

[c] EY (energy yield)= HHV biochar * Biochar yield/HHV seaweed

6.5 Conclusion and future work

This chapter further developed the biorefinery concept, using brown macroalgae *Ascophyllum nodosum* as an example, by assistance of microwave technology. A step-by-step process was designed to obtain fucoidan, alginates, sugars and biochar (alga residue) consecutively. The chemical composition and properties of each product were also analysed. The results show that fucoidan and also pre-extracted fraction inhibited strong antioxidant activities, sugars obtained contained large proportion of fermentable sugars such as glucose, xylose, etc., and biochar obtained had high energy densification. All this indicated that *Ascophyllum nodosum* could be potentially used as feedstock for a biorefinery process to produce valuable chemicals.

However, all the work are based on the lab scale, it is necessary to scale up the process and to investigate the economic feasibility in industrial scale.

Chapter 7: Short Project: Microwave Assisted Drying of *Ascophyllum nodosum*

7.1 Introduction

Ascophyllum nodosum has traditionally been used as an animal feed supplement, soil conditioner agent, fertilizer as well as human nutritional supplements(Kadam *et al.*, 2015). Being marine in nature, fresh seaweed usually contains a large amount of water, about 75-85%(S. Gupta *et al.*, 2011). Therefore, drying is an essential step before it can be used in industrial processing.

Seaweed is generally sun dried, and other drying methods such as oven-drying and freeze-drying has also been reported(Wong & Cheung, 2001). Recently, microwave assisted of drying has drawn intensive attention as a fast and efficient drying method. A broad spectrum of fruits and vegetables including potato, grapes, apple, mushroom, carrot, banana, chili and garlic have been studied by microwave drying(Krokida & Maroulis, 1999; Lombrana *et al.*, 2010; Sharma & Prasad, 2006). However, little information about microwave drying of seaweed is available. In this chapter, fresh *Ascophyllum nodosum* was dried by microwave heating.

7.2 Method

A ROTO SYNTH Rotative Solid Phase Microwave Reactor (Milestone) was used for the drying process. This is a multimode microwave reactor and the apparatus set up is shown in Figure 7.1. 300 g fresh seaweed was put into the sample vessel for the drying process. Three drying conditions were applied, including atmospheric pressure drying, vacuum (500 mbar) assisted drying and gas flow (0.4 L/min argon) assisted drying.

Conditions were chosen to remove ~80% water from the fresh seaweed, leading to optimize water content of 10-15%, ideal for use as cattle feed.

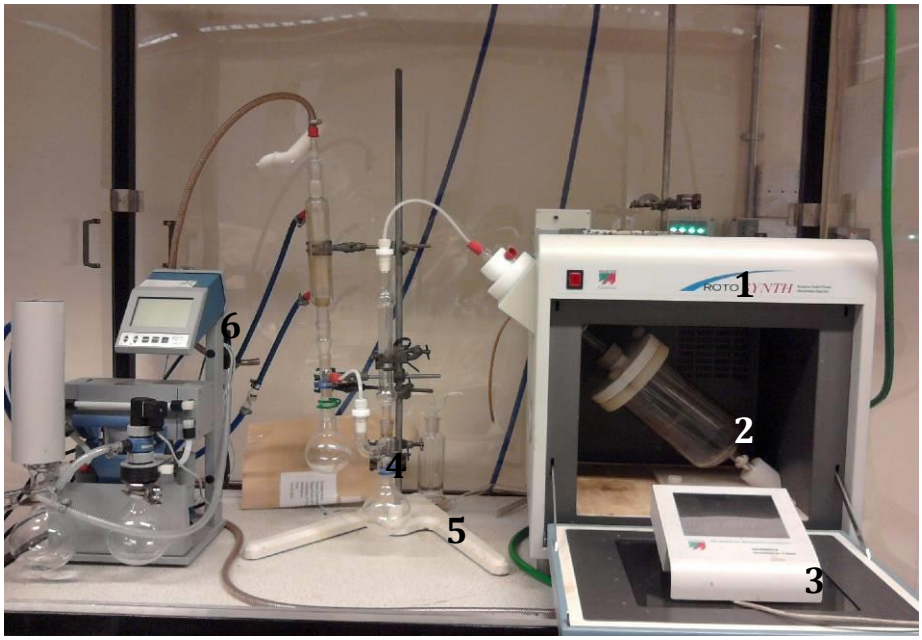


Figure 7.1 Set-up for drying process. (1) Microwave reactor, (2) sample vessel, (3) system control and monitoring console, (4) and (5) round bottom flasks for collection water, (6) vacuum pump.

7.3 Results and discussion

Figure 7.2 shows the generation of water from seaweed under atmospheric pressure drying and vacuum assisted drying with different powers. Apparently, for both methods, the higher the power was, the shorter the time was. Especially when 1200 W was applied, the drying process could be finished in 10 min. Soysal's research about microwave assisted drying of parsley also demonstrated that the drying rate increased with increased microwave output power(Soysal, 2004). Hot air drying is the most widely used drying method, however, this method generally requires about two-thirds of the total drying time for removing the final one third of the moisture content(Y. Y. Xu *et al.*, 2004). As can be seen in Figure 7.2, when applied microwave heating at 1200 W, the rate of water remove was constantly high, although there was slight decrease at the end of drying when the power was 600 W and 800 W. This indicates that microwave with high power could remove the moisture content of biomass efficiently.

In addition, vacuum condition could accelerate the process, and under the lower power,

this effect was stronger. The possible reason may be that water is easier to evaporate under lower pressure, thus is easier to be extracted and removed from the system, accelerating the process.

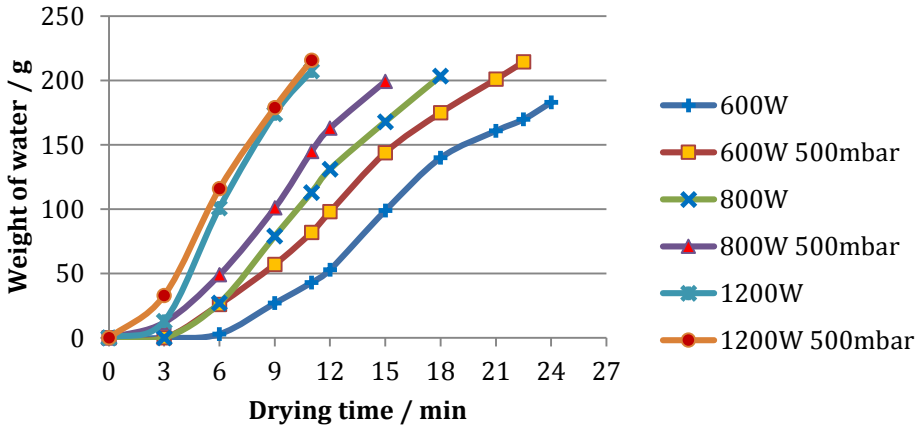


Figure 7.2 Comparison of atmospheric pressure drying and vacuum assisted drying under different power

Drying process was also done with the gas (argon) flow of 0.4 L/min. Table 7.1 shows the comparison of three process conditions. It can be observed that the gas flow could also accelerate the drying process according to weight of residue. However, the water collected from gas flow condition (170.06 g) was less than the other two conditions. The reason is probably that the condenser was not cool enough to catch all the water vapors, and some of them went into atmosphere with gas flow. Table 7.1 also shows the temperature range within which the majority of water was removed. As can be seen, for atmospheric pressure condition and gas flow condition, water was removed at temperature range 100-110 ^{o}C, while vacuum drying condition lowered the drying temperature about 20 ^{o}C, which could help with protecting the thermally labile compounds in the seaweed.

Table 7.1 Comparison of three drying methods

	Atmosphere drying	Gas flow drying (0.4 L/min)	Vacuum drying (500 mbar)
Weight of water (g)	183.17	170.06	214.51
Weight of Residue (g)	105.97	92.27	74.36
Temperature range (°C)	100-108	103-109	80-94

Drying condition：600W, 24 min

It is also worth mentioning that the water from all three methods was colourless, indicating no or little destruction of seaweed (Figure 7.3). However, the characterization of dried product needs to be done to further confirm the quality. Krokida *et al.* investigated the microwave drying of apple, carrot, potato and banana, and concluded that microwave drying could increase the product porosity and prevent the colour damages, could decrease the maximum stress and maximum strain of dehydrated products while increasing their elasticity and decreasing their viscous nature(Krokida & Maroulis, 1999).

Figure 7.3 Water from drying processes

7.4 Conclusion and future work

According to the data above, microwave drying is a fast and efficient method to dry seaweed. Both vacuum assisted drying and gas flow assisted drying could contribute to the processes.

For future work, dried seaweed need to be characterize for the product quality. Conventional drying method also could be done to make a comparison with microwave drying.

Chapter 8: Conclusion and Future Work

Biomass is a sustainable and important source of chemicals and fuels for bio-based industry, which is potential alternative of petro-based industry. The biorefinery is a key process for the development of bio-based industry. A variety of biomass has been studied as feedstock for biorefinery process, among which, food crops and lignocellulosic materials have been the major focus. Macroalgae, a world wide available resource with high productivity, is a desirable biomass feedstock for biorefinery. The high carbohydrate content of macroalgae (generally more than 50%) makes it a good candidate for chemicals and fuels.

This project successfully developed the biorefinery concept using macroalgae *Ascophyllum nodosum* as feedstock, and produced valuable chemicals (e.g. alginate, fucoidan, and sugars) as well as fuels (e.g. bioethanol and biochar) from *Ascophyllum nodosum* by assistance of microwave extraction and microwave hydrolysis technologies. Different reaction parameters were tested to optimize the biorefinery process, and products were characterised to identify the key properties. Major results include:

- Fucoidan could be extracted in 5-30 min by microwave heating from 90 °C to 150 °C, and the highest yield (16.08%) was obtained at 120 °C, 15 min， which is much higher than the value ranging from 1 % - 8 %, reported for the same seaweed species *Ascophyllum nodosum* by other researchers(Foley *et al.*, 2011; Nakayasu *et al.*, 2009; Rioux *et al.*, 2007). Compositional analysis, GPC, HPAEC and IR analysis were employed for characterization of extracted fucoidan. Fucose was the main monosaccharide of fucoidan extracted at 90 °C while glucuronic acid was the main monosaccharide of fucoidan extracted at 150 °C. Both the molecular weight and sulfate content of extracted fucoidan increased with decreasing extraction temperature. All fucoidans exhibited antioxidant activities as measured by DPPH scavenging and reducing power, among which fucoidan extracted at 90 °C was highest, and the value is comparable with literatures(Dore *et al.*, 2013; Ye *et al.*, 2008).

- Alginate could be extracted successfully under low temperature (60-90 ^{o}C) - open vessel system and high temperature (100-140 ^{o}C) - closed vessel system, respectively. The results show that high temperature extraction had higher yield (25-30%) in less time (5-30 min) compared with low temperature extraction (20-25%, 15-120 min). M_w from both extraction were similar, 180 kDa – 220 KDa, which is in line with Rioux *et al.*(Rioux *et al.*, 2007) and Fourest *et al.*'s(Fourest & Volesky, 1997) research who obtained molecular weight of 177.3 kDa and 132 kDa for the same seaweed species *Ascophyllum nodosum*. In addition, four different purification methods were investigated to identify the impact on the purity of alginate. Similar to Gomez *et al.*'s research(Gomez *et al.*, 2009), ethanol route gave highest alginate yield, molecular weight as well as shortest purification steps. Furthermore, this work demonstrates that HCl and $CaCl_2$ route could help remove the co-extracted fucoidan, thus resulted in higher purity alginate.

- Microwave assisted hydrolysis provided a fast and efficient saccharification process. The optimal condition for saccharification was 0.4 M H_2SO_4, 3.13% (w/v) of biomass loading, reaction temperature at 150 ^{o}C for 1 min holding time. A total of 127 mg/g monosaccharides of seaweed were released and 20.8 mg/g ethanol of seaweed was obtained. The ethanol concentration and conversion efficiency were 5.57 g/L and 60.7% (based on glucose, galactose and mannose) respectively. In addition, seaweed residue recovered after hydrolysis process had HHVs from 19-24 MJ/kg, with energy densification ranging from 1.4 to 1.7; this can also be potentially used as solid fuel.

- A step-by-step process was designed to obtain fucoidan, alginates, sugars and biochar (alga residue) consecutively. The yields of fucoidan, alginates, sugars and biochar were 14.09%, 18.24%, 10.87% and 21.44%, respectively. Properties of the separately obtained components were similar to those described above.

To make the story better, there is much more work can be done. For instance:

- Investigate the relation between fucoidan structure and antioxidant activities to unravel the precise mechanism. Other bioactivities of fucoidan such as anticoagulant, antithrombotic, antiinflammatory, antitumoral and etc. can be tested.

- The rheological properties of alginate can be studied，as the industrial application of alginate depends mainly on its ability to thicken aqueous solutions and forms gels.

- Analyse and remove the inhibitors in fermentation medium to achieve a better bioethanol production.

- Economic assessment of the contribution of the components parts should be carried out to estimate the optimal balance of processes for the biorefineries.

- If possible, scale up this biorefinery process to make it economically feasible.

Chapter 9： Materials and Methods

9.1 Materials and reagents

Brown macroalgae *Ascophyllum nodosum* was obtained from Bod Ayre Products Ltd, Shetland, UK. The fresh seaweed was dried by microwave heating and ground. A sieve selected different particle sizes.

Organic solvents dichloromethane and ethanol were both analytical grade > 99% and were purchased from Fisher Scientific UK Limited. Liquid nitrogen and nitrogen were purchased from the BOC group. Concentrated sulfuric acid (~98%), concentrated HCl (~37%), 0.1 M HCl were obtained from Fisher Scientific UK Ltd. Analytical reagent grade of NaOH, $CaCl_2$, Na_2CO_3, K_2SO_4, KOH, $Ba(OH)_2$ and NH_4CH_3COO were purchased from Fisher Scientific UK Ltd. Analytical reagent grade of phenol, sulfamate acid, trifluoroacetic acid, *m*-hydroxydiphenyl, 3-(Trimethylsilyl)-1-propanesulfonic acid sodium salt, alginate sodium, glucose, fucose, xylose, galactose and glucuronic acid were purchased from Sigma-Aldrich UK Limited. Polyethylene glycol/Polyethylene oxide calibration standard of molecular weight 200~1,200,000 $gmol^{-1}$ was purchased from Sigma-Aldrich UK Ltd.

9.2 Experimental methods

9.2.1 Extraction of fucoidan

9.2.1.1 Microwave assisted extraction of fucoidan

Microwave assisted extraction of fucoidan was carried out using Discover and Explorer SP, CEM Corporation. Extraction procedure was described below and summarized in Figure 9.1.

Ascophyllum nodosum (30 g) was extracted with 80% ethanol (300 ml) under mechanical stirring at room temperature for 18 h and then 70 ^oC for 4 h to remove pigments, proteins,(Rioux *et al.*, 2007) mannitol and some salts (Foley *et al.*, 2011). The residue was recovered by centrifugation and dried. The resulting seaweed (25.6 g)

then was split into 30 parts for the following process. 0.85 g *Ascophyllum nodosum* was suspended in 15 ml 0.1 M HCl solution and put in a 35 ml microwave tube. Then the microwave tube was placed into the CEM Discover reactor for microwave irradiation at different temperatures for different times. After irradiation, the suspension was centrifuged to separate the residual alga, which was dried at 80 ^{o}C and weighed to determine the residual amount obtained. Subsequently, 2% (w/v) $CaCl_2$ solution was added to the liquid fraction and the mixture was maintained overnight at 4 ^{o}C for alginate removal. The fraction obtained by ionization of $CaCl_2$ was separated by centrifugation. Double volume of ethanol absolute was added to the resultant filtrate and the ethanol-precipitated polysaccharides were recovered by centrifugation, dried at 40 ^{o}C. The supernatant after fucoidan precipitation was concentrated to 5 ml and further analysed.

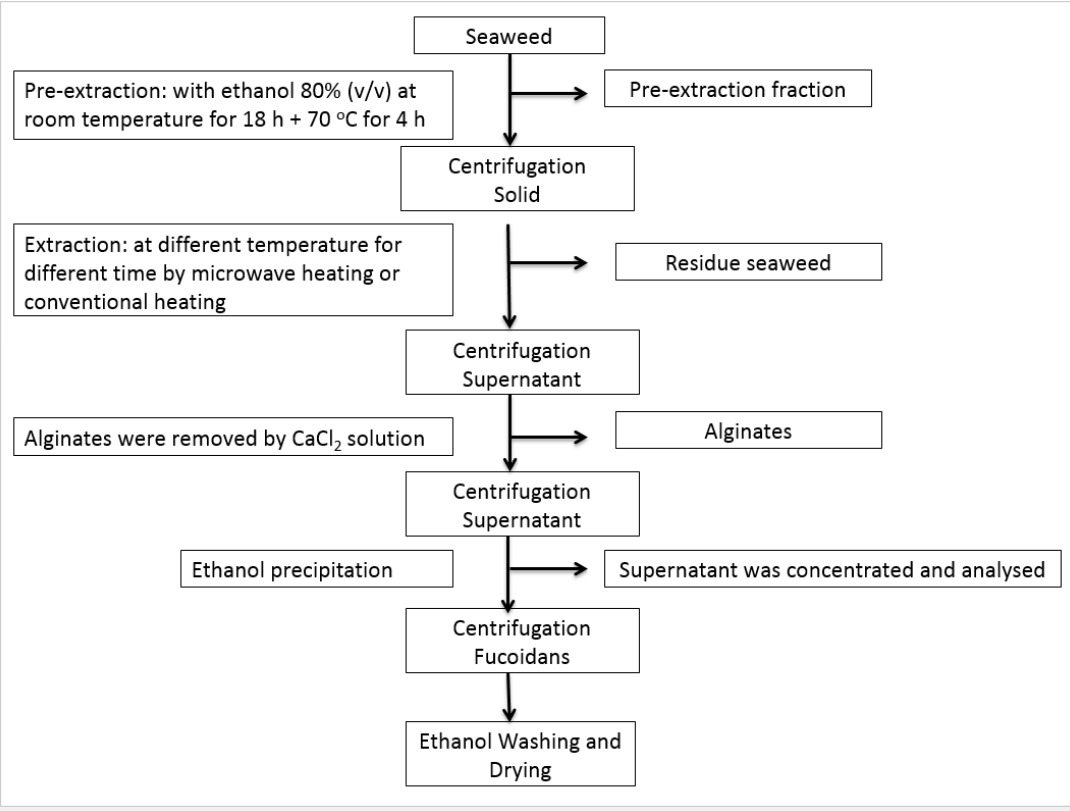


Figure 9.1 Schematic diagram of fucoidan extraction process

9.2.1.2 Extraction of fucoidan by conventional method

To make a comparison, pretreated seaweed was also extracted using conventional method described by Rioux *et al.*(Rioux *et al.*, 2007), with slight modifications. Fucoidan was extracted with 0.1 M HCl in a 50 mL round bottom flask at 70 °C (3 * 3 h) by conventional hotplate heating (Figure 9.2), and flowing separation was same as section 9.2.1.1

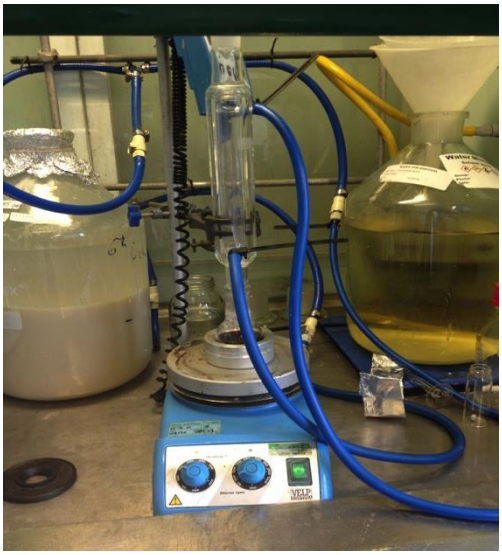


Figure 9.2 Apparatus for conventional heating method

9.2.2 Extraction of alginate

9.2.2.1 Microwave assisted open vessel extraction of alginate

The extraction process was similar with the ones reported by C. G. Gomez *et al.* (Gomez *et al.*, 2009) and Swee-Yong Chee *et al.* (Chee *et al.*, 2011) (Figure 9.3), with some modifications.

1 g dry seaweed was put into about 20 ml HCl (pH = 4). This mixture was stirred for 15 min at room temperature and then the seaweed was washed with around 20 ml distilled water twice. The purpose of this pre-treatment was to moisten the seaweed and to make the alginate more readily soluble in an alkaline solution as well. It is well

known that the acid treatment degrades the acetal bonds on the polymeric chain, so acid pretreatment can be used to control the viscosity of final product. In this case, pH=4 was chosen to obtain a high viscosity according to Hernandez-Carmona *et al.*(Gustavo Herna´ndez-Carmona, 1999). Moistened seaweed was placed in a 50 mL round bottom flask to which then was added 15 ml 0.1mol/L Na_2CO_3 and put into CEM Discover microwave reactor (Figure 9.4), and a condenser was connected to the round bottom flask to avoid water loss during the process. Then the suspensions were irradiated under microwave for different time and temperature. The extracted sodium alginate from the initial material was diluted to 50 mL with distilled water and then the insoluble material was separated by centrifugation, obtaining the sodium alginate in the supernatant.

For separation step, aqueous solution of sodium alginate obtained in the extraction step was directly precipitated, under stirring, by addition of ethanol until reaching a proportion 1:1 in volume, respectively. Thus, the insoluble polymer was separated and then washed with ethanol. Finally, the biopolymer was dried at 40 ^{o}C until constant mass and then ground to powder using mortar and pestle.

The supernatant after alginates precipitation (about 100 mL) were concentrated to 5 mL by evaporation and then were analysed to determine the total carbohydrate and uronic acid content.

The residual alga, which was dried at 80 ^{o}C, was weighed to determine the residual amount obtained.

Alginate Extraction Process

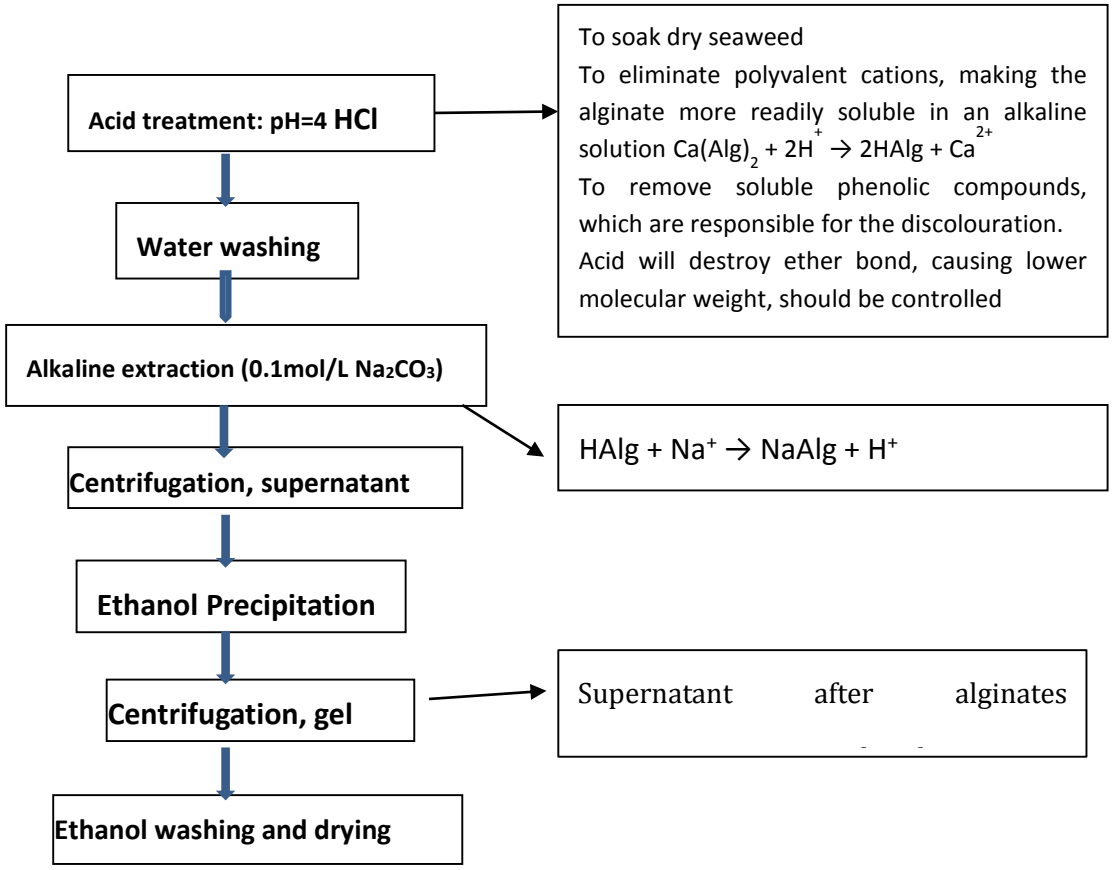


Figure 9.3 Schematic diagram of alginate extraction process

Figure 9.4 CEM Discover microwave reactor

9.2.2.2 Microwave assisted closed vessel extraction of alginate

The method for extraction processes in closed vessel was similar to that in open vessel system. The difference is that a 35 mL microwave tube was used instead of a 50 mL round bottom flask and no condenser was connected. Due to high temperature and closed system, pressure built up during the processes. The method for separation and supernatant analysis was the same as open vessel system.

9.2.3 Microwave assisted acid hydrolysis of *Ascophyllum nodosum*

The microwave assisted hydrolysis process was investigated by varying different process parameters: different acid concentration (0.01 M-0.4 M), temperature (120-180 ^{o}C), biomass loading (solid/liquid ratio: 0.6% - 6%, w/v) and reaction time (0-30 min). The dried seaweed powder was subjected to different concentrations of acidic solution in a standard CEM Discover microwave reaction tube (35 ml). The sample was subsequently inserted into the microwave and irradiated under dynamic mode to enable the system to achieve the desired temperature. Temperature and pressure were recorded during this process using the microwave pressure head and the inbuilt IR sensor. After irradiation, the suspensions were centrifuged to separate the residual alga, which was washed with distilled water and dried at 80 ^{o}C until constant weight.

9.2.4 Fermentation

S.cerevisiae ATCC no200062 was used for fermentation. The yeasts were cultured in ATCC medium (yeast extract 10.0 g/L, bacto-peptone 20.0 g/L, glucose 20.0 g/L) until the OD_{600} reached 0.5. 2 g freeze dried hydrolysate was dissolved in 10 mL distilled water, 0.25 mL 2 M sodium acetate and 2 mL 10 × ATCC medium no glucose (100 g/L yeast extract, 200 g/L bacto-peptone) was added, the mixture was sterilized at 121 ^{o}C for 30 min. Then 2 mL prepared yeasts was added and the final volume was made up to

20 mL with sterile water. The sample was then incubated in a shaking incubator at 37 ^{o}C with a shaking speed of 130 rpm for a total time of 72 h. Samples were withdrawn at different time intervals and were centrifuged at 3000 rpm for 10 min. The supernatant obtained after centrifugation was then analyzed for bioethanol and residual sugar content. Fermentation was done in duplicate.

9.3 Analytical methods

9.3.1 Moisture content determination

Approximately 5 g of seaweed was put inside aluminum dishes and placed into a 383 K oven (Carbolite Ltd). The seaweed was weighed periodically until constant weight was achieved. The moisture content was calculated using the equation below:

$$Moisture\ content\ (\%) = \frac{(Mass\ of\ original\ sample - Mass\ of\ dried\ sample)}{Mass\ of\ original\ sample} \times 100$$

9.3.2 Ash content determination

Approximately 5 g of seaweed was put inside a porcelain crucible and placed into a Barnstead Thermolyne 6000 furnace. The temperature of furnace was increased with a rate of 5 ^{o}C min^{-1} until 600 ^{o}C, holding for 4 hours. After cooling down, the ash was weighed. The ash content was calculated using the equation below.

$$Ash\ content\ (\%) = \frac{Mass\ of\ ash}{Mass\ of\ original\ sample} \times 100$$

9.3.3 Carbohydrate content determination by phenol - H_2SO_4 method

Total carbohydrate content was measured using the phenol-H_2SO_4 method (Dubois *et al.*, 1956) by a Jasco V-550 UV/Vis spectrophotometer. The total carbohydrate content was calculated by reference to the sugar standard (0-150 μg/mL fucose) at 480 nm (Figure 9.5).

Procedure: 2 ml of sugar solution that had a concentration between 10-150 μg/mL was pipetted into a colourimetric tube, and 0.05 ml of 80% (w/w) phenol solution was added. Then 5 ml of concentrated sulfuric acid was added rapidly, the stream of acid being directed against the liquid surface rather than against the side of the test tube in order to obtain good mixing. The tube were allowed to stand 10 minutes, then they were shaken and placed for 10 to 20 minutes in a water bath at 25 - 30 °C before readings were taken. Blanks were prepared by substituting distilled water for sugar solution.

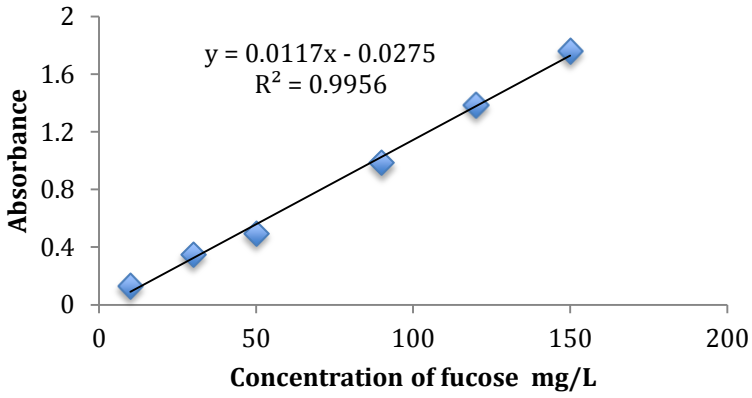


Figure 9.5 Standard calibration curve for carbohydrate content determination

To determine the total carbohydrate content of raw materials, the seaweed powder was initially treated by 2 M trifluoroacetic acid (TFA) for 2 h at 121 °C, then residual seaweed was treated by 72% sulfuric acid for 4 h at room temperature, followed by dilute acid (diluted down to 3.2% sulfuric acid) for 4 h at 120 °C. The sugar content was then measured by phenol-H_2SO_4 method.

9.3.4 Lipid content determination

Approximately 5 g of seaweed was extracted in a Soxhlet extractor, using about 150 mL hexane for 6 hours. The solvent was then removed by rotary evaporator. The lipid in the flask was weighed. The lipid content was calculated using the equation below:

$$Lipid\ content\ (\%) = \frac{Mass\ of\ lipid}{Mass\ of\ original\ sample} \times 100$$

9.3.5 Protein content determination

Protein content of seaweed sample was calculated by converting the nitrogen content (obtained from CHN analysis), determined by micro-Kjeldahl method (6.25*N)(Marinho-Soriano *et al.*, 2006). The protein content was calculated using the equation below:

$$Protein\ content\ (\%) = Percentage\ of\ nitrogen \times 6.25$$

9.3.6 Phenolic content determination

The phenolic content was measured by extracting the seaweed powder with 80% ethanol under mechanical stirring at room temperature for 18 h and then 70 °C for 4 h, and then the extract was measured by the Folin-Ciocalteau (FCR) method. The phenolic content was calculated by reference to the gallic acid (0-500 mg/L gallic acid) at 758 nm by a Jasco V-550 UV/Vis spectrophotometer (Figure 9.6).

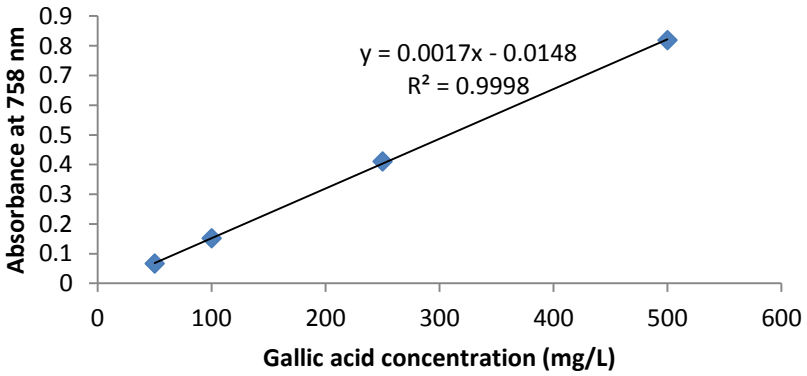


Figure 9.6 Standard calibration curve for phenolic content determination

Procedure: 0.5 ml sample or blank (distilled water) was placed in 50 ml volumetric flask. 35 ml distilled water was added, followed by 2.5 ml FC reagent. Then the solution

was swirled to mix and incubated for at least 1 and not more than 8 min at room temperature. 7.5 ml 20% (w/w) sodium carbonate solution was added, then distilled water was added to the 50 ml line and the solution was mixed and incubated 2 hours at room temperature. 2 ml was transferred to glass cuvette and the absorbance was measured at 758 nm.

9.3.7 Uronic Acid Content Determination

Total uronic acid content was measured using the method of Filisetti-Cozzi and Carpita (Filisetticozzi & Carpita, 1991) by a Jasco V-550 UV/Vis spectrophotometer. The uronic acid content was calculated by reference to the glucuronic acid (0-150 mg/L glucuronic acid) at 525 nm (Figure 9.7).

Procedure: samples containing up to 150 µg uronic acids were dissolved in 0.4 ml of water in glass tubes, and 40 µl of 4 mol/L sulfamic acid-potassium sulfamate (pH 1.6) was added and mixed thoroughly. Concentrated H_2SO_4 containing 75 mmol/L sodium tetraborate (2.4 ml) is then added. The solutions were heated to near 100 °C for 20 min by heating block with the tubes capped. The tubes were placed in an ice bath to quickly cool the reaction mixture to ambient temperature. After cooling, 80 µl of 0.15% (w/v) m-hydroxydiphenyl in 0.5% (w/v) NaOH was overlaid and then stirred vigorously. The pink colour developed to completion in about 5-10 min. Blanks were prepared by substituting distilled water for uronic acid solution.

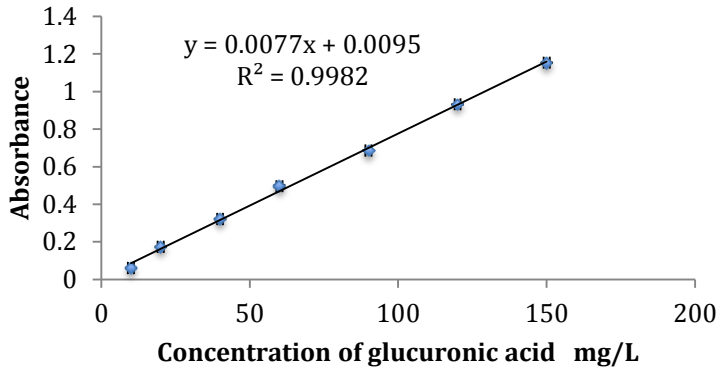


Figure 9.7 Standard calibration curve for uronic acid content determination

9.3.8 Sulfate content determination by $BaCl_2$-gelation method

The sulfate content of fucoidan was quantified using the $BaCl_2$-gelation method by a Jasco V-550 UV/Vis spectrophotometer, using Na_2SO_4 as the standard (Figure 9.8) after hydrolysing fucoidan in 1 M HCl for 5 h at 110 °C(Dodgson & Price, 1962).

Procedure: to the 0.2 mL sulfate containing solution was added 3.8 mL of 4% trichloroacetic acid followed by 1 mL of the $BaCl_2$-gelation reagent. After mixing, the whole was allowed to stand for 10-20 min at room temperature before measuring. A reagent blank was prepared in the same way except that 0.2 ml of water was substituted for the sulfate-containing solution.

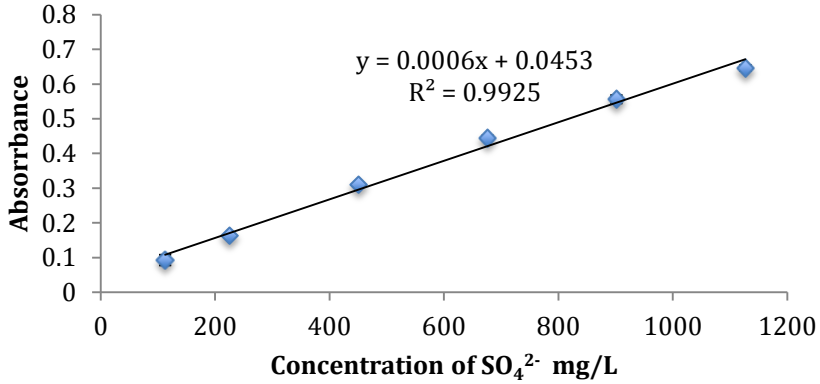


Figure 9.8 Standard calibration curve for sulfate content determination

$BaCl_2$-gelation reagent preparation: 2 g gelation was dissolved in 400 mL of hot water (60 – 70 ^oC) and allowed to stand at 4 ^oC for at least 6 hours and preferably over night. 2 g Barium chloride was dissolved in the semi-gelatinous fluid and the resultant cloudy solution was allowed to stand for 2 -3 hours before use.

9.3.9 Gel permeation chromatography (GPC) analysis

Polysaccharides weight average, M_w, was determined by GPC-ELSD (Gel permeation chromatography - evaporative light scattering detector). The column used was a TSKgel $GMPW_{XL}$ (7.8 mm * 300 mm). The mobile phase consisted of 0.1 mol/L NH_4OAc solution filtrated on 0.22 μm filters. The flow rate was 0.5 ml/min and analyses were performed at room temperature. 5 mg sample was resuspended in 1 ml distilled water. This mixture was allowed to solubilize briefly and then filtered on 0.22 μm filters. The gel permeation column was calibrated using polyethylene glycol/polyethylene oxide standards. The data was processed by WinGPC UniChrom GPC Software made by Polymer Standards Service (PSS).

9.3.10 Bioethanol concentration determination by gas chromatography (GC)

Bioethanol concentration was analysed by gas chromatography (HP 6890 series, Hewlett Packard. Inc., USA) using a flame ionization detector (FID) with a Stabilwax column (Crossbond Carbowax polyethylene glycol; length, 30 m; inner diameter, 0.25 mm; film thickness, 0.25 μm). Following operating condition was used: detector temperature of 225 ^oC; injector temperature of 225 ^oC; oven temperature was increasing from 100 (2.0 min) to 175 ^oC at 10 ^oC/min. Helium was used as the carrier gas and 1-propanol was used as internal standard.

9.3.11 High performance anion exchange chromatography (HPAEC) analysis

The monosaccharide compositions were separated by high-performance anion-exchange chromatography on a Dionex Carbopac PA-10 column with integrated amperometry detection. The separated monosaccharides were quantified by using external calibration with an equimolar mixture of nine monosaccharide standards (arabinose, fucose, galactose, galacturonic acid, glucose, glucuronic acid, mannose, rhamnose, and xylose) that were subjected to acid hydrolysis in parallel with the samples.

To determine the monosaccharide composition of fucoidan, 5 mg fucoidan was hydrolysed with 2 M trifluoroacetic acid for 2 h at 121 °C (Arnous & Meyer, 2008) and then analysed by high-performance anion-exchange chromatography.

Sample preparation: 500 µL sugar solution or fucoidan solution after TFA treatment was evaporated completely in centrifugal evaporator with fume extraction. To the vial, 500 µL propan-2-ol was added, mixed and evaporated. Repeat the propan-2-ol washing, and then resuspend the sample in 200 µL water, mix well. The supernatant was filtered with 0.45 µm PTFE filters and put into vials by syringes.

9.3.12 High performance liquid chromatography (HPLC) analysis

The quantification of furfural and hydroxymethyfurfural in the fermentation medium were done by high-performance liquid chromatography (HPLC) on an ACE C18 column (250 * 4.6 mm) with an evaporative light scattering detector (ELSD). The mobile phase was mixture of MeCN and H_2O (1:3). The flow rate was 0.8 ml/min and analyses were performed at 30 ^{o}C.

9.3.13 FT-IR analysis

Fourier transform infrared spectroscopy (FT-IR) was used to collect infrared spectra of the polysaccharides. A Bruker VERTEX 70 infrared spectroscopy and ATR probe with Golden gate attachment were used to perform this analysis. The software used was Opus. Samples spectra were collected between 400 and 4000 cm^{-1} at a resolution of 4 cm^{-1} and a scan rate of 16. The spectra were subsequently analysed to determine possible bond types and functional group.

9.3.14 NMR analysis

For alginates ^{1}H NMR analysis, around 10 mg sample was dissolved in 1.5 mL D_2O and spectra were recorded on a JEOL-ECS 400 spectrometer at 353K. Chemical shifts are given in ppm relative to internal standard 3-(Trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) (Torres *et al.*, 2007) at 0.00 ppm. Baseline correction was carried out prior to integration.

9.3.15 ICP analysis

Inductively Coupled Plasma (ICP) analysis was performed using the departmental service utilising Agilent 7700x. Sample & Skimmer Cones was Ni, and analysis was run in He mode.

9.3.16 CHN analysis

Elemental analysis of carbon, hydrogen and nitrogen ratios were performed using the departmental service utilising Exeter Analytical (Warwick, UK) CE440 Elemental Analyser (calibrated against acetanilide with S-benzyl-thioronium chloride internal standard).

9.3.17 Antioxidant activity test

9.3.17.1 DPPH radical scavenging ability test

Scavenging activity of 1,1-diphenyl-2-picrylhidrazyl (DPPH) radical was determined according to Dore *et al.'s* method (Dore *et al.*, 2013). 0.1 mL of sample was added to 1.5 mL 0.1 mM ethanol solution of DPPH. After 30 min at ambient temperature, absorbance was measured at 517 nm. The scavenging activity of DPPH radicals was calculated using the following equation: scavenging activity (%)=(1-$A_{sample}/A_{control}$) * 100, where $A_{control}$ is absorbance of the ethanol solution of DPPH without sample (which was replaced by distilled water) and A_{sample} represents absorbance of the ethanol solution of DPPH with tested samples.

9.3.17.2 Reducing power assay

The reducing power was determined according to the method of Ferreira *et al*(Ferreira *et al.*, 2007), with slight modifications. 0.125 mL sample was mixed with 0.125 ml of phosphate butter (pH 7) and 0.125 mL of 1% potassium ferricyanide. The mixture was incubated at 50 ^{o}C for 20 min. The reaction was terminated by the addition of 0.125 mL of 10% trichloroacetic acid (TCA) to the reaction mixture. The solution was then mixed with 0.5 mL distilled water and 0.1 mL of ferric chloride, and the absorbance was measured at 700 nm: higher absorbance indicates higher reducing power.

9.3.18 Calorific value analysis

The calorific value of the seaweed was determined by a Parr 6200 bomb calorimeter, made by Scientific & Medical Products Ltd., UK.

List of Abbreviations

ABE	Acetone, Butanol and Ethanol
AD	Anaerobic Digestion
DCM	Dichloromethane
DPPH	1,1-diphenyl-2-picrylhidrazyl
ED	Energy Densification
ELSD	Evaporative Light Scattering Detector
EPA	Environmental Protection Agency
EY	Energy Yield
FCC	Federal Communications Commission
FCR	Folin-Ciocalteau Reagent
FF	Furfural
FID	Flame Ionization Detector
FT-IR	Fourier Transform Infrared
FTS	Fischer-Tropsch Synthesis
Fuc	Fucose
Gal	Galactose
GC	Gas Chromatography
GHG	Greenhouse Gases
Glu	Glucose
GluA	Glucuronic Acid
GPC	Gel Permeation Chromatography
HHV	Higher Heating Value
HMF	Hydroxymethyfufural
HPEAC	High Performance Anion Exchange Chromatography
HPLC	High Performance Liquid Chromatography
ICP-AES	Inductively Coupled Plasma
MAD	Microwave Assisted Drying

MAE	Microwave Assisted Extraction
Man	Mannose
MAP	Microwave Assisted Pyrolysis
Mn	Number Average Molar Mass
Mw	Mass Average Molar Mass
MW	Microwave
NMR	Nuclear Magnetic Resonance
PI	Polydispersity Index
Rha	Rhamnose
SA	Supernatant After Alginate Precipitation
SCWG	Supercritical Water Gasification
SF	Supernatant After Fucoidan Precipitation
SH	Supernatant From Hydrolysis
TCA	Trichloroacetic Acid
TFA	Trifluoroacetic Acid
U.K.	United Kingdom
U.S.A.	United States of America
Xyl	Xylose

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